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Molecular and genetic characterisation of sea bass caspases.

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Molecular and genetic characterisation of sea bass caspases.

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De acordo com o disposto no Decreto-Lei nº 74/2006 de 24 de Março, esclarece-se serem da nossa responsabilidade a execução das experiências que estiveram na origem dos resultados apresentados, assim como a sua interpretação, discussão e redação. Nesta tese foram apresentados os resultados contidos nos artigos já publicados e seguidamente discriminados:

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Durante esta longa jornada contei com a ajuda de muitas pessoas

que directa ou indirectamente tornaram esta tese possível.

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Abbreviations List

AIF	Apoptosis-inducing factor
Apaf-1	Apoptotic protease activating factor 1
ASC	Apoptosis-associated speck-like protein containing CARD
ATP	Adenosine-5'-triphosphate
Bad	Bcl-2-associated death promoter
Bak	Bcl-2-antagonist/killer
Bax	Bcl-2–associated X protein
Bcl-2	B-cell lymphoma 2
$Bcl\text{-}_{XL}$	B-cell lymphoma extra large
Bid	BH3 interacting domain death agonist
Bik	Bcl-2-interacting killer
Bim	Bcl-2-like-11
BIR	Baculoviral IAP Repeat
CARD	Caspase-associated recruitment domain
CASc	Carboxyl-terminal catalytic domain
CTD	Casp-9-Carboxyl-terminal divergent
DAMP	Danger-associated molecular pattern
DED	Death effector domains
DIABLO	Direct Inhibitor of Apoptosis-Binding protein with Low pI
DISC	Death-inducing signalling complex
DNA-PK	DNA dependent protein kinase
ECPs	Extracellular products
FADD	Fas Adaptor Death Domain or Fas-associated death domain
Fas	TNF receptor superfamily, member 6
FLICE	FADD–like ICE
FLIP	FLICE inhibitory protein
IAPs	Inhibitors of apoptosis proteins
ICAD	Inhibitor of caspase-activated DNase
ICE	Interleukin-1 beta converting enzyme
IL-18	Interleukin-18
IL-1β	Interleukin-1 beta
LPS	Lipopolysaccharide
Mlc-1	Myeloid cell leukemia-1
NF-κB	Nuclear factor kappa light-chain enhancer of activated B cells

NLR	Nod-like receptor
NLRP3	NLR family, pyrin domain containing 3
Omi/Hrt2	High temperature requirement factor A2
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PARP1	Poly(ADP-ribose) polymerase
Phdp	*Photobacterium damselae* ssp. *piscicida*
PYD	Pyrin N-terminal homology domain
RACE	Rapid amplification of cDNA ends
RGD	Integrin-recognition motif
ROS	Reactive oxygen species
SMAC	Second mitochondria-derived activator of caspase
SREBP	Sterol regulatory element-binding protein
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRADD	Tumor necrosis factor receptor type 1-associated death domain
XIAP	X-linked inhibitor of apoptosis

Abstract

Caspases are **c**ysteine **asp**artyl-specific prote**ases** that have been highly conserved throughout evolution. They became known by their involvement in apoptosis and inflammation, although their participation in other biological processes is presently well established. Nevertheless, they are still usually classified as apoptotic or pro-inflammatory. Apoptotic caspases have been further subdivided into (i) upstream initiator caspases (caspase-2, -8, -9 and -10) and (ii) downstream executioner or effector caspases (caspase-3, -6 and -7). Initiator caspases are more specialised to cleave their own precursors, downstream effector caspases and a small number of other substrates, whereas effector caspases cleave cellular proteins, leading to cellular destruction, although they can also activate other caspases. Pro-inflammatory caspases (caspase-1, -4, -5, -11 and -12) play a crucial role in the processing of inflammatory cytokines.

Caspases are expressed as pro-enzymes that are composed of a NH_2-terminal pro-domain highly variable in sequence and length and involved in the regulation of the pro-enzyme activation, a large subunit (p20) containing the active site cysteine within a conserved QAC(G/R/Q/)G motif, and a small subunit (p10). Some pro-caspases have also a short linker between the large and small subunits. Pro-caspases are activated by proteolytic processing at the carboxyl side of a specific aspartic acid (Asp) residue in response to several internal or external stimuli, resulting in the removal of the NH_2-terminal pro-domain and the formation of a heterotetramer composed of two p20 and two p10 subunits with two active sites, that seem to function independently, at opposite ends of each molecule. Similarly, caspases have high specificity to cleave their substrates at a specific Asp residue that must be in the context of a tetrapeptide motif. The tetrapeptide motifs recognised by different caspases differ significantly, explaining the diversity of their biological functions.

Activation of initiator apoptotic and pro-inflammatory caspases occurs at multiprotein complexes, known as DISC for caspase-8, apoptosome for caspase-9, and inflammasome for caspase-1. By increasing the local concentration of the pro-caspases, those multiprotein platforms allow the auto-activation of the caspases through a proximity-induced dimerization mechanism. Effector caspases do not need such a complex mechanism of activation because they are usually activated through proteolytic processing by previously activated caspases or other proteases, such as Granzyme B. Therefore, caspases form a cascade of proteolytic events, where the initiator caspases start the dead effector machinery whereas the effector caspases mostly cleave critical

cellular substrates, leading to the biochemical and morphological alterations of the apoptosing cells.

Caspase activity is tightly regulated at different levels, from gene expression to several steps of the caspase production, processing and activity. The activation of the apoptotic program, in response to a death signal, is regulated by an accurate balance between the pro-apoptotic and anti-apoptotic mechanisms, in which the Bcl-2 family members play a crucial role by regulating the mitochondrial outer membrane permeabilization (MOMP), which allows the release of several regulatory proteins from the mitochondrial intermembrane space. Along with the regulation through different protein factors, the caspase activities can also be regulated by the presence of isoforms.

While the involvement of caspases in several biological processes was well established in mammals when this work started, the information on fish caspases was scarce, with only few fish caspases known and characterised. Given the importance of fish research to support the much needed development of aquaculture and to study diverse mammalian normal and pathological issues, and given the participation of apoptosis and inflammation in many biological processes and pathologies in vertebrates, research on fish caspases is of obvious relevance. This thesis was developed at the Fish Immunology and Vaccinology group of the IBMC that, during the last years, has been using sea bass to study the *Photobacterium damselae* ssp. *piscicida* pathogenicity mechanism as an example of a bacterial infection where the pathogen subverts the apoptotic and pro-inflammatory machineries of the host. The work plan of this thesis was developed in this context, aiming at cloning, sequencing and genetically characterise sea bass caspases representing all functional classes.

The initiator caspases of the extrinsic and intrinsic pathways, caspase-8 and -9, respectively, as well as the central executioner of apoptosis, caspase-3, were identified in sea bass. At the molecular level, the obtained sequences of sea bass caspase-8, -9 and -3 retain the motifs that are functionally important, such as the pentapeptide active-site motif (QACXG) and the cleavage sites at the aspartic acids that give rise to the characteristic organisation in NH_2-terminal pro-domain and large and small subunits. In sea bass caspase-8, two death effector domains (DED) were predicted by bioinformatics analysis. The same approach identified a caspase recruitment domain (CARD) in the NH_2-terminal pro-domain of sea bass caspase-9. Both DED and CARD are important domains involved in protein-protein interactions necessary to mediate dimerization and consequent activation of caspase-8 and -9, respectively. It was also shown that sea bass caspase-8, -9 and -3 were expressed at low levels in several organs of non-stimulated sea bass, and that their expression was up-regulated in the spleen in response to apoptotic stimuli. These data added to the understanding of the biology of fish apoptotic

caspases and allowed the development of tools to study the mechanisms of apoptosis in sea bass.

In this work, the sea bass caspase-1 gene was also cloned, sequenced and characterised. It was shown that sea bass caspase-1 auto-processing is similar to the one of its mammalian homologue, resulting in active p24/p10 and p20/p10 heterodimers. The presence of alternative spliced variants of caspase-1 in sea bass was also reported. The existence of caspase-1 isoforms in fish and in mammals suggests that they have been evolutionarily maintained and therefore are likely to play a regulatory role in the inflammatory response. The involvement of caspase-1 in the processing of inflammatory cytokines in non-mammalian vertebrates has been a matter of debate. This work contributed to clarify this issue, by showing that sea bass and avian IL-1β are specifically cleaved by caspase-1 at different but phylogenetically conserved aspartates, distinct from the cleavage site of mammalian IL-1β.

In conclusion, the sea bass counterparts of mammalian apoptotic caspases-8, -9 and -3 as well as the pro-inflammatory caspase-1, were sequenced and characterised. This allowed the production of new tools that were used to study the involvement of sea bass caspases in apoptotic processes and to biochemically characterise the sea bass caspase-1. In the future, these tools can also be used to better understand the role of caspases in apoptotic and inflammatory processes in fish, as well as to investigate their participation in other biological processes. Additionally, the recognition that the caspase-1 from a teleost fish is able to specifically process the proIL-1β into its mature form opens new doors to the study of the role of caspase-1 in immunological processes in non-mammalian vertebrates. In recent years, the importance of the inflammasomes in different pathologies, such as autoinflammatory and infection diseases, has been revealed. In several infections of mammals, it has been reported that the activation of inflammasomes is accompanied by the induction of pyroptosis, in an attempt to limit the pathogen replication. However, so far there are no data regarding inflammasomes or pyroptosis in any species of fish. Exploring the molecular mechanisms of caspase-1 activation at the inflammasomes as well as the subversion of the inflammasome pathways by pathogens appear as new lines of research in fish. Finally, it is worth mentioning that there are new insights in the regulation of caspases by their isoforms in mammals and thus, the study of the role of sea bass caspase-1 isoforms in the regulation of caspase-1 activity is of great interest.

Resumo

As caspases são proteases cisteínicas com especificidade para aspartatos extremamente conservadas ao longo da filogenia. Tornaram-se conhecidas principalmente devido ao seu envolvimento na apoptose e inflamação embora, hoje em dia, a sua participação noutros processos biológicos esteja bem documentada. Ainda assim, as caspases continuam a ser classificadas como apoptóticas ou pró-inflamatórias. As caspases apoptóticas podem ser subdivididas em (i) iniciadoras (caspase-2, -8, -9 e -10) e (ii) executoras ou efetoras (caspase-3, -6 and -7). As caspases iniciadoras são mais especializadas em clivar os seus próprios precursores, as caspases efetoras e um pequeno número de outros substratos, enquanto que as caspases efetoras estão sobretudo envolvidas na clivagem de vários substratos celulares, levando às alterações morfológicas típicas da apoptose. As caspases pro-inflamatórias (caspase-1, -4, -5, -11 e -12) têm uma função crucial no processamento de citoquinas inflamatórias.

As caspases são sintetizadas como pro-enzimas compostas por um pro-domínio amino-terminal extremamente variável em sequência e tamanho e envolvido na regulação da ativação da pro-enzima, uma subunidade grande (p20) contendo a cisteína reativa no contexto de um motivo QAC(G/R/Q/)G conservado, e uma subunidade pequena (p10). Algumas pro-caspases também têm um pequeno fragmento de ligação entre as subunidades grande e pequena. Em resposta a diversos estímulos externos ou internos, as pro-caspases são ativadas por processamento proteolítico específico no lado carboxilo de um resíduo de ácido aspártico (Asp), resultando na remoção do pro-domínio e na formação de um hetero-tetrâmero com dois locais ativos, composto por duas subunidades p20 e duas subunidades p10. As caspases clivam os seus substratos num resíduo Asp específico que tem de estar no contexto de um motivo tetrapeptídico. Diferentes caspases reconhecem motivos tetrapeptídicos distintos, estando esta diferença na base da diversidade das funções biológicas das caspases.

A ativação das caspases apoptóticas iniciadoras e das caspases pró-inflamatórias ocorre em complexos multiproteicos, conhecidos como DISC para a caspase-8, apoptosoma para a caspase-9, e inflamasoma para a caspase-1. As referidas plataformas multiproteicas conduzem a um aumento da concentração local das pro-caspases, permitindo a auto ativação das mesmas por um mecanismo de dimerização induzido por proximidade. As caspases efetoras não necessitam de um mecanismo tão complexo de ativação, uma vez que são normalmente ativadas por processamento proteolítico efetuado por caspases previamente ativadas ou por outras proteases, como por exemplo a Granzima B. Desta forma, as caspases formam uma cascata de eventos

proteolíticos, onde as caspases iniciadoras despoletam a maquinaria efetora da morte, enquanto que as caspases efetoras clivam principalmente substratos celulares, levando à ocorrência das alterações bioquímicas e morfológicas características da apoptose.

A atividade das caspases encontra-se altamente regulada a vários níveis, desde a sua expressão genética até aos vários passos da sua produção, processamento e atividade. A ativação do programa apoptótico, em resposta a um sinal de morte, está regulada por um balanço preciso entre os mecanismos pro-apoptóticos e anti-apoptóticos, no qual os membros da família Bcl-2 têm um papel fundamental através da regulação da permeabilização da membrana externa mitocondrial (MOMP), a qual permite a saída de diversas proteínas reguladoras do espaço intermembranar da mitocôndria. Para além da regulação efetuada através de diferentes fatores proteicos, as atividades das caspases também são reguladas por isoformas.

Aquando do início do presente trabalho, o envolvimento das caspases dos mamíferos em diversos processos biológicos estava bem documentado. No entanto, a informação relativa às caspases dos peixes era bastante limitada, e apenas algumas caspases de peixes tinham sido identificadas e caracterizadas. Dada a importância da investigação em peixes, não só para apoiar o desenvolvimento da aquacultura mas também como contribuição para o estudo de diversos aspetos fisiológicos e patológicos em mamíferos, e dada a participação da apoptose e inflamação em vários processos biológicos e patologias de vertebrados, a investigação das caspases em peixes é, obviamente, relevante. O presente trabalho foi desenvolvido no grupo de Imunologia e Vacinologia de Peixes do IBMC que, durante os últimos anos, tem vindo a usar o robalo para estudar o mecanismo de patogenicidade da *Photobacterium damselae* ssp. *piscicida* como um exemplo de uma infeção bacteriana onde o agente patogénico subverte as maquinarias apoptótica e pro-inflamatória do hospedeiro. O plano de trabalho desta tese foi desenvolvido nesse contexto, tendo como objetivo a clonagem, sequenciação e caracterização genética de representantes de todas as classes de caspases no robalo.

As caspases iniciadoras da via extrínseca e intrínseca, caspase-8 e -9, respetivamente, assim como a principal caspase executora da apoptose, a caspase-3, foram identificadas no genoma do robalo. A nível molecular, as sequências da caspase-8, -9 e -3 obtidas retêm os motivos que são funcionalmente importantes, tais como o motivo catalítico pentapeptídico (QACXG) e os locais de processamento (em ácidos aspárticos) que resultam na organização característica das caspases em pro-domínio, grande e pequena subunidades. Na caspase-8 de robalo, dois domínios efetores de morte (DED) foram previstos por análise bioinformática. A mesma abordagem permitiu identificar um domínio de recrutamento de caspases (CARD) no pro-domínio da caspase-9 de robalo. Tanto o DED como o CARD são domínios importantes envolvidos em interações

proteína-proteína necessários para mediar a dimerização e consequente ativação das caspases-8 e -9, respetivamente. Dados obtidos durante este trabalho revelaram baixos níveis de expressão das caspases-8, -9 e -3 em diversos órgãos de robalo não estimulados e um aumento de expressão das mesmas no baço em resposta a um estímulo apoptótico. Este trabalho originou informação que contribuiu para uma melhor compreensão da biologia das caspases em peixes e que permitiu o desenvolvimento de ferramentas para estudar os mecanismos da apoptose em robalo.

Neste trabalho, o gene da caspase-1 de robalo foi também clonado, sequenciado e caraterizado. Foi mostrado que a caspase-1 de robalo é auto-processada de forma semelhante à da sua homóloga de mamíferos, resultando em heterodímeros ativos de p24/p10 e p20/p10. A existência de variantes da caspase-1 originadas por processamento alternativo em robalo foi também reportada. A existência de isoformas da caspase-1 em peixes e em mamíferos sugere que foram mantidas durante a evolução filogenética e portanto muito provavelmente desempenham uma função reguladora na resposta inflamatória. O papel da caspase-1 no processamento de citoquinas inflamatórias em vertebrados que não mamíferos tem vindo a ser objeto de intenso debate. Os resultados aqui apresentados contribuíram para clarificar essa questão, uma vez que mostraram que as IL-1β de robalo e de aves são clivadas especificamente pela caspase-1 em aspartatos conservados filogeneticamente, distintos do local de clivagem presente na IL-1β de mamíferos.

Em conclusão, as caspases apoptóticas-8, -9 e -3 assim como a caspase-1 do robalo, foram sequenciadas e caracterizadas. A identificação destas caspases em robalo permitiu a produção de novas ferramentas que foram usadas para estudar o envolvimento das caspases de robalo em processos apoptóticos e para caracterizar bioquimicamente a caspase-1. No futuro, estas ferramentas poderão ser aplicadas na clarificação do envolvimento das caspases do robalo em mais situações envolvendo processos apoptóticos e/ou inflamatórios assim como no estudo de outras funções desempenhadas pelas caspases. Adicionalmente, a demonstração de que a caspase-1 de um peixe teleósteo processa a proIL-1β na sua forma matura abre portas ao estudo do papel da caspase-1 em processos imunológicos em vertebrados que não mamíferos. Nos últimos anos, tornou-se evidente a importância dos inflamasomas em diversas patologias, tais como doenças auto-imunes e infeciosas. Em diversas infeções de mamíferos, foi mostrado que a ativação dos inflamasomas é acompanhada pela indução de piroptose, como tentativa do hospedeiro controlar a replicação do agente infecioso. No entanto, em peixes, não existem quaisquer dados no que respeita a inflamasomas ou piroptose. A clarificação dos mecanismos moleculares envolvidos na ativação da caspase-1 ao nível dos inflamasomas nos peixes, assim como a subversão os inflamasomas por diferentes

agentes patogénicos, apresentam-se como novas linhas de investigação que podem vir a ser exploradas no futuro. Por último, é de salientar que há dados recentes sobre novos mecanismos de regulação das caspases de mamíferos pelas suas isoformas e, assim sendo, o estudo do papel das isoformas da caspase-1 do robalo na regulação da atividade da caspase-1 é de grande importância.

CHAPTER I

Introduction*

* Parts of this chapter were adapted from dos Santos NM, do Vale A, **Reis MI**, Silva MT. Fish and apoptosis: molecules and pathways. Current Pharmaceutical Design (2008) 14(2):148-69 (Annex I).

Introduction

1. Caspases

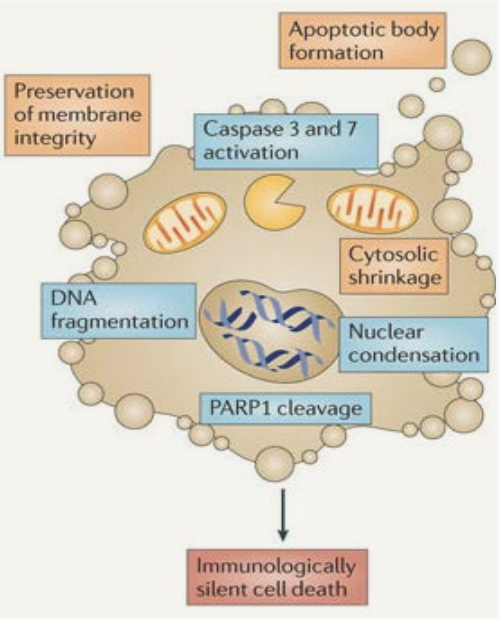
The family of intracellular **C**ysteine **Asp**artyl-Specific Prote**ases**, known as caspases**,** is highly conserved in multicellular organisms and their members share common features such as having high specificity to cleave their substrates after aspartic acid (Asp) residues [1]. Caspases are usually described as being the key players responsible for the morphological and biochemical changes occurring during apoptosis (Box 1). Nevertheless, when the first caspase was identified, there was no indication of its involvement in apoptosis. In 1992, the mammalian interleukin-1 beta (IL-1β) converting enzyme (ICE or caspase-1) was cloned and described as being responsible for the processing of pro-inflammatory cytokines, in particular interleukin-1 beta, into their active forms [2, 3]. The association of a caspase to cell death was reported for the first time one year later, when the ced-3 gene, the mammalian caspase-9 homolog, was cloned in the nematode *Caenorhabditis elegans* and was shown to encode a protein that was implicated in cell death [4]. The crucial role of caspases in apoptosis and inflammation is well recognised almost since their discovery and recent and very elegant studies have, associated apoptotic caspases with the regulation of multiple cellular processes beyond cell death, such as cell migration, proliferation and differentiation, thus amplifying the functions of the apoptotic caspases to non-apoptotic purposes [5-9]. Nowadays, the idea that caspases are important proteins involved in a wide range of biological reactions besides cell death is well established [6, 8, 9]. For this reason, the interest to understand what drives the activation and regulation of caspases increased and currently caspases are among the most studied proteins in science biology, with thousands of scientific papers on the subject being publish every year.

1.1. Classification

All caspases share similarities in amino acid sequence, structure and substrate specificity [10]. These proteases are constitutively expressed as zymogens (pro-enzymes) of about 30 to 55 kDa and are composed of a NH_2-terminal pro-domain, which is highly variable in sequence and length and is involved in the regulation of the pro-enzyme activation, a large subunit of approximately 20 kDa (p20) containing the cysteine active site within a conserved QAC(G/R/Q/)G motif, and a C-terminal small subunit of approximately 10 kDa (p10) (Figure 1). Some pro-caspases have also a short linker of about 10 amino acids between the large and small subunits [10-13]. Functionally, caspases can be classified as apoptotic or pro-inflammatory.

Box 1. Apoptosis

Apoptosis [14] is a genetically controlled and evolutionarily conserved form of active cell death, with an increase in complexity during phylogeny. Apoptosis is critical in the elimination of redundant, ectopic, damaged, mutated or infected cells and, therefore, plays an essential role in normal development, tissue homeostasis and immunity [7, 15-17]. Cells dying by apoptosis shrink and loose the connections with the surrounding cells. In general, organelles remain intact in the cytoplasm, despite some observations that describe an increase in endoplasmic reticulum size, the release and aggregation of ribosomes, the enlargement of the cisternae to form vesicles and vacuoles and the occurrence of changes in the mitochondria network [18, 19]. The main changes in the nucleus involve the chromatin condensation (pyknosis), DNA fragmentation by endonucleases into small fragments and nuclear fragmentation (karyorrhexis) [19]. Finally, the blebbing of the plasma membrane of the cell that has already lost the contact with their neighbours becomes perceptive. In some cases, the cell breaks into small fragments of various sizes containing the cellular contents and surrounded by membrane (apoptotic bodies) [19]. Apoptotic cells and apoptotic bodies are, *in vivo*, engulfed and digested mainly by macrophages, resulting in the elimination of the dying cells before they lyse and, therefore, without induction of an inflammatory response [14, 19-22]. In mammals, apoptosis may follow two principal pathways (Box 2): (i) the extrinsic/death-receptor pathway, initiated at the tumour necrosis factor (TNF) family receptors or at the dependence receptors [23] and (ii) the intrinsic/Bcl-2-regulated/mitochondrial pathway, regulated by the Bcl-2 family proteins and mainly characterised by mitochondrial membrane permeabilization (MMP) [23].



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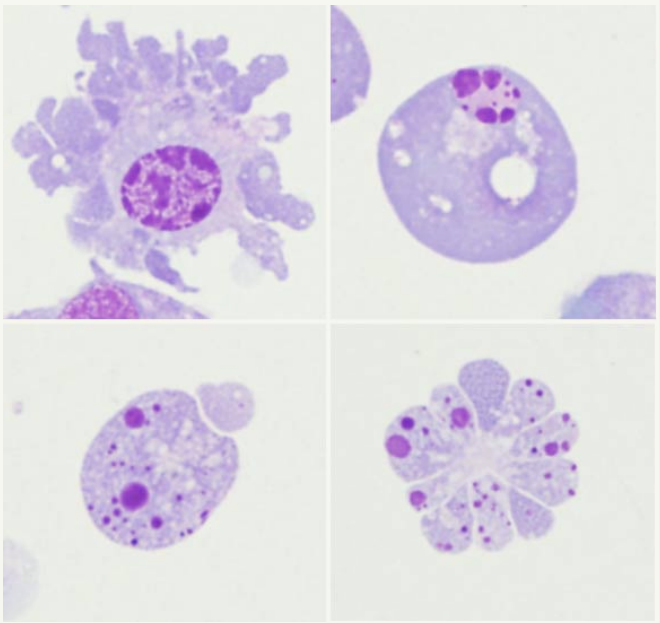


Cells undergoing apoptosis in response to the apoptogenic toxin AIP56 [25].

Apoptotic caspases can be further subdivided into: i) upstream initiator caspases (caspase-2, -8, -9 and -10) and (ii) downstream executioner or effector caspases (caspase-3, -6 and -7). The pro-forms of the initiator caspases possess large NH_2-terminal pro-domains, containing either death effector domains (DED) (caspases-8 and -10) or a caspase-associated recruitment domain (CARD) (caspases-2 and -9); these domains are responsible for the interaction with upstream adaptor molecules and thereby

have a relevant role in the activation of these caspases. The initiator caspases are more specialised to cleave their own precursors, downstream effector caspases and a small number of other substrates [13, 26]. The executioner or effector caspases have pro-forms containing only short NH_2-terminal pro-domains thought to be involved in subcellular targeting. Effector caspases cleave and inactivate cellular proteins, which will lead to cellular destruction, although they can also activate other caspases.

Pro-inflammatory caspases (caspase-1, -4, -5, -11, -12, -13 and -14) are synthesised as pro-forms with large NH_2-terminal pro-domains containing CARD, and their main role lies in cytokine maturation rather than apoptotic activity [10, 13, 27].

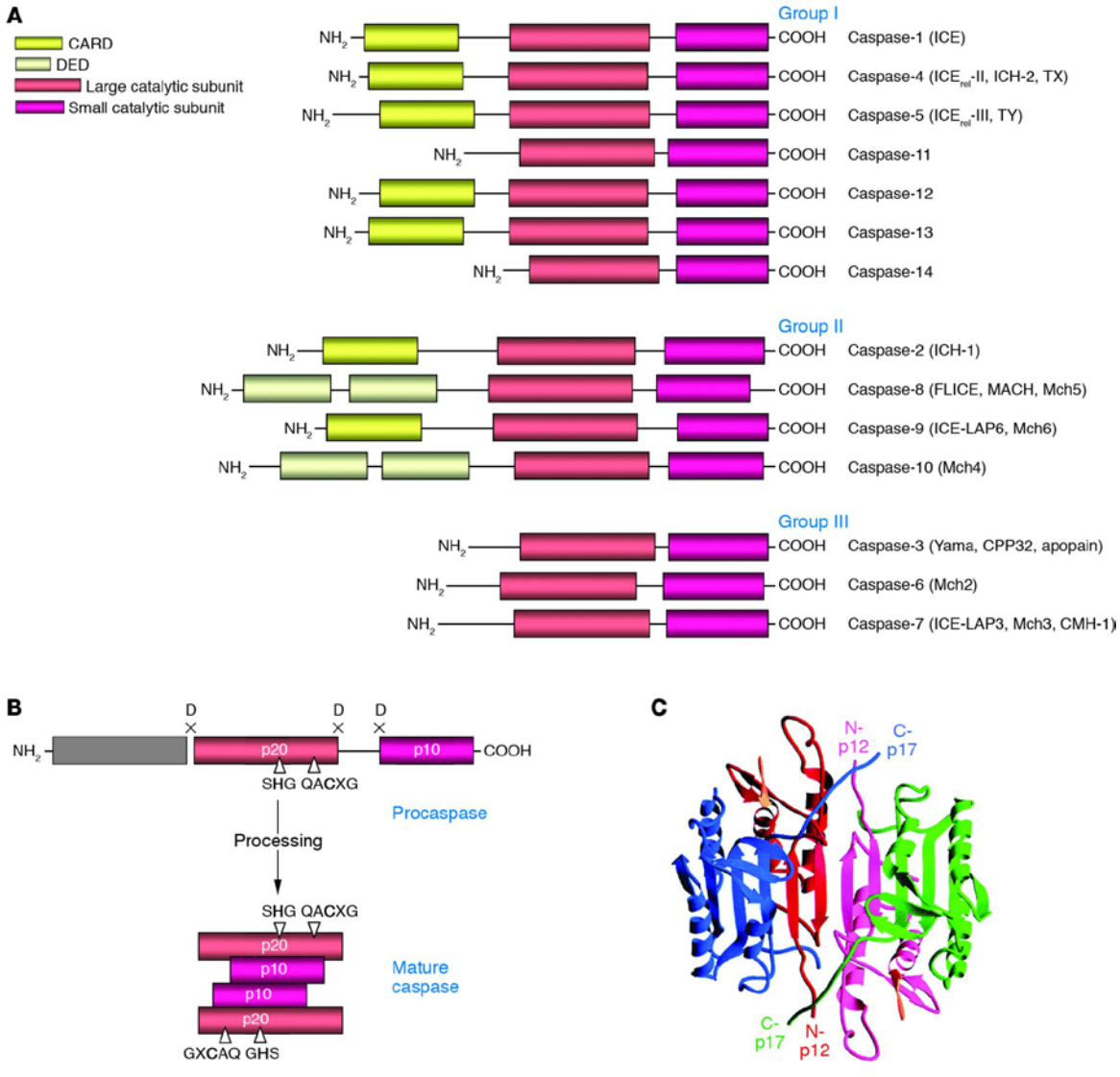


Figure 1. Structure and classification of the caspase family. (A) Diagrammatic representation of the three major groups of caspases: pro-inflammatory (group I), apoptotic initiator (group II), apoptotic executioner (group III). (B) Schematic representation of pro-caspase activation. (C) Three-dimensional structure of the caspase-3 heterotetramer composed of two heterodimers p17/p12. NH_2- and CH_2- termini of the small and large subunits are indicated. Adapted from [28].

The caspase zymogens are present in almost every nucleated animal cells and become active in response to several internal or external stimuli [7, 29]. Although most pro-caspases have a cytosolic location, when apoptosis is activated the caspases can be localised in several organelles of the cell, including mitochondria, endoplasmic reticulum or microsomal fraction, in order to cleave their substrates [30]. The caspases' substrates are cleaved at a specific Asp residue that must be in the context of tetrapeptide motifs, i.e., at least four amino acids contiguous to the cleavage site must be recognised for efficient catalysis. In the tetrapeptide motifs, the NH_2-terminal residue is designated P_4 and the target Asp is P_1. The tetrapeptide motifs recognised by different caspases differ significantly, explaining the diversity of their biological functions [10, 12]. So far, approximately 400 substrates of mammalian caspases were identified and are gathered at the online Caspase Substrate Database Homepage (CASBAH), being most of them related to apoptosis [31]. Caspase substrates are usually cleaved at a single site, resulting in the loss or change in function, meaning that substrate cleavage by caspases is not only specific but also highly efficient [10, 12, 32].

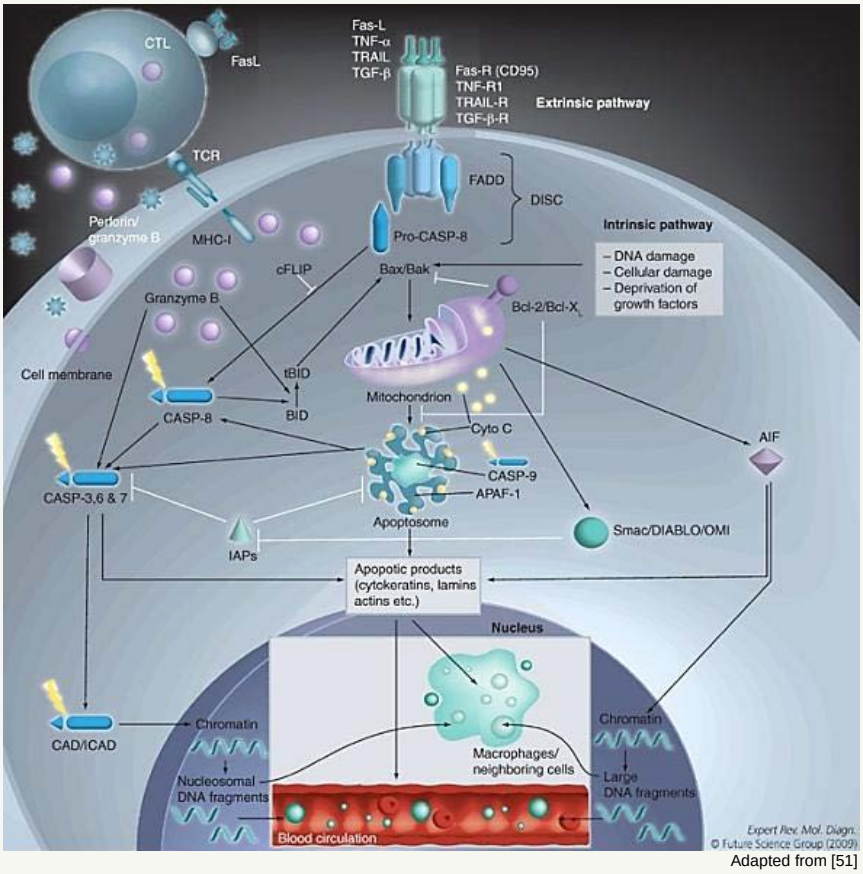
1.1. Functions

Caspases play an essential role in the two principal apoptotic pathways (Box 2), contributing to the cell death and to the apoptotic phenotype. They inactivate protective pathways and activate downstream reactions, leading to cellular destruction. Caspases cleave key structural components of the cytoskeleton, destroy DNA, disrupt the nuclear structure, induce the cell to display signals that mark it for phagocytosis and disintegrate the cell into apoptotic bodies. Accordingly, the caspase inhibition using synthetic or natural inhibitors abolishes apoptosis and a deletion in caspase genes leads to defects in apoptosis, revealing a definitive role for caspases in this process [10, 12].

The identification of activated caspases in the absence of cell death led to the idea that the apoptotic caspases have other functions beyond the apoptotic context [33]. For instance, it was shown that caspase-3 is involved in the differentiation of embryonic, hematopoietic and neural stem cells (by facilitating neurogenesis), as well as in maturation of dendritic cells [34-36]. Non-apoptotic functions of caspase-9 were also described in patients with Alzheimer disease [37], in muscle differentiation [38] and in aged olfactory bulb of mice [39]. In addition, caspase-8 is involved in non-apoptotic signalling pathways and defects in this caspase are the cause of some autoimmune diseases, revealing a significant non-apoptotic role of caspase-8 in the regulation of cell migration and in the proliferation of immune cells [40]. Humans with caspase-8 deficiency have immunodeficiency caused by alterations in the activation of T- and B-lymphocytes, and natural killer cells [41, 42]. The maintenance of genomic integrity and suppression

Box 2. Pathways for the induction of apoptosis

Apoptosis may follow two principal pathways:



Adapted from [51]

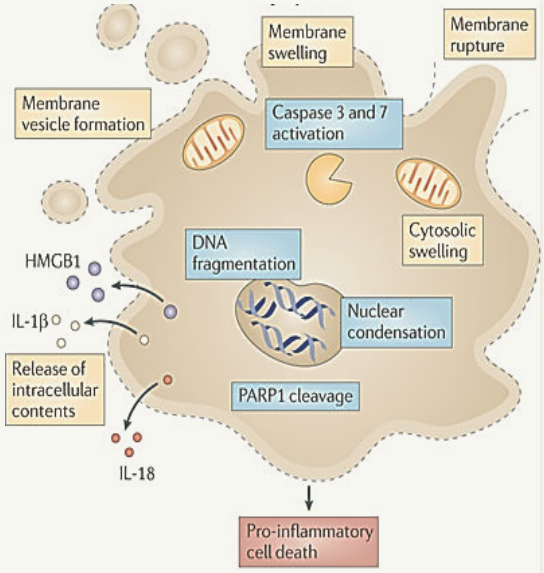
(i) The **extrinsic pathway** is initiated at tumour necrosis factor (TNF) super family receptors at the cell surface or at the dependence receptors at the cell surface (like UNC5A-D and DCC) [23]. One example of an extrinsic apoptotic pathway is the apoptosis occurring after the binding of the Fas ligand to Fas receptors (a member of the TNF-R superfamily of death receptors) on the surface of the target cells. After ligand engagement, Fas interacts with the adaptor molecule FADD (Fas-associated death domain protein) and pro-caspase-8 is recruited to the "death-inducing signalling complex" (DISC), a multiprotein complex that is rapidly assembled at the cytoplasmic portion of the Fas. The death effector domain (DED) of pro-caspase-8 associates with the DED of FADD resulting in the activation of caspase-8. Caspase-8 is released from the complex in its active form and activates the downstream caspases, including caspase-3, -6, and -7, leading to the characteristic morphological changes of apoptosis (Box 1 and [7, 43, 44]). (ii) The **intrinsic pathway** can be activated through several stress types, including irradiation, ultraviolet light, and cytotoxic drugs, among others [23, 45]. The cascade of events leading to caspase activation initiates when the apoptotic signal induces the release of several molecules, including cytochrome c, from the mitochondrial intermembrane space into the cytosol. Once in cytosol, cytochrome c binds to the Apoptotic Protease-Activating Factor-1 (Apaf-1), and multiple pro-caspase-9 molecules are recruited to the complex. This multiprotein complex, named apoptosome, is constituted by seven molecules of each Apaf-1, cytochrome c, (d)ATP and pro-caspase-9 [46]. The apoptosome facilitates the trans-processing of caspase-9 zymogens via the proximity-induced dimerization mechanism. The active caspase-9 is able to efficiently cleave and activate downstream executioner caspases such as caspase-3, which will subsequently cleave many important intracellular substrates, leading to apoptotic cell death [7, 43, 44]. Although the two pathways may function independently, cross-talk can occur between them at multiple levels. Caspase-8 cleavage of Bid, a pro-apoptotic member of the Bcl-2 family (Box 5), leads to the activation of this molecule. The truncated Bid translocates to the mitochondria, where it promotes the dimerization of Bax and Bak that form an oligomeric pore in the outer mitochondrial membrane allowing the exit of cytochrome c to the cytosol. Other caspases, such as caspase-3, as well as other proteases (like Granzyme B) have been shown to cleave and activate Bid, meaning that this is not a feature restricted to caspase-8 [47-50].

of necroptosis are also part of the diverse functions of caspase-8 [52, 53].

The involvement of pro-inflammatory caspases in cytokine maturation is well established and was first recognised for caspase-1 [54-56]. Nevertheless, the processing of inflammatory cytokines are no longer the only reported functions of this group of caspases. For instance, caspase-1 has been implicated in pyroptosis (Box 3), a new form of cell death that is induced in macrophages infected with *Salmonella typhimurium* [57] and surprisingly, is also responsible for processing more than 40 different proteins [58]. The wide range of caspase-1 substrates includes the executioner caspase-7 [59] as well as glycolytic enzymes, such as GAPDH (glyceraldehyde 3-phosphate dehydrogenase) [58].

Box 3. Pyroptosis

Pyroptosis [57] is a subroutine of cell death that involves the activation of caspase-1 observed in macrophages and dendritic cells infected with several classes of pathogens [24]. Pyroptosis has been described as a powerful mechanism to eliminate intracellular pathogens. In this context, caspase-1 mediates host defence responses and prevents pathogen replication by inducing pyroptotic cell death [60-62]. Morphological features of pyroptosis are distinct from the ones of apoptosis and include the increase in cell size and loss of plasma-membrane integrity in cells where the nucleus and the mitochondria remain intact. During pyroptosis, the pro-inflammatory cytokines IL-1β and IL-18 are processed and released, and chromosomal DNA is cleaved, although in a different pattern from apoptosis [61, 62].



Adapted from [24]

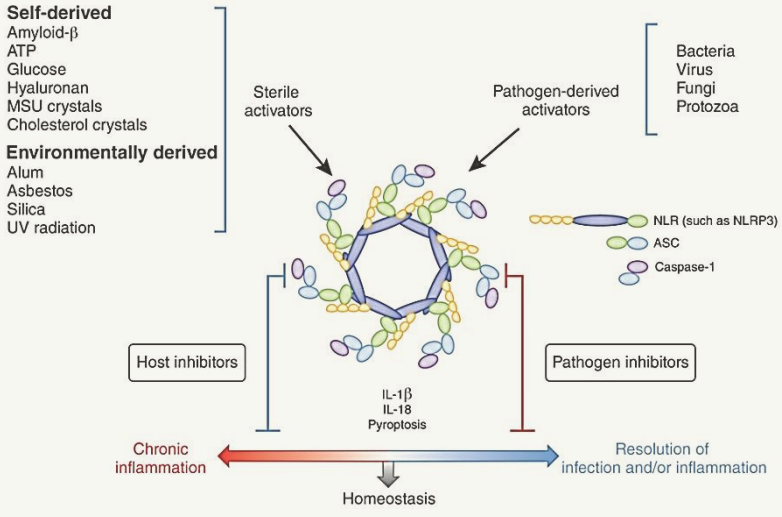
1.2. Activation and regulation

The caspase zymogens become active in response to several internal or external stimuli. The proteolytic processing of the pro-enzymes to form the active caspases occurs on the carboxyl side of Asp residues, resulting in the removal of the NH_2-terminal pro-domain and the formation of a heterotetramer composed of two large and two small subunits with two active sites, that seem to function independently, at opposite ends of each molecule (Figure 1) [10, 12]. Exceptions have been reported for DRONC from *Drosophila melanogaster* [63], the mammalian caspase-9 homolog, and sea bass caspase-1 (Chapter VI), where cleavage at a glutamate residue may also occur. The fact that caspases are activated by proteolytic processing at Asp residues and also cleave their substrates at Asp residues, suggests that caspases can auto-activate (by auto-proteolysis) or can be activated by enzymes with similar specificity.

The activation of caspases requires the formation of a heterotetramer composed by the association of two heterodimers of p20/p10 [10, 11, 29, 43]. The initiator caspases exist as inactive monomers and are activated by dimerization at specific molecular platforms by a process that does not requires the removal of the linker region [10, 11, 29, 43, 64, 65]. The proximity-induced dimerization mechanism explains the activation process of pro-caspase-9 at the apoptosome and pro-caspase-8 at the DISC [64, 65]. In response to a death stimulus, these caspases are activated by a mechanism that involves the assembly of a molecular platform and the association with a regulatory subunit and, subsequently, the recruitment of the initiator pro-caspases through their pro-domains, such as DEDs or CARDs. In this complex mechanism, the caspase is suggested to be activated through conformational change.

Box 4. Inflammasomes

The inflammasomes are multiprotein complexes with more than 700 kDa where caspase-1 is activated. The assembly of the inflammasomes is initiated upon recognition of several pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) by members of the NOD-like receptor (NLR) or the HIN-200 receptor families. The apoptosis-associated speck-like protein (ASC), which contains a caspase recruitment domain (CARD) and a pyrin domain (PYD), is an essential protein in the inflammasomes. The adaptor molecule ASC interacts with the NLR and with caspase-1 through the PYD and CARD, respectively. Several types of inflammasomes have been described, according to the receptor that recognises the activator molecule. In the case of the inflammasome that starts with NLRP3, which can be activated by UV-irradiation, microbial PAMPs or endogenous DAMPs, the PYD of the receptor NLRP3 interacts with the PYD of the ASC, which in turn allows the interaction of its CARD with the CARD of caspase-1, leading to its activation [24, 66, 67]. As a result of caspase-1 activation, the inflammasomes promote an inflammatory response through the activation of the pro-inflammatory cytokines, IL-1β and IL-18, and are involved in the induction of pyroptosis (Box 3). The inflammasomes, by inducing immune responses that limit pathogen progression, constitute an important intracellular defence mechanism against several pathogens, including bacteria, viruses, fungi and parasites. Therefore, it is not surprising that several successful pathogens have developed strategies to interfere with the activation of the inflammasomes, disarming the innate immune defences of the hosts [56, 68, 69]. Besides their central role in the innate immune responses, recent studies have implicated the inflammasomes in metabolic diseases and carcinogenesis, as well as in the regulation of the intestinal microbial ecology [56, 68, 69].



Adapted from [68]

The local concentration of the pro-caspase increases and a conformational change is induced, producing a microenvironment that allows the pro-caspase to aggregate and interact with specific adaptor proteins, like FADD/MORT1 or Apaf-1, leading to activation through proximity-induced dimerization [10, 11, 29, 43]. For this reason, initiators pro-caspase-8 or -9, which possess weak protease activity, when in high concentrations are able to cleave other pro-caspase-8/-9 molecules. As for the initiator caspases -8 and -9, caspase-1 is also activated by an identical dimerization mechanism in a multiprotein complex known as inflammasome (Box 4 and [10, 68]).

In contrast to the initiator caspases that exist as inactive monomers, the effector caspases exist in the cells as inactive dimers and need to be catalytically cleaved within their linker region between the large and small subunit for activation. The proteolytic processing and activation of effector caspases is mainly performed by previously activated initiator caspases, but also by other proteases that cleave theirs substrates at an aspartic acid, such as Granzyme B [70-72]. As a result, the tetrapeptides recognised by the initiator caspases normally match the sequences of the cleavage sites separating the large and small subunits of the zymogens of the effector caspases [10, 29, 43]. Thereby, the effector caspases do not need such a complex mechanism of activation as the initiator caspases [10, 11, 29, 43]. Therefore, caspases form a cascade of proteolytic activities in which the initiator caspases start the dead effector machinery and the effector caspases complete most of the proteolysis of critical substrates that will lead to apoptosis.

The accidental activation of a caspase could have a devastating effect and, for that reason, the process of activation must be tightly regulated. The regulation of caspases occurs at different levels, from gene expression to several steps of the caspase production, processing and activity. Upon a cell death signal, the activation of the apoptotic program is regulated by an accurate balance between the pro-apoptotic and anti-apoptotic mechanisms, in which the Bcl-2 family members (Box 5) play a crucial role [73]. The mitochondrial outer membrane permeabilization (MOMP) is an important mechanism that allows the release of several regulatory proteins from the intermembrane space of mitochondria, and is ensured by the pro-apoptotic members of Bcl-2 family, Bax and Bak (Box 5). One example of a released pro-apoptotic molecule is the apoptosis-inducing factor (AIF) that was reported to induce a caspase independent apoptosis-like cell death [74]. Under different apoptosis-inducing conditions, the mature AIF protein, normally localised in the mitochondrial intermembrane space, translocates through the outer mitochondrial membrane into the cytosol and into the nucleus, inducing nuclear chromatin condensation, as well as large scale DNA fragmentation [74]. Other examples of pro-apoptotic molecules that are released from the mitochondria are the Smac/Diablo, the serine protease Omi/Hrt2 and the endonuclease G [74-76]. The protein Smac/Diablo

is normally localised at the mitochondrial intermembrane space and upon an apoptotic stimuli is released to the cytosol where it can sequester and neutralise the anti-apoptotic effect of the inhibitors of apoptosis proteins (IAPs) allowing the activation of caspases to carry on [47, 77].

The extrinsic and intrinsic apoptotic pathways can be negatively regulated by IAPs, that act as competitive inhibitors that bind to the active site of caspases and thus, regulate their activation and activity [47, 78]. The most studied member of the IAP family is XIAP (X-chromosome-encoded inhibitor of apoptosis proteins) that contains three BIR (Baculoviral IAP Repeat) domains at the NH2-terminus. The BIR1 and 2 domains are involved in the inhibition of caspase-3 and -7 while caspase-9 activity is inhibited by the BIR3 domain [47, 79].

The most well characterised inhibitors of the extrinsic pathway are c-FLIPs (cellular FLICE inhibitory proteins). The c-FLIP family was originated by tandem duplication of caspase-8 and -10 genes having two DEDs as these caspases and thus, compete with the ligation of caspase-8 to the DEDs of the death receptors [80]. Therefore, the inhibition of caspase-8 activation by c-FLIPs results from a defective recruitment of caspase-8 to the DISC [81-83]. Although the inhibitory role of c-FLIPs has been widely documented, one of the isoforms, $FLIPE_L$, has shown to induce survival or death depending on its concentration levels [84].

Along with the regulation through different protein factors, the caspase activity can also be regulated by the presence of isoforms, which are products that derive from the full-length form by alternative splicing. For instance, one mechanism of caspase-3 regulation relies on caspase-3s, a short isoform of caspase-3 that is originated by alternative splicing [85]. This short version of caspase-3 lost the exon six, resulting in a complete absence of the short subunit and also of the 95 amino acids at the C-terminal of the large subunit, including the active centre. Caspase-3s was found to be expressed in several human tissues and has approximately 20 kDa. Caspase-3s competes with caspase-3 activation at the apoptosome complex and consequently, the formation of an efficient active caspase-3 is affected. When both caspases are expressed together in cells that received an apoptotic stimulus, the cleavage of PARP1 and the DNA fragmentation is prevented and therefore, the apoptotic process is compromised [85].

Human caspase-9 also has alternative variants that have an inhibitory role in the activation of caspase-9 [86, 87]. Caspase-9b is a small variant of caspase-9 traduced from a cDNA missing exons 3, 4, 5 and 6. This results in a protein that has the pro-domain and the small subunit intact but lacks most of the large subunit, including the catalytic site. *In vitro* binding assays demonstrated that caspase-9b inhibits caspase-9 activation by competing to the CARD-CARD interactions with Apaf-1-caspase-9 and thus, by

preventing the formation of a functional apoptosome [86, 87]. A caspase-9 isoform, Casp-9-Carboxyl-terminal divergent (CTD), was also identified in rat and CTD was shown to have an alternative peptide sequence replacing the C-terminal of the full-length caspase-9. Like the previous discussed caspase isoforms, CTD is expressed in several tissues and was implicated in the regulation of cell death, since it interferes with the activation of caspase-9 [88].

Another important mechanism for regulating the caspase-8 is the multiple splicing of caspase-8 gene. Caspase-8 co-expressed with caspase-8 isoforms possessing incomplete caspase regions resulted in decreased cell death [89, 90]. For example, caspase-8L, modulates the caspase-8 activity by the homotypic binding to FADD. This isoform exerts its dominant effect by preventing the coupling of pro-caspases-8 and subsequent apoptosis by the receptor pathway [91].

In human, four additional isoforms of caspase-1 were identified: the isoform β and γ, lacking part of the pro-domain, and the isoforms δ and ε, where most of the pro-domain and the region containing the active site have been deleted [92]. Like the caspase isoforms described above, these isoforms may have a regulatory role in the activation process of caspase-1. For instance, in the isoform ε most of the pro-domain and all the p20 subunit were deleted, meaning that this isoform has only the p10 subunit with 19 different amino acids in the NH_2-terminus. This incomplete p10 prevents the formation of an active tetramer by competing with the p10 subunit of caspase-1 [92].

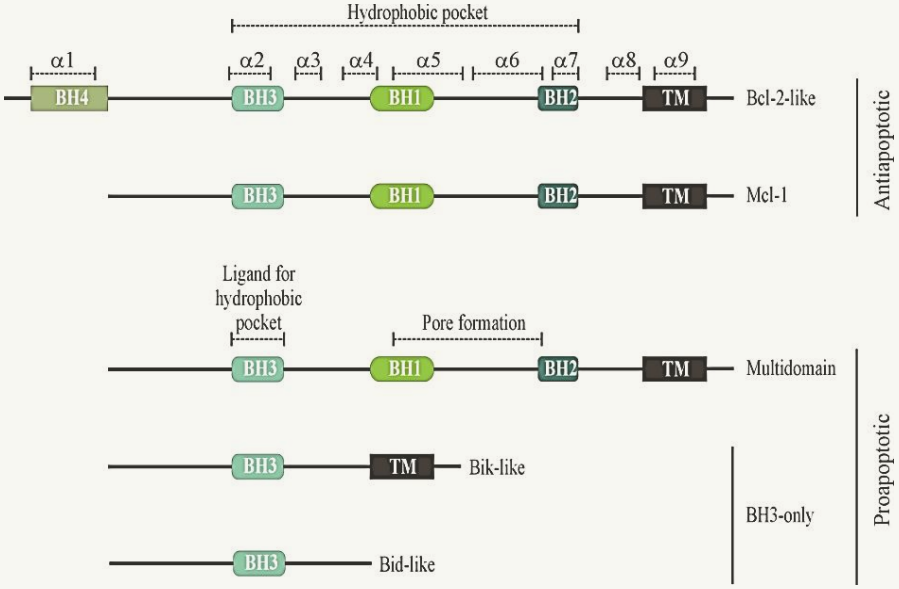
2. Fish caspases

A high conservation at the molecular and functional levels has been described between the players of the apoptotic machinery in invertebrates (*Caenorhabditis elegans* and *Drosophila*) and mammals [93, 94]. However, fish represent an excellent and advantageous model for the study of vertebrate development and disease [95-98], bridging the gap between the *C. elegans/Drosophila* and mouse/human models [99]. Moreover, contrary to *C. elegans* and *Drosophila*, fish can be used for studying the development and function of vertebrate-specific organs and have a fully developed immune system similar to that of mammals [100]. Despite the increasing interest in caspases and their evolutionarily conserved function, until the beginning of the 21st century, the information on caspases was limited to mammals (human and mouse), *Drosophila*, *C. elegans* and to a few reports on chicken [101], frog [102], and a few fish species [103-105]. Since then, the number of sequenced caspases from different species has been increasing. At the moment, 11 and 10 caspase genes were identified in the human and mouse genomes, respectively. In humans, the 11 genes encode caspase-1, -2, -3, -4, -5, -6, -7, -8, -9, -10 and -14. The mouse caspases are functional orthologs of

Box 5. Bcl-2 family

Bcl-2 family members have been extensively studied in mammalian systems, mainly due to their importance as key regulators of apoptosis, playing a central role in some pathological conditions, including cancer, neurodegenerative disorders and autoimmune diseases [45, 73, 106]. Briefly, Bcl-2 family members include proteins with sequence and structural similarity in the so-called Bcl-2 homology (BH1 to BH4) domains. Bcl-2 proteins regulate the response to diverse cytotoxic conditions such as UV- and γ-irradiation, anti-neoplastic drugs, virus and bacteria, but normally have limited impact on the signals induced by the death receptors of the TNF family [107]. Bcl-2 members can be anti-apoptotic (like Bcl-2 and Bcl-x_L) or pro-apoptotic (such as Bad, Bik, Bid, Bim). The anti-apoptotic members are mainly characterised by containing three to four BH domains and for inducing cell survival by sequestering/inhibiting pro-apoptotic scaffold or adaptor proteins and pro-apoptotic members needed for activation of caspases, and/or by maintaining organelle integrity [45, 73, 108]. Pro-apoptotic members can be subdivided into BH3-only proteins ("activators" and "de-repressors/facilitators"), which, as the name stands for, only contain the third BH domain (Bid, Bim, Bad) [109-111], or multidomain pro-apoptotic proteins (Bax and Bak). These share a high degree of structural similarity with pro-survival Bcl-2 family members, containing two to three BH domains, and all having the BH3 domain responsible for the pro-apoptotic activity [76, 108]. At least in some instances, cell death can be prevented at the mitochondrial level by anti-apoptotic Bcl-2 members which, depending on their relative abundance, can inhibit the release of apoptogenic molecules into the cytosol [108]. In addition, Bcl-2 family members are also involved in the regulation of several types of pro-apoptotic signals that are generated in organelles other than mitochondria, such as nucleus, endoplasmic reticulum and lysosomes [45].

That fish have functionally conserved Bcl-2 family members, similar to the ones found in mammals, has been shown by several lines of evidence. Fish have, in their genomes, homologs of virtually all mammalian Bcl-2 family members regulated by similar transcriptional and posttranslational mechanisms. Most of the data related with the Bcl-2 family members of fish refer to zebrafish although their presence in other fish species was also reported such as Bax and Bcl-$_{XL}$ in trout [112], Bcl-2 in gudgeon [113], and Bcl-2 and mlc-1 in carp [114]. Bcl-2 family members in other fish species can also be found in non-redundant and EST (Expressed Sequence Tags) public databases as well as genomic sequence databases (Table 2 in Annex 1). Several fish Bcl-2 family genes have two copies (Table 2 in Annex 1 and [115]) resulting from the early whole-genome duplication in the teleost lineage after the divergence from tetrapods about 450 million years ago [116-118].



human caspases and maintain the same names with the exception of caspase-11 and -12 that are functional orthologs of caspase-4 and -5, respectively. The other exception is caspase-10 which is absent in the mouse genome [6, 11].

2.1. Fish apoptotic initiator caspases

Caspase-8 (also known as FLICE/MACH1/Mch5) is an essential component of the extrinsic/death-receptor pathway (Box 2 and [23]). The identification and characterisation of caspase-8 from medaka, stickleback, zebrafish and sea bass (Chapter III and [119-121]) have recently been reported. At the genomic level, whereas zebrafish caspase-8 genes show a similar structure as their mammalian equivalents, being organised in 10 exons and 9 introns with coincident splice junctions sites [119, 122], the sea bass, medaka and stickleback caspase-8 genes present 12 exons and 11 introns, with the additional exons located at the first death effector and protease domains (Chapter III and [120]). Although results from Southern blot analysis suggested that the sea bass caspase-8 gene is a single copy gene (Chapter III), the caspase-8 gene is apparently duplicated in the genome of several species of fish (Table 2 in Annex 1). Chromosome mapping showed that the chromosomal segment around the zebrafish, medaka, fugu and stickleback caspase-8 genes has an organisation syntenic with that of other vertebrates [119, 120]. Fish caspase-8 molecules revealed about 35% identity and 55% similarity to the human counterpart, which together with the above data supports that fish caspase-8 genes are orthologs of mammalian caspase-8. As their amphibian and mammalian homologs, fish caspase-8 possess two DEDs at their NH_2-terminal pro-domains, and a carboxyl-terminal catalytic domain (CASc) including conserved amino acids essential for catalytic activity and the characteristic pentapeptide active site QACQG (QACRG in zebrafish caspase-8b) (Chapter III and [119, 120]). That zebrafish caspase-8 associates with the adaptor molecule FADD through homophilic DED interactions was shown by detecting zebrafish caspase-8 and human FADD in immunoprecipitates of HEK293T cells co-expressing HA-tagged human FADD with Flag-tagged zebrafish caspase-8 [119]. Additionally, exogenous expression of zebrafish caspase-8 in *caspase-8*-deficient mouse embryonic fibroblast (MEF) cells triggered cell death with or without ligation of Fas, the former showing an increased sensitivity, with 80% of the transfectants dying following Fas ligation. This indicates that zebrafish caspase-8 directly transmits apoptotic signals via Fas to downstream molecules, including, as shown in zebrafish caspase-8-expressing HeLa cells, caspase-3; the same holds true for TNFα induced-cell death [119]. Similarly, exogenous expression of medaka caspase-8 induced apoptotic death in transfected HeLa cells, but apoptotic death was inhibited by either zVAD-fmk, the anti-apoptotic cowpox virus serpin cytokine response modifier A (CrmA) [123], or the pan-caspase inhibitor p35

[120]. Furthermore, co-injection of zebrafish FLIP, or CrmA, reduced zebrafish caspase-8a-mediated embryonic mortality, confirming that caspase-8 functions similarly in zebrafish and mammals [124]. The long splice form of the extrinsic pathway inhibitor cellular FLIP (cFLIP) is a key regulator of the extrinsic pathway of caspase-8 activation, inhibiting the recruitment and activation of caspase-8 by death receptors [78, 82]. Like mammalian FLIP, which contains two DEDs that interact with the DED in FADD, zebrafish FLIP is structurally similar to caspase-8 and -10 and lacks an active-site cysteine [124]. Inhibition of caspase-8-mediated apoptosis in early zebrafish embryos has been also shown to occur through interactions of Yaf2 with the DED of caspase-8, with implications on cell survival during zebrafish embryogenesis [121]. Yaf2 is a member of the Rybp/Yaf2 protein family, with Rybp (also known as DEDAF) being described in mammals as interacting with DED-containing proteins, including FADD, caspase-8, caspase-10, and DEDD, for promoting Rybp pro-apoptotic activity [125]. Finally, induction of extensive apoptosis in zebrafish embryos injected with zebrafish caspase-8a mRNAs [124] as well as detection of caspase-8 transcription by RT-PCR in both maternal and zygotic transcripts during zebrafish embryonic development, with ubiquitous expression in whole embryos [119], suggested that caspase-8 plays an important role during embryonic development of fish embryos similarly to mammalian/amphibian embryos. Moreover, in sea bass treated with *Photobacterium damselae* ssp. *piscicida* (*Phdp*) culture supernatants (known to induce apoptosis of sea bass macrophages and neutrophils), the expression levels of caspase-8 in the spleens were increased (Chapter III). The involvement of sea bass caspase-8 in the *Phdp* infection model is further supported by the detection of caspase-8 activity in sea bass peritoneal phagocytes treated with recombinant AIP56 [126]. The above data confirm that, like its mammalian homolog [90, 127], fish caspase-8 is an initiator caspase recruited to the DISC via binding to the adaptor protein FADD, transmitting apoptotic signals from death receptors to downstream effector caspases; it can also mediate cross-talk between the extrinsic and intrinsic apoptotic pathways, since fish Bid sequences reveal conservation of the caspase-8 cleavage site.

Caspase-10, similarly to caspase-8, contains two DEDs and a CASc, and is involved in the Fas ligand-mediated cell death pathway [10, 47]. Both caspases are not clearly distinguished since, for instance, the difference in the activation process is not well studied in detail [128]. Recent studies demonstrated that caspase-10 inhibition prevents apoptosis mediated by the TRAIL-R2 receptor in human immune cells, proving that caspase-10 has an important role in apoptosis mediated by this receptor [129]. Despite *caspase-10*-like genes with high degree of similarity to human *caspase-10* have been reported for fish and can be found in the databases (Table 2 in Annex 1), the only

characterisation of a fish caspase-10 belongs to the Japanese flounder [130]. The gene has 6.6 kb and is organised in 11 exons and 10 introns similar to the human caspase-10. Additionally, the sequence conserves characteristic features such as the DEDs, the large and small subunits and the pentapeptide active site (QACQG), suggesting that it may have a similar function as the human caspase-10. Although the sequence of Japanese flounder caspase-10 revealed 35.3% and 32.6% similarity to human caspase-8 and -10, respectively, the phylogenetic tree clusters the fish caspase-10 with other caspase-10 sequences. The Japanese flounder caspase-10 mRNA was found to be expressed in several tissues and an over-expression in the cell line HINAE resulted in the induction of apoptosis [130]. Since, in addition to caspase-8 and caspase-10, other components essential for forming the DISC, as well as death ligands and the BH3-only member Bid, have been identified in fish (Annex 1), these data strongly point to the existence of a structural and functional conserved extrinsic/death receptor apoptotic pathway in vertebrates.

Although caspase-2 was one of the first caspases to be discovered and has been implicated in the regulation of cell cycle progression [131], its role in apoptosis remains a matter of considerable debate [131]. Caspase-2 genes have been identified in fish (Table 2 in Annex 1 and [99, 124]), but no detailed structural and functional characterisation of a fish caspase-2 has been reported so far. The fact that fish caspase-2 gene presents a conserved synteny with the human ortholog and that fish caspase-2 contains a CARD in its pro-domain (Figure 2), suggests that this protease plays a similar role in the apoptotic process of fish and mammals.

Caspase-9 (also known as ICE-LAP6/MCH-6/Apaf-3) is a member of the CED-3 subfamily, bearing high similarity to caspase-3, and is a key component of the intrinsic/mitochondrial death-pathway (Box 2 and [23, 132, 133]). *Caspase-9* genes have been identified in some species of fish (Table 2 in Annex 1), and the primary structures of sea bass (Chapter IV), and large yellow croaker [134] caspase-9 were characterised. BLAST analysis revealed homology between fish caspase-9 and their vertebrate equivalents, and, as expected, fish caspase-9 presented higher similarity (>90%) and identity (>84%) with other fish counterparts than with that of non-fish vertebrates (46-48% identity and 59-64% similarity). Sea bass and large yellow croacker *caspase-9* genes are organised in 10 exons and 9 introns, which, when compared with the human homolog, represents an extra exon located in the predicted pro-domain (Chapter IV and [134]). Southern blot analysis of sea bass genomic DNA suggested that, in fish, *caspase-9* gene exists as a single copy gene, an hypothesis that is supported by the fact that only a single *caspase-9* gene has been identified in other fish species (Table 2 in Annex 1 and [124]). Additionally, fish caspase-9 genes likely preserve a conserved synteny with the human

ortholog, as indicated by the reported analysis of the zebrafish *caspase-9* [124]. Moreover, fish caspase-9 conserves characteristic caspase-9 features (Figure 2) [10] such as a CARD in the NH_2-terminal pro-domain, a large (p20) and small (p10) subunits, a

caspase family signature, and all important catalytic residues, including the active site pentapeptide QACGG (Table 2 in Annex 1). The presence of CARD in fish caspase-9 is relevant, taking into account that fish apoptotic protease-activating factor 1 (Apaf-1) was identified and contains CARD, a nucleotide-binding oligomerization domain (NO) and WD-40 repeats (Table 2 in Annex 1 and [99]). This suggests that, like mammals [135], fish might regulate caspase-9 activity in an Apaf-1/cytochrome *c*-dependent manner within the apoptosome context [75, 136]. In addition, a homolog of Nod1, an Apaf-1-like molecule that regulates apoptosis and NF-κB activation [137, 138], was found in zebrafish [99]. As with the mammalian equivalents [47, 78, 132, 139], further regulation of fish caspase-9 may be also achieved through inhibition by IAPs, as both IAP and XIAP homologs have been identified in fish (Table 2 in Annex 1 and [99, 124]). Structural studies suggest that the NH_2-terminal BIR2 linker region of XIAP occupies the catalytic site in active caspase-3 and -7, whereas the XIAP BIR3 domain is required for inhibiting caspase-9 [47, 78, 132, 139].

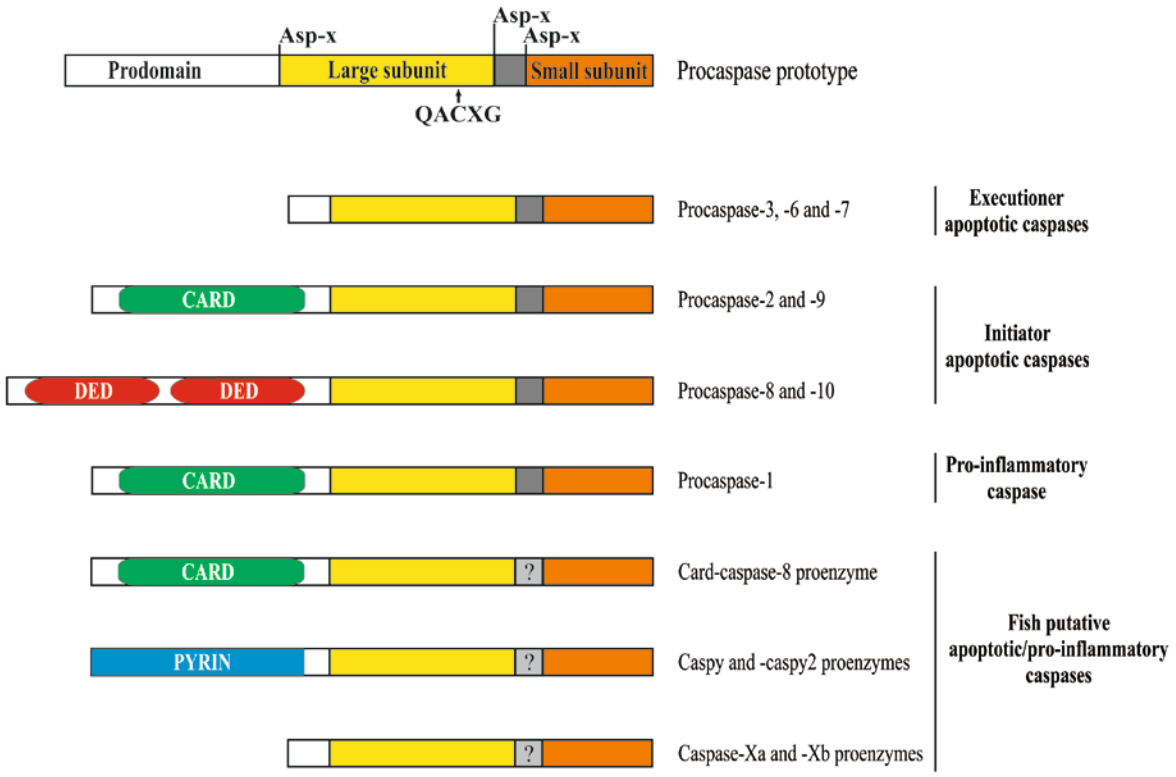


Figure 2. Fish apoptotic and inflammatory caspases. Diagrammatic representation of the domain architecture of the executioner and initiator apoptotic caspases, pro-inflammatory caspase-1 and putative apoptotic/pro-inflammatory caspase pro-enzymes of fish.

The identification of Smac/DIABLO homologs and several serine proteases highly similar to HtrA2/Omi in fish (Table 2 in Annex 1) also indicates that the inhibitory effect of IAPs may be neutralised in a similar way as in mammals, i.e., during apoptotic signalling, Smac/DIABLO and HtrA2 are released from the mitochondria into the cytoplasm where they bind to XIAP and prevent its inhibitory activity [47, 78, 132, 139]. Indications of caspase-9 function and involvement in apoptosis in fish were obtained by detection of caspase-9 transcription by RT-PCR in head kidney of sea bass infected with the apoptotic-inducing bacteria *Phdp*. The increased expression of caspase-9 correlated with the presence of apoptotic cells and putative caspase-9 activity in the head kidney, the latest detected by a luminescence assay using a proluminescent substrate containing the caspase-9 specific cleavage tetrapeptide LEHD (Chapter IV). Recent studies in large yellow croaker suggested that the intrinsic apoptotic pathway might be implicated in the immune response in spleens and kidneys stimulated with poly(I:C), since the levels of caspase-9 expression and the enzyme activity increase in these conditions [134]. Taken together, the above data represent strong evidence that apoptotic processes involving caspase-9 as well as the mechanisms of caspase-9 regulation are evolutionarily conserved among vertebrates, pointing to the existence of a conserved functional intrinsic apoptotic pathway in vertebrates.

2.2. Fish apoptotic executioner caspases

Caspase-3 is the main downstream effector caspase that, once activated by initiator caspases, except caspase-2 [10, 132, 140], cleaves the majority of the cellular substrates in apoptosing cells [10, 132, 140]. Caspase-3 is the most well characterised caspase in fish, including zebrafish [103, 141], salmon [142], sea bass (Chapter V) and large yellow croaker [143]. BLAST search revealed a close relation between fish caspase-3 and the caspase-3 molecules of other vertebrates, which is supported by the relatively high percentage of amino acid identities (50-80%) (Chapter V and [103]). Fish *caspase-3* presents smaller introns than the human equivalent, and presents a 6 exon/5 intron (7 exon/6 intron in zebrafish) structure, while the human gene has an 8 exon/7 intron structure. However, they all conserve an identical gene organization within the coding region. Although Southern blotting has suggested that sea bass *caspase-3* is a single copy gene (Chapter V), it is likely that it is present in all fish genomes as a duplicate gene, as two *caspase-3* genes have been identified in other fish species (Table 2 in Annex 1). Whether the duplicates have acquired distinct spatiotemporal functions requires further investigation. As their mammalian homologs, fish caspase-3 present a short NH_2-terminal pro-domain and a catalytic domain containing both caspase p20 and p10 subunits, a

caspase family signature and all important catalytic residues, including the characteristic pentapeptide active-site QACRG (Figure 2) (Chapter V and [103, 142, 143]). The apoptotic function of fish caspase-3 has been widely demonstrated. Thus, high proteolytic activity towards the mammalian caspase-3 and -7 substrate Ac-DEVD-MCA was reported for the recombinant zebrafish caspase-3, whereas it had low activity against the caspase-1, caspase-6/ -8, and caspase-9 substrates (Ac-YVAD-MCA, Ac-IETD-MCA and Ac-LEHD-MCA, respectively), indicating a similar substrate specificity to that of mammalian caspase-3 [103]. Moreover, overexpression of the zebrafish caspase-3 in cultured fish cells and zebrafish embryos induced apoptosis that could be repressed by the inhibitor of mammalian caspase-3 Z-DEVD-FMK [103]. Zebrafish embryos microinjected with a zebrafish caspase-3 expression vector also had high Ac-DEVD-MCA-hydrolysing activity. In the same study, Yabu et al also showed that the expression of the zebrafish *caspase-3* gene was regulated in both a tissue- and stage-specific manner, with caspase-3 mRNA from maternal origin being present in early embryogenesis and after gastrulation by zygotic genome activation, especially in the pectoral fin bud, optic vesicle and hindbrain [103]. Finally, an increase of caspase-3 expression was observed in the head kidney of sea bass infected with the apoptotic-inducing bacteria *Phdp*, which was accompanied by the occurrence, in that organ, of cells with apoptotic morphology and activated caspase-3 (Chapter V). In the large yellow croaker it was shown that caspase-3 expression and activity are increased in spleen and kidney stimulated with poly(I:C) [134, 143].

Caspase-7 is highly similar to caspase-3 (Figure 2) and has similar substrate specificity [10, 132]. However, at least for apoptosis induced via the intrinsic pathway, a combined function of caspase-3 and -7 is apparently required for cell death execution [10, 132, 144]. Caspase-7 has been identified in several fish species as a duplicated gene (Table 2 in Annex 1) and its primary structure has been recently characterised in salmon [142]. The caspase-7 gene from salmon is organised in a 5 exon/4 intron structure, differing from the mouse and human counterparts, which have a 6 exon/5 intron structure [142]. *Caspase-7* mRNA was exclusively detected by whole-mount *in situ* hybridization in the lens of salmon embryos, while *caspase-3b*, *caspase-6a* and *-6b* were expressed in multiple organs of heat-stressed and control salmon embryos. Moreover, cell death induced in heat-stressed salmon embryos could be correlated with the expression of caspase-7, as well as caspase-6 and -3, in a similar way as in mouse embryos [142].

Although structurally similar to caspase-3 and -7, caspase-6 (Figure 2) has a somewhat different substrate specificity, its optimal substrate being VEHD rather than DEVD, the preferred caspase-3 and -7 substrate [145]. Caspase-6 was demonstrated to have an important role in neurodegenerative diseases, such as Huntington and Alzheimer diseases, through the involvement in neuronal degeneration and death [146]. In fish, it has

been characterised in trout [104] and salmon [142], being identified as a duplicate [142] or even triplicate [124] gene (Table 2 in Annex 1). As caspase-3 and -7, fish caspase-6a and -6b have a relatively short NH_2-terminal pro-domain followed by a catalytic C-terminal domain comprising the large and small subunits, the caspase family signature as well as the amino acids involved in catalysis, including the QACRG catalytic site, and those forming the P1 carboxylate binding pocket. Caspase-6a and -6b share high identity (98% amino acid identity between salmon caspase-6a and -6b) as well as high identity with other vertebrate counterparts (>60% amino acid identity). At genomic level, salmon *caspase-6a* presents a 7 exon/6 intron organization, as its human homolog, whereas the insertion of an intron within the first exon generated an additional exon in the *caspase-6b* gene, similar to the chicken *caspase-6* gene [142]. In human *caspase-6*, intron 3 is involved in activation by binding of the pro-apoptotic transcription factor p53 [147]. However, as hypothesised by Takle et al [142] for salmon, the different genomic structure of *caspase-6a* and *-6b* may cause different regulation of the two genes that may be worth investigating. RT-PCR analysis revealed that the trout *caspase-6* gene is expressed in brain, blood, gill, liver, head kidney and spleen [104]. In salmon, caspase-6a and -6b were also expressed in multiple tissues of heat-exposed and control embryos, although cardiac expression of caspase-6a and -6b was only observed in heart of the heat-stressed embryos, with an apparent correlation with cell death [142]. Trout caspase-6 expression in head kidney leukocytes could be specifically increased by addition of LPS, although only in the presence of and following a pre-incubation with cortisol [104]. This effect was mediated by glucocorticoid receptors, as incubation with the glucocorticoid receptor blocker RU486 abrogated the expression of caspase-6 in LPS-stimulated trout leukocytes. Moreover, a marked up-regulation of caspase-6 expression in the trout head kidney was observed upon *in vivo* induction of confinement stress. Although both glucocorticoids and stress are regarded as inducers of apoptosis in fish [148, 149], no correlation of the observed induction of caspase-6 with cell death was reported by Laing et al [104]. The above data clearly indicate that fish possess a caspase cascade, similar to that characterised in mammals.

2.3. Fish putative apoptotic caspases

A caspase-8-like gene, showing high similarity to human and other vertebrate *caspase-8* and *caspase-10* sequences, but possessing a CARD at their NH_2-terminal pro-domains instead of DEDs (and, therefore, tentatively named *card-caspase-8*), has been reported in fish (Figure 2) [105, 119, 120]. The *card-caspase-8* is not a third *caspase-8/-10* gene as the one identified in the frog and chicken genomes; instead, it apparently results from replacement of its DED by a CARD upon a tandem duplication of the

ancestral *caspase-8* gene over the course of evolution [119, 120]. In addition to the CARD in its pro-domain, card-caspase-8 contains the caspase family signature, the active catalytic site, and the large (p20) and small (p10) subunits at the CASc. Moreover, card-caspase-8 exhibits pro-apoptotic activity [105, 120]. However, it would be interesting to find out whether fish card-caspase-8 is activated by interacting with death receptors via a CARD-containing adapter molecule such as RAIDD or by recruitment to large multiprotein complexes (as caspase-2) [10, 131, 132], or by heterotypic interaction (DED-CARD) with FADD. Other caspases mostly homologous to mammalian caspase-8 (zebrafish caspase-Xa and -Xb), but containing a QACRG active site and lacking obvious NH_2-terminal DEDs (Figure 2), have also been reported (Table 2 in Annex 1 and [142]).

2.4. Fish inflammatory caspases

Caspase-1, also known as ICE (interleukin-1β converting enzyme), is an inflammatory caspase that normally exists in the cytoplasm and is involved in inflammatory processes and in pyroptosis (Box 3) [68, 150]. As previously mentioned, caspase-1 was the first cysteine protease of the caspase family to be identified and it was also the first in which the three-dimensional structure has been solved, revealing that active caspases are heterotetramers originated from the inactive zymogens [151, 152]. The first caspase-1 homologue identified in fish was the sea bass caspase-1 (Chapter VI) followed by the caspase-1 of sea bream [153]. Both fish caspase-1 retain the CARD in the NH_2-terminal pro-domain and the caspase catalytic domain as their mammalian counterpart (Chapter VI and [153]), indicating that a strikingly conserved structure has been kept throughout evolution. In addition, the sea bass recombinant caspase-1 (Chapter VI) is auto-catalytically converted *in vitro* into two active heterodimeric complexes consisting of p10 paired either with p24 or p20 as described for human caspase-1 [3, 154-156]. Similarly to what has been described for human caspase-1, alternative splicing isoforms were also found in sea bass, all containing the pro- and p20 domains, including the fully conserved active site QACRG sequence. The differences introduced by the alternative splicing events in the sea bass caspase-1 isoforms are all located downstream from the active site (Chapter VI), contrary to some of the human isoforms [92]. A regulatory role in the activation of caspase-1 have been described for proteins containing CARD, such as COP, INCA, ICEBERG and caspase-12, that compete through CARD-CARD interactions for the activation of caspase-1 at the inflammasome complex [67]. All the sea bass caspase-1 isoforms kept the CARD at the pro-domain and thereby could play a role in the regulation of the inflammatory response. The gilthead sea bream caspase-1 is constitutively expressed in some immune tissues and the use of a caspase-1 specific inhibitor reduces the endogenous caspase-1 activity in the sea bream

fibroblast cell line SAF-1 and in primary leukocytes [153]. Additionally, it was reported that processing of IL-1β by a gilthead sea bream fibroblast cell line is abrogated by a specific caspase-1 inhibitor [157]. However, until recently, the recognition site for caspase-1 processing was missing in the IL-1β molecules reported so far in non-mammalian vertebrates, including fish, and the ability of non-mammalian inflammatory caspases to process IL-1β has been a matter of debate. In the present thesis (Chapter VI) it was shown that sea bass caspase-1 cleaves sea bass IL-1β at the aspartate residue 100, which is absolutely conserved in all IL-1β sequences reported so far (Figure S3 in Chapter VI). This could suggest that this aspartate residue would be the non-mammalian caspase-1 cleavage site. However, analysis of the *in vitro* cleavage of avian IL-1β using human and sea bass caspase-1 suggests that avian IL-1β is cleaved at aspartate residue 80 present in a IL-1β stretch where aspartates are also well conserved in most of the IL-1β sequences reported so far (Chapter VI). Thus, it is very likely that IL-1β cleavage is a class-specific, or perhaps even a species-specific event, and that mammalian IL-1βs have acquired a specific caspase-1 cleavage site during phylogeny.

Two pyrin domain-containing caspases (Figure 2), caspy and caspy2, as well as the zebrafish ortholog of apoptosis-associated speck-like protein containing CARD (ASC), were also identified in zebrafish [158]. Whether these caspase molecules play initiator or executioner roles in apoptosis is not clear, although the presence of long pro-domains and, at least for caspy, the need for oligomerization for its activity, mostly resemble initiator caspases. Both caspy and caspy2 contain long NH_2-terminal pyrin domains [159] and a C-terminal caspase catalytic domain, the latest including conserved amino acids residues essential for proteolytic catalysis, and residues essential for interaction with critical core residues of substrates, as well as the pentapeptide active site QACRG. Whereas the NH_2-terminal pyrin domain of caspy and caspy2 were most homologous to that present in zebrafish ASC (87% similarity) and to that of human pyrin-domain containing Nod-family protein Cryopyrin/PYPAF1 (46% similarity), respectively, the catalytic domains shared closer similarity with that of human caspase-1 and -5, respectively [158]. Additionally, the pyrin domain of caspy2 exhibits homology not only to pyrin domains but also to the CARDs of *Xenopus* caspase-1 (49% similarity) and bovine caspase-13 (45% similarity) [158]. As its mammalian counterparts, zebrafish ASC is composed of an NH_2-terminal pyrin domain linked to a C-terminal CARD [158]. Functionally, both caspy and caspy2 induced apoptosis when transfected into mammalian cells, an effect attenuated by p35 and inhibited by zVAD-fmk, whereas only caspy was inhibited by the human caspase-1 inhibitor CrmA. Moreover, caspy and caspy2 preferentially cleave the caspase-1 substrate AcYVAD-AMC or the caspase-5 substrate AcWEHD-AFC, respectively [158], suggesting that both are active enzymes with different

substrate specificities. Finally, co-immunoprecipitation and expression assays showed that only caspy interacts with zebrafish ASC through a homophilic pyrin-pyrin domain interaction, involving the same speck [158]. However, proteolytic processing of caspy and induction of caspy-dependent apoptosis can only be achieved by enforced oligomerization of zebrafish ASC, which is presumably induced through an interaction between the C-terminal CARD of zebrafish ASC and a putative upstream regulator yet to be identified, therefore indicating that ASC is an adaptor molecule [158]. Finally, morpholino antisense oligonucleotide-based knockdown of caspy in zebrafish embryos was shown to affect development of the jaw and posterior pharyngeal arches with no other abnormalities identified in any other part of the body, suggesting that caspy is involved in the execution of a program of neural crest cell death required for proper developmental and assembly of cartilage [158]. However, it cannot be ruled out that caspy is involved in processing the zebrafish IL-1/IL-18 and/or another factor that is required for normal development of pharyngeal arch cartilages [158]. So far, the mammalian caspases that play similar roles to those played by caspy and caspy2 in fish have not been identified.

As a concluding remark, there is compelling evidence that fish do contain functional homologs of virtually all members involved in the apoptotic machinery (Table 2 in Annex 1), making fish excellent models for studying apoptosis-related genetic or infectious disorders as well as for drug development.

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CHAPTER II

Project Aims

Project Aims

Over the years, the idea that caspases are important proteins, playing a relevant role in inflammation and apoptosis, kept the attention of the scientific community. Additionally, recent discoveries attributed to caspases several non-apoptotic functions and, consequently, highlighted the significance of caspases and the interest in this cysteine protease family. In mammals, the structural features of caspases, along with their activation and regulation mechanisms are widely understood. However, novel interactions of caspases with other proteins and new aspects of their regulation and participation in different acting fields are constantly being discovered, raising unprecedented questions and making this a very challenging and fascinating area of research.

As reviewed in the introduction section, fish possess caspases and many studies point to an evolutionary conserved regulation of caspases and to the existence, in fish, of apoptotic and inflammatory processes similar to the ones described in mammals. Nevertheless, when the work for this thesis started, the knowledge about fish caspases was still very insipient, with only few fish caspases known and characterised. This work was developed at the Fish Immunology and Vaccinology group of the IBMC that, during the last years, has been using sea bass to study the *Photobacterium damselae* ssp. *piscicida* pathogenicity mechanism as an example of a bacterial infection where the pathogen subverts the apoptotic and pro-inflammatory machineries of the host. This thesis was developed in this context, aiming at cloning, sequencing and genetically characterise sea bass caspases representing all functional classes. Therefore, the main objectives of the present thesis were:

- To clone, sequence and genetically characterise sea bass caspases representing all functional classes, including the apoptogenic caspase-8, -9 and -3, as well as the pro-inflammatory caspase-1.
- To use the information obtained from the above studies to follow the involvement of those caspases in physiological and/or immunogenic/pathological processes at genomic and proteomic level.

CHAPTER III

Molecular cloning of sea bass (*Dicentrarchus labrax* L.) caspase-8 gene and its involvement in *Photobacterium damselae* ssp. *piscicida* triggered apoptosis.

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ABSTRACT

Caspase-8 is an initiator caspase that plays a crucial role in some cases of apoptosis by extrinsic and intrinsic pathways. Caspase-8 structure and function have been extensively studied in mammals, but in fish the characterization of that initiator caspase is still scarce. In this work, the sea bass counterpart of mammalian caspase-8 was sequenced and characterized, and its involvement in the apoptogenic activity of a toxin from a fish pathogen was assessed. A 2472 bp cDNA of sea bass caspase-8 was obtained, consisting of 1455 bp open reading frame coding for 484 amino acids and with a predicted molecular weight of 55.2 kDa. The sea bass caspase-8 gene has 6639 bp and is organized in 11 introns and 12 exons. Several distinctive features of sea bass caspase-8 were identified, which include two death effector domains, the caspase family domains p20 and p10, the caspase-8 active-site pentapeptide and potential aspartic acid cleavage sites. The sea bass caspase-8 sequence revealed a significant degree of similarity to corresponding sequences from several vertebrate taxonomic groups. A low expression of sea bass caspase-8 was detected in various tissues of non-stimulated sea bass. Furthermore, it is shown that stimulation of sea bass with mid-exponential phase culture supernatants from *Photobacterium damselae* ssp. *piscicida* (*Phdp*), known to induce selective apoptosis of macrophages and neutrophils, resulted in an increased expression of caspase-8 in the spleen, one of the main affected organs by *Phdp* infection.
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1. Introduction

Caspases are cysteine proteases playing a central role in apoptosis and in the proteolytic processing of pro-inflammatory cytokines. Functionally, caspases can be classified as upstream initiator apoptotic caspases (caspases-2, -8, -9 and -10), downstream executioner or effector apoptotic caspases (caspases-3, -6 and -7) and pro-inflammatory caspases (caspase-1, -4, -5, -11 and -12) whose main role lies in cytokine maturation rather than apoptotic activity (reviewed in [1]). Caspase-8 is an initiator caspase involved in the early steps of apoptosis by the receptor pathway triggered by Fas, TNFR1, and related death receptors of the TNF superfamily (reviewed in [1]). Once activated, caspase-8 is able to directly activate, by proteolytic cleavage, downstream effector caspases such as procaspases-3 and -7, leading to the cell dismantling and death. Caspase-8 can also amplify the apoptotic signal through an indirect pathway involving the cleavage and activation of BID [2]. Truncated BID translocates to the mitochondria, triggering the release of cytochrome c into the cytosol where it will lead to the activation of Apaf-1/caspase-9 apoptosome [3].

Besides the role of caspase-8 in apoptotic death signalling, in recent years, several non-apoptotic functions of caspase-8 have been reported (reviewed in [4]). Caspase-8 was found to play a non-apoptotic role in embryonic development, a critical role during monocyte differentiation into macrophages and in the regulation of T cell development (reviewed in [4]). The emerging roles of caspase-8 show the importance of this molecule and its participation in the balance between survival and apoptotic cell death. Caspase-8 structure and function have been extensively studied in mammals, but information about the role of caspase-8 in fish remains scarce. Given the importance of fish research to support the much needed development of aquaculture and to study diverse mammalian normal and pathological issues, and given the participation of apoptosis in development, homeostasis and in many pathologies of vertebrates (reviewed in [5,6]), research on fish apoptosis is of obvious relevance as recently discussed [7,8]. In this work, we sequenced and characterized the sea bass caspase-8 gene and studied the expression of caspase-8 in organs of resting and stimulated fish.

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Table 1
Primers used in this study.

Primer	Sequence[a]	Application
AUAP2	5′-GACTCAGGACTTCAGGACTTAG-3′	cDNA amplification
APv2	5′-GACTCAGGACTTCAGGACTTAG(T_{17})(AGC)-3′	cDNA synthesis
T7	5′-TAATACGACTCACTATAGGGCGA-3′	cDNA sequencing
SP6	5′-CTATTTAGGTGACACTATAGAATAC-3′	cDNA sequencing
CASP8FW3	5′-GATGTHTTKRYTGARATGGA-3′	cDNA amplification
CASP8FW4	5′-GTCTGTGTSCTYTCMCACGG-3′	cDNA amplification
CASP8RV3	5′-GTMTAVTTGGGYTCHGGCAT-3′	cDNA amplification
DLCASP8FW1	5′-GTCTCTCTTCCTCTGAAAGT-3′	cDNA amplification
DLCASP8FW2	5′-GGAGCTATGTGAAGGAGTGT-3′	cDNA and genomic amplification
DLCASP8FW3	5′-CACCTACAATGCGGCTGTAA-3′	Genomic amplification
DLCASP8FW4	5′-CAGCCAACAGGTTCCTGTAT-3′	Genomic sequencing
DLCASP8FW5	5′-CACATCCAAGGGATGTAAGT-3′	Genomic sequencing
DLCASP8FW6	5′-GATGATGATGCCTTGGTGGT-3′	Probe amplification; expression
DLCASP8RV1	5′-GACATGGCACATATCCTCTC-3′	cDNA amplification
DLCASP8RV2	5′-GTGAAGGGCTTTGTCACATC-3′	cDNA amplification
DLCASP8RV5	5′-CCTCTGTATGTAGCGCTGTA-3′	cDNA amplification
DLCASP8RV6	5′-AGTCAGTGCCTGCTGTTGAT-3′	cDNA and genomic amplification
DLCASP8RV7	5′-TAAGTCCAACTCTATGATAA-3′	Genomic amplification
DLCASP8RV9	5′-TGCAATCCTGCCACACTGTT-3′	Genomic sequencing
DLCASP8RV10	5′-AGGACGAGCTTCTTGGTGAG-3′	cDNA and genomic sequencing
DLCASP8RV11	5′-TCATCCCGCTCCGAGCTTTC-3′	Probe amplification; expression
DLBACTINFW	5′-ATCGTGGGGCGCCCCAGG-3′	Expression
DLBACTINRV	5′-CTCCTTAATGTCACGCACGATTTC-3′	Expression
DLBACTINFW2	5′-GGCCAGAAGGACAGCTACGTT-3′	Expression
DLBACTINRV2	5′-AGCCACGCTCTGTCAGGATCT-3′	Expression

[a] M = A/C; Y = C/T; S = C/G; V = GCT; H = AGT; R = A/G; K = G/T.

2. Materials and methods

2.1. Fish

Sea bass were obtained from a local fish farm and kept in a recirculating, UV-treated salt-water (30–35%) system at 20 ± 1 °C, and fed at a ratio of 2% body weight per day. For collection of organs fish were euthanized with a lethal dose of 2-phenoxyethanol (Panreac; >5 ml/10 L).

2.2. Cloning and sequencing of the cDNA

Sea bass were inoculated intraperitoneally with the Apoptosis Inducing Protein of 56 kDa (AIP56), an exotoxin produced by *Photobacterium damselae* ssp. *piscicida* (*Phdp*) that induces apoptosis in sea bass macrophages and neutrophils [9]. Head kidneys from 3 fishes were collected 3 h after AIP56 inoculation. The total RNA from head kidneys was extracted according to MicroPoly(A)Pure™ (Ambion®). The first-strand cDNA synthesis was performed according to the BioScript RNase H Minus (BIOLINE), using approximately 500 ng of total RNA as the template and the adaptor/anchor primer APv2 (Table 1, Fig. 1) at 42 °C for 60 min.

Degenerate primers, CASP8FW4 and CASP8RV3 (Table 1, Fig. 1), based on conserved regions obtained by multiple alignment (CLUSTAL W) of caspase-8 amino acids sequences from different vertebrates (*Homo sapiens*, *Rattus norvegicus*, *Xenopus laevis*, *Gallus gallus*, *Takifugu rubripes*, *Danio rerio*, *Ictalurus punctatus*, *Tetraodon* sp., *Oryzias latipes*) were used in initial PCR amplification of the cDNA (2 μl) in a 50 μl reaction (1.5 mM $MgCl_2$, 5 μl 10× PCR buffer, 40 mM dNTP mix, 0.4 μM of each primer and 1.25 units of Taq DNA polymerase) with the following conditions: 1 cycle of 94 °C for 2 min; 35 cycles of amplification (94 °C for 45 s; 56 °C for 1 min; 72 °C for 30 s); and 1 cycle of 72 °C for 5 min. A PCR product (~500 bp) were purified (Gel extraction Kit, Qiagen), ligated overnight at 4 °C and used to transform Escherichia coli XL-1 competent cells according to the pGEM-T Easy Vector Systems (Promega). The transformants were plated on LB/Agar medium containing ampicillin (200 μg/ml) and IPTG/X-GAL (50 μg/ml), and incubated at 37 °C overnight. The plasmid DNA (200–300 ng) of one positive colony was extracted (QIAprep Spin Miniprep Kit, Qiagen) and sequenced at MWG (www.mwg-biotech.com) using the vector primers T7 and SP6 (Table 1).

Based on the partial caspase-8 sequence obtained, several sea bass specific primers (so-called DLCASP8) were designed (Table 1, Fig. 1) and used to move upwards to the 5′ end. The degenerate primer CASP8FW3 and the specific primer DLCASP8RV1 were used in a first PCR amplification of the same cDNA (2 μl) used to obtain the first fragment in 50 μl reaction (1.5 mM $MgCl_2$, 5 μl 10× PCR buffer, 40 mM dNTP mix, 0.4 μM of each primer and 1.25 units of Taq DNA polymerase) with the following conditions: 1 cycle of 94 °C for 2 min; 30 cycles of amplification (94 °C for 45 s; 52 °C for 1 min; 72 °C for 1 min); and 1 cycle of 72 °C for 5 min. A semi-nested PCR was performed with the specific primer DLCASP8RV2, following identical PCR conditions used in the first amplification reaction. A PCR product (~600 bp) was purified, cloned and sequenced as described above. Specific reverse primers (DLCASP8RV5 and DLCASP8RV6, Table 1, Fig. 1) were designed based on the sequenced fragment and used for reaching the 5′-UTR following a 5′ RACE System Kit (Invitrogen). The PCR product was purified, cloned and sequenced as previously described.

Full-length caspase-8 cDNA was obtained after two amplifications of the same cDNA mentioned above and with specific forward primers designed in the 5′-UTR (DLCASP8FW1 and DLCASP8FW2, respectively) and the anchored primer AUAP2 (Table 1, Fig. 1) in a 50 μl reaction (1.5 mM $MgCl_2$, 5 μl 10× PCR buffer, 40 mM dNTP mix, 0.4 μM of each primer and 1.25 units of Taq DNA polymerase) with the following conditions: 1 cycle of 94 °C for 2 min; 35 cycles of amplification (94 °C for 45 s; 57 °C for 30 s; 72 °C for 1 min 30 s; and 1 cycle of 72 °C for 5 min). The PCR product was cloned using the same strategy as describe above. The plasmid DNA of three positive clones was extracted and sequenced as describe above with primers T7, SP6 and DLCASP8RV10 (Table 1, Fig. 1).

2.3. Genomic DNA cloning and sequencing

Sea bass genomic DNA was extracted from sea bass erythrocytes as described [10] and used as template in a PCR with specific caspase-8 primers designed in the 5′- and 3′-UTRs. The first part of sea

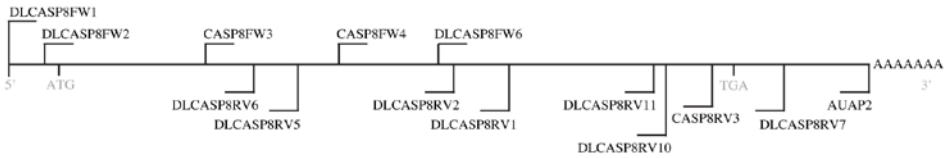

Fig. 1. Schematic representation showing the sea bass caspase-8 cDNA and the relative position of the primers used in this study. Primers DLCASP8FW3, DLCASP8FW4, DLCASP8FW5 and DLCASP8RV9 were designed in Introns and were not included in this scheme (see Table 1).

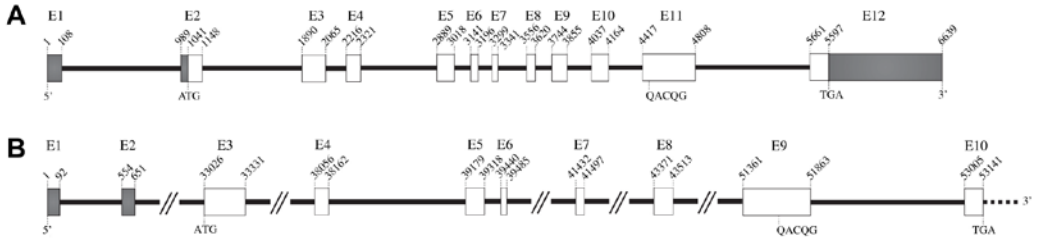

Fig. 2. Schematic representation of genomic organisation of caspase-8 from sea bass (A) and human (B). The genomic organisation of human caspase-8 (Gene ID: ENSG00000064012, Transcript ID: ENST00000432109) was obtained from the ensembl Genome site (http://www.ensembl.org). The exons are indicated by an E above the boxes and introns by a solid line (introns larger than 1200 bp are represented by a solid line interrupted by two lines). Coding nucleotides and untranslated regions are represented by white and black boxes, respectively. Values above boxes represent nucleotide numbers.

bass caspases-8 gene was obtained after an amplification of sea bass genomic DNA with DLCASP8FW2 and DLCASP8RV6 (Table 1, Fig. 1) in a 50 µl reaction (1.5 mM $MgCl_2$, 5 µl 10× PCR buffer, 40 mM dNTP mix, 0.4 µM of each primer and 1.25 units of Taq DNA polymerase) with the following conditions: 1 cycle of 94 °C for 2 min; 35 cycles of amplification (94 °C for 45 s; 57 °C for 30 s; 72 °C for 1 min 40 s); and 1 cycle of 72 °C for 5 min. The second part of the sea bass caspases-8 gene was obtained with the primers DLCASP8FW3 and DLCASP8RV7 (Table 1, Fig. 1) and with the same conditions as described for the first part, except for the annealing temperature (55 °C). The two bands were purified and cloned as mentioned previously. Three positive clones were sequenced for each purified product with the primers DLCASP8FW4 and DLCASP8RV9 for the first part, DLCASP8FW5 and DLCASP8RV10 for the second part, and T7 and SP6 for both bands (Table 1, Fig. 1). The full-length sequence was obtained by assembling the two partial sequences. This was possible because of the existence of a 180 bp contig in the region corresponding to the second DED of sea bass caspase-8 that allowed us to assure that both partial sequences originate from the caspase-8 gene and to exclude the possibility that one of the partial sequences could correspond to a card-caspase 8 (lacking DEDs) described in other fish species [7,11].

2.4. Southern blotting

For Southern blot, sea bass genomic DNA was digested overnight at 37 °C with the restriction enzymes BamHI, SpeI and XbaI (all zero cutters in sea bass caspase-1 gene) and NcoI (double cutter at 4434, beginning of exon 11, and 6409, end of 3′-UTR), all from Fermentas, isolated or combined (50 µl reactions: 9 µg of DNA, 5 µl 10× recommended buffer, 20 units of each enzyme). Electrophoresis of the digested DNA was made in 0.8 % agarose gel in TAE buffer (40 mM Tris, 0.11% acid acetic, 0.2% 0.5 M EDTA pH 8.0) and subjected to Southern blotting [12]. The region corresponding to exon 11 of sea bass caspases-8 was amplified with primers DLCASP8FW6 and DLCASP8RV11 (Table 1, Fig. 1) in a 50 µl reaction (1.5 mM $MgCl_2$, 5 µl 10× PCR buffer, 40 mM dNTP mix, 0.4 µM of each primer and 1.25 units of Taq DNA polymerase), using one of the clones referred in Section 2.2 as template, with the following conditions: 1 cycle of 94 °C for 2 min; 35 cycles of amplification (94 °C for 45 s; 59 °C for 30 s; 72 °C for 20 s); and 1 cycle of 72 °C for 5 min. The purified product was used as probe. Probe labelling, hybridization and post-hybridization stringency washes were performed accordingly to Gene ImagesTM AlhPhos Direct™ Labelling and Detection System (Amersham Biosciences) kit. For signal generation and detection the Chemiluminescent Signal Generation and Detection with CDP-*Star*™ protocol from the same kit was followed.

2.5. Expression analysis

For analysis of basal expression, total RNA was extracted from head kidney, spleen, thymus, intestine, liver and heart of three non-stimulated fish (10 g) and reverse transcribed to cDNA as previously described. The cDNAs were normalized by dilution using a β-actin PCR product as control gene, amplified with 2 µl of template with primers DLBACTINFW and DLBACTINRV (Table 1) in 50 µl reactions with the following conditions: 94 °C for 2 min, 30 cycles of amplification (94 °C for 45 s; 59 °C for 1 min; 72 °C for 30 s); 72 °C for 5 min. Amplifications of caspase-8 transcripts were performed with 4 µl of the cDNA dilutions using specific primers design in exon/intron boundaries of sea bass caspase-8 (DLCASP8FW6 and DLCASP8RV11, Table 1, Fig. 1) and with the same conditions used for the control gene. The gels were stained by soaking (30 min) with ethidium bromide (5 µg/mL TAE) and analyzed by densitometry using the ImageQuant® Molecular Dynamics®.

Analysis of caspase-8 expression was also performed in the spleens of fish injected with a dose of mid-exponential phase culture supernatants from *Phdp* known to induce apoptosis of macrophages and neutrophils [9,13] resulting in approximately 50% of mortality. Groups of 8 fish per time were intraperitoneally injected with *Phdp* culture supernatants and, as control groups, 3 fish per time were injected with PBS. Spleens were collected at 1, 3, 6, 12 and 24 h after injection. Spleens of 12 non-stimulated fish were also collected. Total RNA was extracted from the spleens according to the RNAqueous 4-PCR kit (Ambion) and reverse

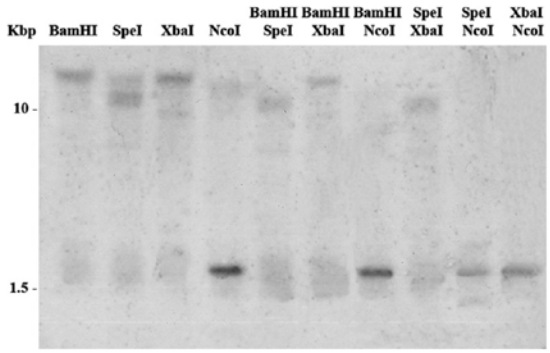

Fig. 3. Southern blot of genomic organisation of sea bass caspase-8. Genomic DNA was digested with BamHI, SpeI, XbaI and NcoI (alone and combined) blotted and hybridized with a probe corresponding to sea bass caspase-8 exon 11. The numbers indicate the size in kilo bases from the Hipper ladder I (Bioline).

transcribed to cDNA using the adaptor/anchor primer APv2 (Table 1) at 42 °C for 60 min following the BioScript RNase H Minus (BIOLINE) protocol. All cDNAs were diluted to 1/10 and used in Real-Time PCR to measure the caspase-8 expression. Real-time PCR was performed using iQ™ SYBR Green Supermix on an iQ™5 real-time detection system (BIO-RAD) according to the manufacturer's instructions with the following conditions: denaturation at 95 °C for 3 min, 40 cycles of amplification (denaturing at 95 °C for 10 s;

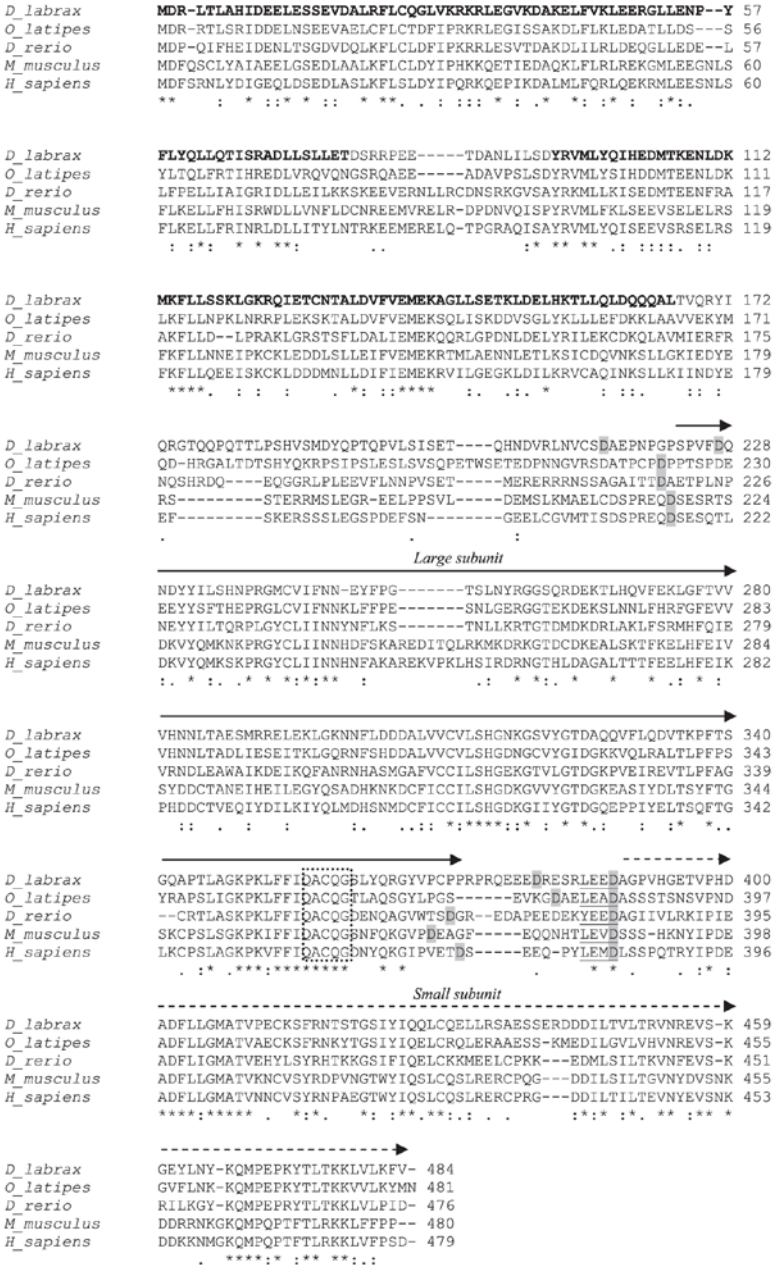

Fig. 4. Multiple alignment of the caspase-8 amino acid sequence of sea bass and other vertebrate. The pentapeptide active-site motif (QACRG) is boxed in a discontinuous line and the caspase tetrapeptide recognition site is underlined. The human large and small subunits are represented by ⟶ and ⇢ respectively. The putative cleavage sites at aspartic acid residues, which separates the prodomain, large and small subunits, are shaded in gray and were indicate based on the cleavage sites described for human casapase-8. The two DED predicted by Prosite for the sea bass caspase-8 are shown in bold. The numbers indicate the amino acid positions and dashes indicate gaps introduced to optimize similarity between sequences. Asterisks denote identical residues and ":" and "." chemical similarity between amino acids.

annealing at 57 °C for 30 s; extension at 72 °C for 30 s) following by melting curve analysis, and the primers DLBACTINFW2/DLBACTINRV2 for β-actin and DLCASP8FW6/DLCAP8RV11 for caspase-8 (Table 1, Fig. 1). Data were normalized for β-actin and results presented as arbitrary units.

2.6. Sequence analysis

The sea bass caspase-8 amino acid sequence was deduced using expasy translate tool (http://www.expasy.org). Full nucleotide and protein sequences from sea bass caspase-8 were compared to several caspases-8 sequences currently available in the GenBank database retrieved using the BLAST program (http://www.ncbi.nlm.nih.gov). The multiple alignments were made using CLUSTAL W program (http://www.ebi.ac.uk/clustalw/index.html). The caspase-8 domains and possible N-glycosylation sites were based on PROSITE predictions (http://www.expasy.org/prosite). Molecular weight was calculated by expasy compute pI/Mw tool (http://www.expasy.org). The neighbour-joining phylogenetic tree was constructed using MEGA 3.1 program [14], using p-distance parameter and complete deletion of gaps. The phylogenetic tree was tested for reliability using 1000 bootstrap replications. The percentages of similarity and identity were calculated by pair-wise alignments by the program needle at http://www.ebi.ac.uk, with first and extending gap of 10 and 0.5, respectively.

3. Results and discussion

In this study, we identified and characterized the sea bass caspase-8. The full-length sea bass caspase-8 cDNA (2472 bp) (NCBI accession number: FJ225665) shows significant homology with caspase-8 of other vertebrates, as revealed by BLAST analysis. Overall, this cDNA fragment consists of a 1455 bp open reading frame (ORF), 160 bp of the 5′-UTR and 840 bp of the 3′-UTR, besides a polyadenylation tail. The 3′-UTR includes a canonical polyadenylation signal (AATAAA) 18 nucleotides upstream the polyadenylation tail. The nucleotide sequence was translated into 484 amino acids with a predicted molecular weight of 55.2 kDa.

In sea bass genome, the caspase-8 gene (NCBI accession number FJ225664) has 6639 bp and is organized in 12 exons and 11 introns (Fig. 2), with the donor and acceptor exon/intron splice junctions conforming the GT/AG rule [15], and in a similar fashion as the one presented in medaka and stickleback caspase-8 genes [11], but different from the one of zebrafish [11,16], which has a 10 exon/9 intron organisation similar to that of human caspase-8 gene [17]. The intron lengths of sea bass caspase-8 gene vary from 102 to 880 nucleotides, which are significantly smaller than the ones from the human caspase-8 gene (393–32,348 nucleotides) rendering an approximately 4 and 5 times smaller gene than the mouse and human caspase-8 gene, respectively. The sea bass caspase-8 open reading frame starts in exon 2, which also contains part of 5′-UTR. The two DEDs of sea bass caspase-8 are located in the exons 2 and 3 (first DED), and end of exon 3 and exons 4 and 5 (second DED), mostly resembling the medaka organisation [11], but again differing from the one presented by zebrafish (first DED encoded by exon 2; second DED encoded by exons 3 and 4) [16] and human (first DED encoded by exon 3; second DED encoded by exons 4 and 5) [17] caspase-8 genes. As in medaka and human gene, exons 6, 7 and 8 encode the linker region localized between the DED of prodomain and the large subunit of the active enzyme. On the other hand, the zebrafish caspase-8 gene has 4 exons in the linker region between the DEDs of the prodomain and the large subunit [16]. The large (p20) and the small (p10) subunits of the sea bass caspase-8 molecule are encoded by exons 9–12, with the active site (QACQG) being encoded by exon 11, similarly to the structure presented in the medaka caspase-8 gene [11].

Southern hybridization of sea bass genomic DNA digested with different enzyme combinations (Fig. 3), with a probe corresponding to exon 11 of sea bass caspase-8 yielded a pattern consistent with a single copy gene. However, the existence of caspase-8 like forms found in other fish species (reviewed in [7]) cannot be excluded.

To compare the caspase-8 proteins from sea bass and other vertebrates a multiple alignment was made (Fig. 4). Two death effector domains (DED) profiles in the N-terminus (M^{1} to T^{77} and D^{93} to L^{166}, respectively) were identified by PROSITE as well as the caspase p20 (P^{238} to L^{362}) and p10 (V^{397} to L^{481}) domain profiles. These features of sea bass caspase-8 are similar to those of other caspase-8 molecules [16,18,19]. A caspase family cysteine active-site K^{249}PKLFFIQACQG^{360} and two N-glycosylation sites (N^{284}LTA and N^{418}TST) were also predicted. Caspase-8 shares the active-site pentapeptide QACXG with the other members of the caspases family, having a glutamine (Q) residue as the fourth amino acid of the active-site [20]. Sea bass caspase-8 active-site pentapeptide conserves the canonical sequence QACQG of other caspase-8 active sites (Fig. 4). However, different profiles are present in the caspase-8 sequence of catfish and zebrafish caspase-8Xa, where the fourth amino acid of the active-site is an R instead of a Q and the two dead effector domains of the prodomain are replaced by a CARD domain (reviewed by [7]).

The active form of caspase-8 is generated after proteolytic cleavage of the proenzyme at aspartic acid residues. In humans, caspase-8 becomes active by a first cleavage at Asp^{374}, releasing the small subunit and generating a fragment of 43 kDa (prodomain and large subunit). Posterior cleavages occur at Asp^{216} and Asp^{384}, releasing the prodomain and the linker region from the large subunit [21]. Two potential cleavage sites (Asp^{380} and Asp^{388}), which may be involved in the release of the C-terminal

Table 2
Percentage of similarity, identity, and gaps of caspase-8 and card-caspase 8 (when signalled) from sea bass and other species. Only complete sequences were considered. The results were based in pair alignments of complete sequences from other species with the one from sea bass using the programme needle in the server www.ebi.ac.uk.

Species	*Dicentrarchus labrax*			Accession numbers
	Similarity (%)	Identity (%)	Gaps (%)	
Gasterosteus aculeatus	78.2	64.3	2.9	ENSGACP00000016969
Oryzias latipes	70.7	54.5	5.1	AAS91704
Takifugu rubripes	69.8	57.1	8.0	NEWSINFRUP00000179400
Salmo salar	67.8	52.5	6.9	ACN10768
Danio rerio	56.7	40.4	9.1	AAS91705
D. rerio casp8-like2	41.8	26.3	19.2	XP_685430
D. rerio similar CARD-casp8	40.3	24.4	31.3	CAM14414
O. latipes CARD-casp8	39.1	22.9	30.8	AM144512/EF564191
G. aculeatus CARD-casp8	38.7	26.6	35.3	ENSGACP00000016983
Ictalurus punctatus CARD-casp8	38.3	27.7	35.6	AAT37512
T. rubripes CARD-casp8	36.4	24.8	37.8	SINFRUP00000182829
Mus musculus	56.0	36.6	13.2	CAA07677
Rattus norvegicus	54.0	36.6	11.7	NP_071613
Homo sapiens	53.2	36.1	10.8	AAD24962
Sus scrofa	52.9	35.5	11.8	NP_001026949
Bos taurus	52.9	34.9	12.2	NP_001039435
Canis familiaris	52.7	35.6	15.6	NP_001041494
Gallus gallus	51.1	33.6	12.4	NP_989923
Xenopus laevis	46.8	30.2	18.8	BAA94749
Aedes aegypti	39.0	24.2	25.6	EAT33580
Molgula tectiformes	32.4	19.3	36.9	ABI64126

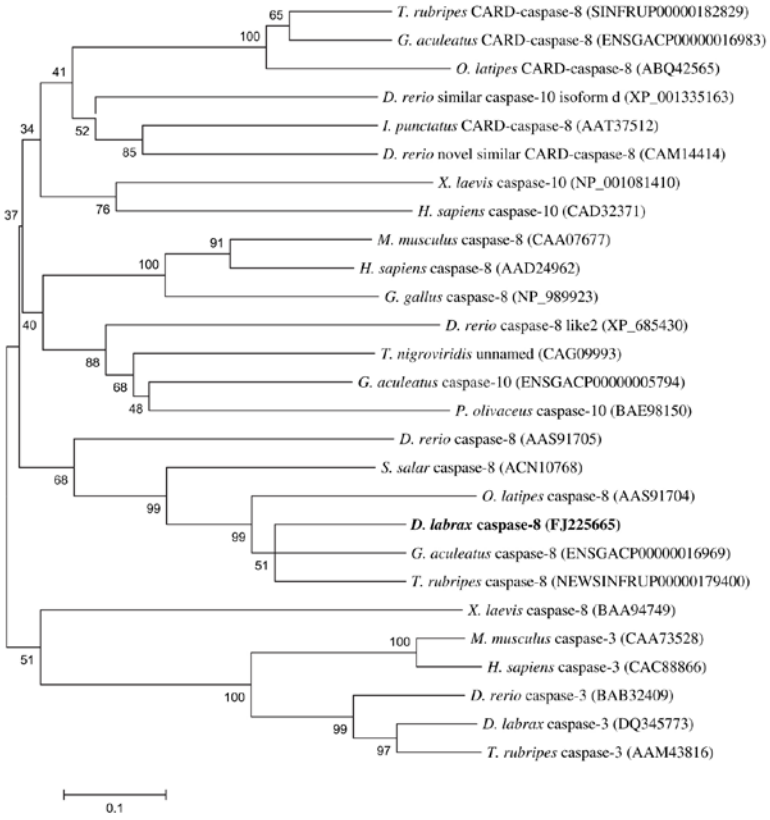

Fig. 5. Neighbour-joining tree (MEGA 3.1) with p-distance and complete deletion of gaps of caspase-8, -10 and -3 (outgroup) amino acid sequences. Only complete sequences were considered. The sequences were aligned using CLUSTALW according to default parameters. The numbers in branches nodes denote the bootstrap percentages for 1000 replicates. Accession numbers are shown in front of each respective species.

subunit (p10), are present in the sea bass caspase-8 (Fig. 4). Nevertheless, only the Asp^{388} is conserved in all the caspase-8 sequences in the context of caspase tetrapeptide (LEXD) recognition site [22] characteristic of caspase-8, -6, -9 and -10. In sea bass caspase-8, two aspartic acid residues (Asp^{215} and Asp^{227}), which may serve as putative cleavage site for releasing of sea bass caspase-8 prodomain, are located in the region that in humans contain the Asp^{216} (Fig. 4).

The sea bass caspase-8 sequence exhibits a very close similarity to the ones of other vertebrates (Table 2), with higher similarity (78.2%) and identity (64.3%) with the caspase-8 from stickleback (*Gasterosteus aculeatus*). Moreover, higher similarity and identity was observed to other caspase-8 from non-fish vertebrates (56.0% similarity/36.6% identity from *Mus musculus* to 46.8% similarity/30.2% identity from *X. laevis*), when compared to CARD-caspase-8 from other fish species (less than 42% similarity/27% identity). The

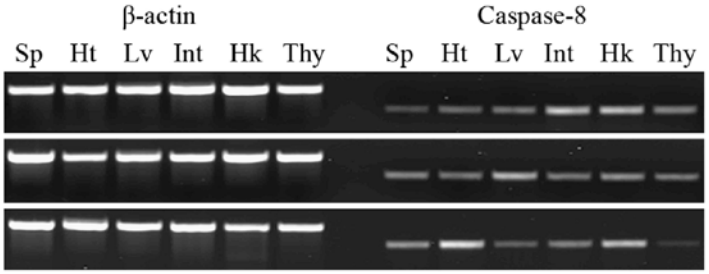

Fig. 6. Basal expression of sea bass caspase-8 in different tissues. RT-PCR was used to detect caspase-8 transcripts in the spleen (SP), heart (Ht), liver (Lv), intestine (Int), head kidney (HK), and thymus (Thy) of three non-stimulated sea bass. Expression of the β-actin gene was used as an internal control.

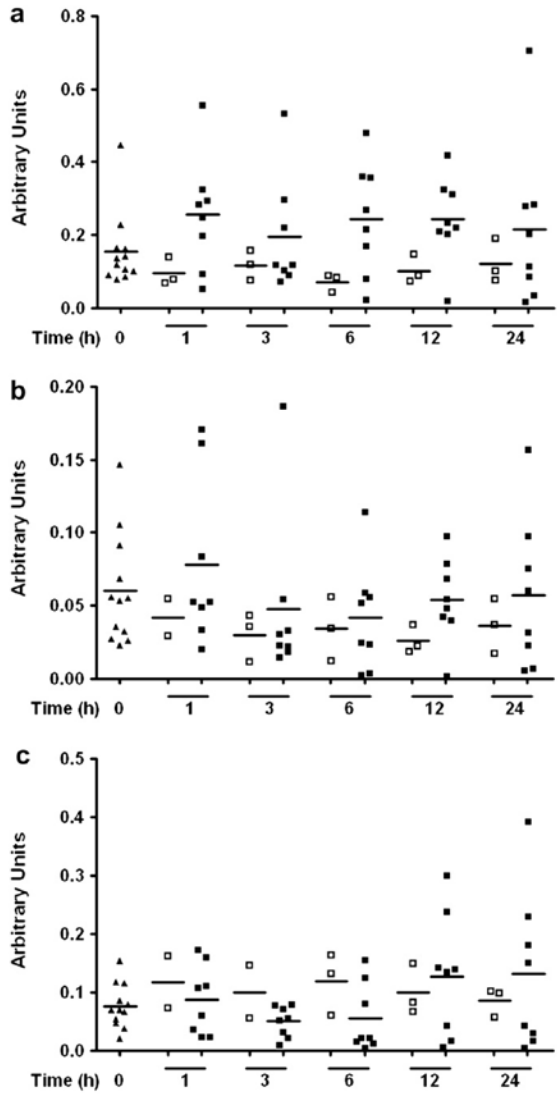

Fig. 7. Real-Time PCR expression analysis of sea bass caspase-8, -3 and -9, in spleens of fish stimulated a known apoptotic stimulus (Phdp ECPs). Data were normalized for β-actin and results presented as arbitrary units. Each symbol corresponds to the value obtained for an individual fish. Filled triangles – non-stimulated fish; open squares – fish injected with PBS; filled squares – fish injected with Phdp ECPs; lines – average.

results from the pair-wise alignments are supported by the neighbour-joining tree data (Fig. 5), which groups the sea bass caspase-8 with the ones of other fish, with closer relation with other Percomorpha fish. Of notice is the apparent common origin of the fish caspase-8, with that of non-fish caspase-8, CARD-caspase-8 and caspase-10 (reviewed in [23]).

Caspase-8 is expressed in several types of tissues. In humans, the highest expression levels occur in peripheral blood leukocytes, spleen, thymus, and liver [24]. In the mouse, a similar distribution of caspase-8 expression is present, where the higher levels were detected in spleen, thymus, liver and kidney [19]. In sea bass, caspase-8 expression was observed in several tissues of non-stimulated fish, all presenting some basal levels of expression (Fig. 6).

Recently, it has been shown that *Phdp* induces selective apoptotic secondary necrosis of macrophages and neutrophils through the action of an exotoxin named AIP56 [9,13,25]. Moreover, in experimental and natural *Phdp* infections, as well as when mid-exponential phase culture supernatants from *Phdp* or recombinant AIP56 are inoculated into the fish, destruction of macrophages by apoptotic secondary necrosis was seen predominantly in the spleen, head kidney and gut lamina propria [25]. In order to assess whether caspase-8 would be involved in the AIP56-induced apoptosis and to ascertain which of the apoptotic pathways would be the initial, a kinetic study of caspase-8, -9 and -3 expression in one of the *Phdp* targeted organs, the spleen, was performed. The results show that in spleens of fish treated with *Phdp* culture supernatants, the expression levels of caspase-8 increased early (1 h post-inoculation), following by a slight decrease (3 h post-inoculation) and a later increase (6–24 h post-inoculation), in a similar fashion of that observed for sea bass caspase-3 (Fig. 7a and b). On the other hand, up-regulation of caspase-9 under these experimental conditions occurs only later in time (Fig. 7c). The involvement of the intrinsic pathway with caspase-9 up-regulation has been previously suggested using a *Phdp* infection model [26], and recently further supported by the implication of mitochondria into the AIP56 apoptosis-induced process, as indicated by the loss of mitochondrial membrane potential, translocation of cytochrome c to the cytosol and the over-production of ROS (Costa-Ramos et al., submitted). Together, the above results suggest the involvement of both the extrinsic and intrinsic pathways in the AIP56-induced apoptosis, possibly initiated by caspase-8 and with the intrinsic/mitochondrial pathway functioning as an amplifying mechanism [27].

In conclusion, the sea bass counterpart of mammalian caspase-8 was sequenced and characterized. Basal expression of caspase-8 was detected in several organs of non-stimulated fish, and was found to be involved in the apoptosis induced by the exotoxin AIP56 produced by *Phdp*. The present identification of sea bass caspase-8 will allow the production of tools and its monitoring in studies of the role of that initiator caspase in sea bass apoptotic processes.

Acknowledgments

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CHAPTER IV

First molecular cloning and characterisation of caspase-9 gene in fish and its involvement in a gram negative septicaemia.

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First molecular cloning and characterisation of caspase-9 gene in fish and its involvement in a gram negative septicaemia

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Abstract

Caspase-9 is an initiator caspase in the apoptotic process whose function is to activate effector caspases that are downstream in the mitochondrial pathway of apoptosis. This work reports for the first time the complete sequencing and characterisation of caspase-9 in fish. A 1924 bp cDNA of sea bass caspase-9 was obtained, consisting of 1308 bp open reading frame coding for 435 amino acids, 199 bp of the 5′-UTR and 417 bp of the 3′-UTR including a canonical polyadenilation signal 10 nucleotides upstream the polyadenilation tail. The sequence retains the pentapeptide active-site motif (QACGG) and the putative cleavage sites at Asp_{121}, Asp_{325} and Asp_{343}. The sequence of sea bass caspase-9 exhibits a very close homology to the sequences of caspase-9 from other vertebrates, particularly with the putative caspases-9 of *Danio rerio* and *Tetraodon nigroviridis* (77.5 and 75.4% similarity, respectively), justifying the fact that the phylogenetic analysis groups these species together with sea bass. The sea bass caspase-9 gene exists as a single copy gene and is organised in 9 introns and 10 exons. The sea bass caspase-9 showed a basal expression in all the organs analysed, although weaker in spleen. The expression of sea bass caspase-9 in the head kidney of sea bass infected with the *Photobacterium damselae* ssp. *piscicida* (*Phdp*) strain PP3, showed increased expression from 0 to 12 h returning to control levels at 24 h. Caspase-9 activity was detected in *Phdp* infected sea bass head kidney from 18 to 48 h post-infection, when the fish were with advanced septicaemia.
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Keywords: Sea bass; *Dicentrarchus labrax*; Apoptosis; Caspase-9; *Photobacterium damselae* ssp. *piscicida*

1. Introduction

Apoptosis (Kerr et al., 1972) is a highly regulated and conserved form of active cell death. The morphological and biochemical changes associated with apoptosis are largely caused by the activation of a family of intracellular cysteine aspartyl-specific proteases known as caspases, which cleave many vital cellular substrates contributing to cell death and to the apoptotic phenotype (Reed, 2000; Samali et al., 1999; Thornberry and Lazebnik, 1998; Wolf and Green, 1999). Two main pathways for activating caspases, thereby triggering apoptosis, have been described (Marsden and Strasser, 2003; Strasser et al., 2000): (i) the death receptor/extrinsic pathway, initiated at the tumour necrosis factor (TNF) family receptors (Salvesen and Dixit, 1997; Wallach et al., 1999; Yuan, 1997) and (ii) the mitochondrial/intrinsic pathway (Green and Reed, 1998; Reed, 1997).

Caspases are among the most specific proteases, with a remarkable specificity for cleavage on the carboxyl side of aspartic acid residues. The cleavage aspartate residues are recognized within the context of tetrapeptide motifs that differ significantly among caspases and explains the diversity of their biological functions (Reed, 2000; Thornberry and Lazebnik, 1998). Functionally, caspases can be classified as: (i) upstream initiator caspases (caspases-2, -8, -9 and -10) (Lawen, 2003; Salvesen and Dixit, 1997) whose proforms possess large NH_2-terminal prodomains, containing either death effector domains (DED) (caspases-8 and -10) or a caspase-associated recruitment domain (CARD) (caspases-2 and -9) (Reed, 2000); (ii) downstream executioner or effector caspases (caspases-3, -6 and -7) (Lawen, 2003; Salvesen and Dixit, 1997), whose proforms contain only short NH_2-terminal prodomains with no apparent function (Reed, 2000); and (iii) pro-inflamatory caspases, whose main role lies in cytokine maturation rather than apoptotic activity (Lawen, 2003; Reed, 2000; Salvesen and Dixit, 1997).

Caspase-9, also known as ICE-LAP6, MCH-6 or Apaf-3, is a member of the CED-3 subfamily, bearing high similar-

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ity to caspase-3. The major difference between caspase-9 and other family members is the pentapeptide active-site QACGG in the large subunit, in which a Gly is found instead of the usual Arg (Duan et al., 1996). Procaspase-9 has a long NH_2-terminal prodomain, containing a CARD, and two potential processing sites between its large and small subunits that in human caspase-9 have been identified in $P_{312}EPD \downarrow A_{316}$ and $D_{327}QLD \downarrow A_{331}$ (Srinivasula et al., 1996). Homophilic interactions between the CARD of caspase-9 and that of the apoptotic protease-activating factor-1 (Apaf-1), driven by cytochrome *c* and ATP/dATP, lead to the assembly of a heptameric complex, the apoptosome, resulting in the proteolytic cleavage of procaspase-9 (Green and Reed, 1998; Wang, 2001). Caspase-9 is the initiator caspase in the mitochondrial/intrinsic apoptotic pathway (Hakem et al., 1998; Li et al., 1997), and whose assignment is to generate the active forms of caspase-3, -6 and -7 and, thereby, transmit the apoptotic signal to the execution phase. Moreover, caspase-9 can also have a significant role in the death-receptor-mediated apoptosis, through the cleavage by caspase-8 of BID into truncated BID, which in turns targets mitochondria to release cytochrome *c* and activates caspase-9 (Gross et al., 1999; Li et al., 1998; Luo et al., 1998; Wang et al., 1996). Both caspase-3 and granzyme B can activate procaspase-9, although processing it to differently sized products (Cohen, 1997). Furthermore, alternative variants of caspase-9 acting as inhibitors of caspase-9 activation and apoptosis, and, thereby, indicating a physiological role in regulating cell death, have been reported (Angelastro et al., 2001; Seol and Billiar, 1999; Srinivasula et al., 1999). In addition, studies indicate that caspase-9 contributes to the disassembly of the cell by disrupting the nuclear-cytoplasmic barrier. It seems that caspase-9 increases the diffusion limit of the nuclear pores, thus allowing caspase-3, and other molecules, to enter or leave the nucleus, by diffusion (Faleiro and Lazebnik, 2000). In relation to the mechanisms that regulate caspase-9 activation, it has been shown that caspase-9 has an X-chromosome-encoded inhibitor of apoptosis proteins (XIAP)-binding sequence, similar to the Smac sequence that binds to the BIR3 domain of XIAP (Srinivasula et al., 2001). Therefore, IAP binding to the exposed motif in caspase-9 results in the inhibition of its activity (Verhagen et al., 2001; Wang, 2001). Nevertheless, this anti-apoptotic effect of IAPs can be neutralized upon release into the cytosol of certain mitochondrial proteins, such as Smac/DIABLO and HtrA2, that sequester the IAPs, thus releasing caspase-9 (Gulbins et al., 2003). It has also been reported that human caspase-9 can be inactivated by Akt phosphorylation, a form of posttranslational modification employed to modulate caspase activity (Chang and Yang, 2000), although, this regulation does not seem to be conserved in all species (Chang and Yang, 2000; Fujita et al., 1999). On the other hand, caspase regulation at the level of gene expression have also been described (Earnshaw et al., 1999).

Although the caspase family appears to play pivotal roles in apoptosis beyond species, information on caspases has been mostly limited to mammals (human and mouse), *Drosophila*, *C. elegans* and to a few reports on chicken (Johnson and Bridgham, 2000), *Xenopus* (Nakajima et al., 2000), and a few fish species (Laing et al., 2001; Long et al., 2004; Yabu et al., 2001). Recently, sea bass caspase-3 gene has been cloned and characterised by our group (Reis et al., 2006). In this manuscript the molecular cloning and characterisation of caspase-9 gene in fish is presented for the first time, as well as its involvement in the apoptotic process triggered by *Photobacterium damselae* ssp. *piscicida* (*Phdp*) in sea bass macrophages and neutrophils.

2. Material and methods

2.1. Fish

Sea bass, *Dicentrarchus labrax*, were kept in a recirculation, UV-treated salt-water (30–35%) system at 20 ± 1 °C, and fed at a ratio of 2% body weight per day. For collection of organs fish were euthanised with a lethal dose of 2-phenoxyethanol (Panreac; >5 ml/10 l).

2.2. cDNA cloning and sequencing

Sea bass peritoneal cells were collected 30 min after intraperitoneal inoculation of 10 μl of extracellular products (ECP) from *Phdp*, containing approximately 40 μg of apoptosis inducing protein (AIP56), diluted in 190 μl sea bass PBS (1.15 mM K_2HPO_4, 7.8 mM Na_2HPO_4, 170 mM NaCl, 13 mM Na_2CO_3) (do Vale et al., 2005) and used for mRNA extraction, according to the MicroPoly(A)Pure™ (Ambion®). cDNA synthesis was performed according to the Superscript™ III First-Strand Synthesis System (Invitrogen—Life Technologies), using 500 ng of the extracted mRNA as the template and the adaptor/anchor primer APv (Table 1). Degenerate primers were designed based on conserved regions from multiple alignments of caspase-9 amino acid (aa) sequences from different vertebrates (*Mus musculus*, *Rattus norvegicus*, *Homo sapiens*, *Xenopus laevis*, *Takifugu rubripes* and *Danio rerio*). Sequences from *T. rubripes* and *D. rerio* were obtained by performing a Blast search at Ensembl Genome Browser (http://www.ensembl.org) using the caspase-9 sequence from *R. norvegicus* as query. Fig. 1 schematizes the relative position of each primer used for obtaining the cDNA and genomic sequences. The cDNA (2 μl) was first amplified with primers CASP9FW1 and CASP9RV1 (Table 1) in a 50 μl reaction (0.2 mM dNTPs mix, 1.5 mM $MgCl_2$, 5 μl 10× buffer, 0.4 μM of each primer and 1.25 units of Taq polymerase) with the following conditions: 94 °C for 2 min; 30 cycles of amplification (94 °C for 45 s; 50 °C for 1 min; 72 °C for 30 s); 72 °C for 5 min. A semi-nested PCR was performed as above but with the primers CASP9FW2 and CASP9RV1 (Table 1). A reamplification was further performed and the PCR product (382 bp) purified (Gel Extraction Kit, Qiagen), ligated overnight at 4 °C and used to transform *E. coli* XL1 competent cells following the pGEM-T easy Vector System (Promega). Transformants were plated on LB agar medium containing ampicilin (200 μg/ml) and IPTG/X-GAL (50 μg/ml each). The plasmid DNA of one positive colony was extracted (High Pure Plasmid Isolation Kit Protocol, Roche®) and used (200–300 ng) with primers T7 and SP6 (Table 1) in sequencing reactions performed according to the Big Dye terminator 2.0 (Applied Biosystems), and analysed in an automatic sequencer.

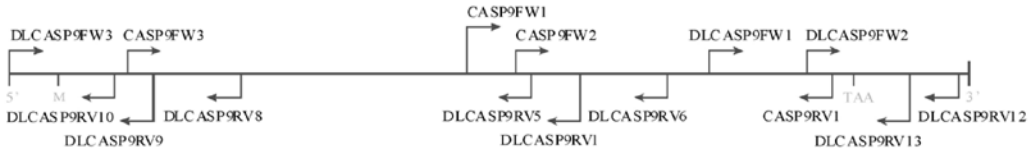

Fig. 1. Schematic representation of the relative position of each primer used for obtaining the cDNA and genomic sequences.

The sequence was used to design specific forward and reverse primers, which were used for obtaining the 3′-untranslated region (UTR) and moving upwards to the N terminal, respectively. In this way, the same cDNA was first amplified as above but with the primers, CASP9FW3 and CASP9RV1 (Table 1). A semi-nested PCR was then performed as above but using as reverse primer DLCASP9RV6 (Table 1), and 59 °C as annealing temperature and 45 s for extension time. A third amplification was performed as above with CASP9FW3 and DLCASP9RV1 (Table 1) but with 57 °C as annealing temperature and 45 s for extension time. Only after a further amplification using CASP9FW3 and DLCASP9RV5 (Table 1) with the same conditions as before, a band with the expected size was obtained. The amplified product was cloned and sequenced as previously described. Primers DLCASP9RV8 and DLCASP9RV9 (Table 1) were then designed and used for reaching the 5′-UTR following the 5′ RACE System Kit (Invitrogen). The PCR product was purified, cloned and sequenced as previously described. However, the obtained sequence did not reach the start codon so a new primer DLCASP9RV10 (Table 1) were designed and the 5′-UTR was reached following the same strategy as above.

Table 1
Degenerate and specific primers used in this study

Primer	Sequence
APv	5′-GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT TT (AGC)-3′
T7	5′-TAA TAC GAC TCA CTA TAG GGC GA-3′
SP6	5′-CTA TTT AGG TGA CAC TAT AGA ATA C-3′
CASP9FW1	5′-AAR YTN TTY TTY ATH CAR GCN TG-3′
CASP9FW2	5′-GAR AAR GAY III GGN TTY GAR GT-3′
CASP9FW3	5′-MGN GAY CAR GCN MGI CAR-3′
CASP9RV1	5′-GAR GAR YTT NCK NAR RAA RTT RAA-3′
DLCASP9FW1	5′-GTT ACC CAT GAC TTG GTC AC-3′
DLCASP9FW2	5′-AGG GCT CTA CAA GCA GAT G-3′
DLCASP9FW3	5′-GCG AAT GCA ACC GAG CAC AA-3′
DLCASP9FW4	5′-TAT TCC CCA CGT ACT GTC CT-3′
DLCASP9FW5	5′-GGA GGA CAG ATA CAG GAA GA-3′
DLCASP9FW6	5′-ACC CTC TCC CAG TGT CCT CT-3′
DLCASP9RV1	5′-ATC GGA CGT GCT CAG AGA-3′
DLCASP9RV3	5′-CGG TCT AGT ATC TCG ACG TA-3′
DLCASP9RV5	5′-GGC ATC CGT CTG ATC GTC TG-3′
DLCASP9RV6	5′-TGA GGC TCT GGC GTC CAG TT-3′
DLCASP9RV8	5′-TGA AGG CAC TCC ACG AAT AG-3′
DLCASP9RV9	5′-GAC TAG CTG CCT AGC CTG AT-3′
DLCASP9RV10	5′-TAG CCT GAT CAC GCC TGG TC-3′
DLCASP9RV12	5′-GAG TCA GCA AAA GTA TTT TA-3′
DLCASP9RV13	5′-ATC AAT GAC AGC TGC TAT TT-3′
DLCASP9RV14	5′-AGT GTC AGC AGA TGG AAT AC-3′
DLCASP9RV15	5′-CGC AGC AGT CAT ACT GTG AA-3′
DLCASP9RV17	5′-CGG TTG TGA CTC ACC TCA G-3′
DLBACTINFW	5′-ATC GTG GGG CGC CCC AGG-3′
DLBACTINRV	5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′

Y = C/T; N = A/C/T/G; I = inosine; R = A/G; H = ACT; M = A/C; K = G/T.

For 3′-UTR, the cDNA was first amplified with primers DLCASP9FW1 (Table 1) and APv in a 50 μl reaction (as above) with the following conditions: 94 °C for 2 min; 30 cycles of amplification (94 °C for 45 s; 52 °C for 1 min; 72 °C for 50 s); 72 °C for 5 min. A semi-nested PCR was then performed as above using primers DLCASP9FW2 (Table 1) and APv, and 56 °C for the annealing temperature and 30 s for the extension time. After reamplification, the PCR product (495 bp) was purified, cloned and sequenced as previously described.

For obtaining clones with complete sequences, the cDNA (2 μl) was first amplified using the primers DLCASP9FW3 and APv (Table 1) in a 50 μl reaction (0.2 mM dNTPs mix, 1.5 mM $MgCl_2$, 5 μl 10× buffer, 0.4 μM of each primer, 1.25 units of Taq polymerase and 0.15 units of Pfu DNA Polymerase) with the following conditions: 94 °C for 2 min; 30 cycles of amplification (94 °C for 45 s; 60 °C for 1 min; 72 °C for 1 min and 20 s); 72 °C for 5 min. The PCR products were reamplified and after a third amplification as before, but using the primer DLCASP9RV13 (Table 1) and 54 °C for the annealing temperature, the amplified product was cloned as above and plasmid DNA from three positive colonies (extracted as before) sequenced at MWG (http://www.ecom.mdwdna.com) with the primers DLCASP9RV1, T7 and SP6 (Table 1).

2.3. Genomic DNA cloning and sequencing

Genomic DNA was isolated from sea bass erythrocytes as described (Stet et al., 1993). In order to achieve the caspase-9 gene, the genomic DNA was used as template with primers DLCASP9FW3 and DLCASP9RV12 (Table 1) in a 50 μl PCR reaction (0.2 mM dNTP mix, 2 mM $MgCl_2$, 5 μl 10× buffer, 0.4 μM of each primer, 2.5 units of Taq polymerase and 0.15 units of Pfu DNA Polymerase), with the following conditions: 94 °C for 2 min; 30 cycles of amplification (94 °C for 45 s; 53 °C for 1 min; 72 °C for 3 min); 72 °C for 5 min. The obtained PCR product was purified using the GFX™ PCR DNA and Gel Band Purification kit (Amersham Biosciences), cloned into pGEM-T easy vector as mentioned above and three positive clones were sent for sequencing at MWG with the primers DLCASP9FW4, DLCASP9RV3, DLCASP9RV14, T7 and SP6 (Table 1).

2.4. Southern blotting

Restriction enzymes *Eco*RI and *Hin*dIII isolated and combined were used to digest genomic DNA in 50 μl reactions (9 μg of DNA, 4 μl 10× buffer B, and 20 units of each

enzyme) overnight at 37 °C. Electrophoresis of the digested DNA was made in 0.8% agarose gel (≈6 mm thick) in TAE buffer (39.95 mM Tris, 0.11% Acid Acetic Glacial, 0.2% 0.5 M EDTA pH 8.0). Depurination of the DNA was performed by rinsing the gel in distilled water and then immersing in 0.25 M HCl for 30 min at RT with gentle shaking. The DNA was then denatured by rinsing the gel in distilled water and then immersing in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 20 min with gentle shaking. The solution was refreshed and incubation occurred for another 20 min. Finally, the gel was rinsed in distilled water and then incubated twice in neutralization solution (1.5 M NaCl, 0.5 M Tris–HCl) for 20 min and shaken as before. The DNA was transferred ON at RT by capillarity to a Hybond™-N+ nylon membrane (Amersham Biosciences) using 20× SSC (3 M NaCl, 0.3 M sodium citrate dehydrated) as transfer buffer. The DNA was crosslinked in a UV crosslinker (Vilber Lourmat).

A fragment of the sea bass caspase-9 cDNA was amplified twice with DLCASP9FW5 and DLCASP9RV15 (Table 1) in 50 μl reactions as before with the following conditions: 94 °C for 2 min; 30 cycles of amplification (94 °C for 45 s; 57 °C for 30 s; 72 °C 30 s); 72 °C for 5 min. The PCR product (754 bp) was extracted (GFX™ PCR DNA and Gel Band Purification kit, Amersham Biosciences) and used as a probe. Labelling of the probe, hybridization and post-hybridization stringency washes were performed accordingly to Gene Images™ AlkPhos Direct™ Labelling and Detection System (Amersham Biosciences) kit. For signal generation and detection the Chemiluminescent Signal Generation and Detection with CDP-*Star*™ protocol from the same kit was followed.

2.5. Expression analysis by RT-PCR

Expression analysis of organs from non-stimulated fish has been performed as described for caspase-3 (Reis et al., 2006) with the exception that amplifications of caspase-9 transcripts were done using 12 μl of the cDNA dilutions and primers DLCASP9FW6 and DLCASP9RV17 (Table 1).

Expression analysis of *Phdp* infected sea bass has been performed as described for caspase-3 (Reis et al., 2006). The same procedure as above was followed to normalise the cDNAs, except that 30 cycles of amplification were used. The caspase-9 transcripts were amplified as above but using 6 μl of cDNA and 30 cycles of amplification. The gels were stained and analysed by as described before (Reis et al., 2006).

2.6. Detection of active caspase-9

Caspase-9 activity in head-kidney tissues was measured using a Caspase-Glo® assay kit (Promega). In this assay, when the proluminescent substrate containing the tetrapeptide LEHD is cleaved by caspase-9, a substrate for luciferase (aminoluciferin) is released resulting in the luciferase reaction and the production of a luminescent signal. Sea bass weighting approximately 21 g were intra-peritoneally infected with 1.3×10^4 CFU of *Phdp* strain PP3 in 100 μl TSB-1, as described above. Head-kidneys were collected from 9 non-infected sea bass and from groups of 9 infected sea bass sampled at 6, 12, 18, 24 and 48 h after i.p. infection. Since fish showing lethargy and loss of equilibrium were found to die within a very short time, fish with these signs of terminal disease were sampled regardless the time post-infection. In this experimental infection, terminal disease occurred between 46 and 96 h and the cumulative mortality reached 90%. Head-kidney extracts were prepared by homogenization and incubation for 20 min on ice with caspase lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM DTT) and subsequently centrifuged (15 min, 13,000 rpm, 4 °C). An equal volume of Caspase-Glo® 9 reagent and head-kidney extracts were added to the wells of a white-walled 96-well plate and incubated at RT for 10 min. For each extract non-specific cleavage of the substrate was checked by adding a 2 μl of a 2.5 mM caspase-9 inhibitor (Caspase-9 Inhibitor II, Calbiochem). The luminescence of each sample was measured in a plate-reading luminometer (Molecular Devices). The protein concentration of the supernatants was previously determined using the MicroBCA protein Assay Kit (Pierce) and the results expressed as relative luminescence units/μg of protein.

2.7. Sequence analysis

The sea bass caspase-9 amino acid sequence was deduced using expasy translate tool (http://www.expasy.org). Full nucleotide and protein sequences from sea bass caspase-9 were compared to several caspase-9 sequences currently available in the GeneBank database retrieved using the BLAST program (http://www.ncbi.nlm.nih.gov). The multiple alignment was made using CLUSTAL W program (http://www.ebi.ac.uk/clustalw/index.html). The caspase-9 domains and possible *N*-glycosylation sites were based on PROSITE predictions (http://www.expasy.org/prosite). Molecular weight was calculated by expasy compute p*I*/Mw tool (http://www.expasy.org). The neighbour-joining phylogenetic tree was constructed using MEGA2 program (Kumar et al., 2001), using p-distance parameter and complete deletion of gaps. The phylogenetic tree was tested for reliability using 1000 bootstrap replications. The percentages of similarity and identity were calculated by pair-wise alignments by the program needle at http://www.ebi.ac.uk, with first and extending gap of 10 and 0.5, respectively.

3. Results

3.1. cDNA sequence

The full-length sea bass caspase-9 cDNA (accession number DQ345775) was obtained by RT-PCR using mRNA extracted from sea bass peritoneal cells collected 30 min after intraperitoneal inoculation of *Phdp* ECP. The amplified DNA fragment of 1924 bp shows significant homology with the caspase-9 of other vertebrates, as revealed by the BLAST result. Overall, this cDNA fragment consists of 1308 bp open reading frame (ORF), 199 bp of the 5′-UTR and 417 bp of the 3′-UTR, including a canonical polyadenylation signal (AATAAA) 10 nucleotides upstream the polyadenylation tail. The nucleotide sequence was translated into 435 amino acids with a predicted molec-

Table 2
Percentage of identity, similarity and gaps of the complete sequence of caspase-9 from other species and sea bass

Species	*Dicentrarchus labrax*			Accession numbers
	Similarity (%)	Identity (%)	Gaps (%)	
D. rerio	77.5	67.0	6.0	AAH97103
Tetraodon nigroviridis	75.4	67.1	12.0	CAG01765
R. norvegicus	63.6	46.5	9.6	NM_031632
M. musculus	63.4	47.1	9.6	NM_015733
H. sapiens	63.3	48.0	8.3	AY214168
X. laevis	62.5	47.4	9.2	AB038172
Canis familiaris	62.4	48.0	7.9	DQ116956

The results were based in pair alignments of some complete sequences from other species with sea bass using the programme needle in the server http://www.ebi.ac.uk.

ular weight of 49.0 kDa. A CARD domain profile in the N-terminus (M_1 to E_{90}) was identified by PROSITE, as well as the caspase p20 (P_{169} to E_{301}) and p10 (A_{344} to S_{431}) domain profiles. A caspase family histidine active site (caspase family signature) H_{234}SQYDCCVVIMLSHG$_{248}$ and a caspase family cysteine active site K_{288}PKLFFIQACGG$_{299}$ as well as an *N*-glycosylation site (N_{12}RTN) were also predicted.

Multiple alignment of sea bass caspase-9 with those from other species shows that the active site pentapeptide QACGG is conserved and located in the large subunit (Fig. 2). Furthermore, it suggests Asp_{325} and Asp_{343} as the two sea bass putative cleavage aspartic acids that separate the large and small subunits (Fig. 2). Due to the much higher variability in the prodomain it is not clear in the alignment in which aspartic acid may occur the cleavage of the prodomain. However, it is likely that the putative sea bass prodomain cleavage site is located at Asp_{121} (Fig. 2) due to its resemblance with the predicted cleavage motif MDVD found in the prodomain of sea bass caspase-3 (Reis et al., 2006). The amino acids residues His_{247}, Gly_{248} and Cys_{287}, which have been predicted in human caspase-9 to be involved in catalysis, as well as the residues Arg_{180}, Gln_{285}, Arg_{355} and Ser_{361}, which form a binding pocket for the carboxylate side chain of P1 aspartic acid (Duan et al., 1996), are also conserved in the sea bass caspase-9 sequence.

3.2. Genomic organisation

The full length sea bass caspase-9 gene (accession number DQ345776) has 2972 bp in size organised in 10 exons and 9 introns (Fig. 3) and with the donor and acceptor exon/intron splice junctions conforming to the GT/AG rule (Breathnach and Chambon, 1981).

The result of the southern blot analysis (Fig. 4) suggests that the caspase-9 gene in sea bass genome is a single copy gene.

3.3. Phylogenetic analysis

The sea bass caspase-9 sequence exhibits a very close homology to the ones of other vertebrates (Table 2), with higher similarity (>75.4%) and identity (67%) with the putative caspases-9 from *T. nigroviridis* and *D. rerio*. In relation to the non-fish vertebrates, the degree of identity ranges from 48.0% (*H. sapiens*/*C. familiaris*) to 46.5% (*R. norvegicus*). Caspase-9 sequence from *R. norvegicus* shows the highest percentage of similarity (63.6%), while that of *C. familiaris* has the lowest percentage of similarity (62.4%). The results from the pair-wise alignments are supported by the neighbour-joining tree data (Fig. 5), which groups all the fish caspase-9 sequences together, apart from the non-fish vertebrates. All caspase-9 sequences are clustered together apart from the caspase-3 sequences.

3.4. Caspase-9 gene expression and detection of activated caspase-9

In control fish, caspase-9 has a low basal expression in all the organs analysed with weaker expression in spleen (Fig. 6). In the head kidney of *Phdp*-infected fish, the results show an increase in sea bass caspase-9 expression at 12 h returning to normal levels at 24 h (Fig. 7), when the fish were with very advanced septicaemia. Apoptotic cells, identified by chromatin condensation, nuclear fragmentation and formation of apoptotic bodies (Wyllie et al., 1980) were seen in infected head kidneys. Caspase-9 activity was detected by a luminescence assay using a proluminescent substrate containing the caspase-9 specific cleavage tetrapeptide LEHD (Fig. 8). Caspase-9 activity was present from 18 to 48 h post-infection, when the fish were with very advanced septicaemia and many were in terminal disease.

4. Discussion

BLAST and Prosite analysis clearly indicate that the obtained sea bass sequence is a caspase-9 homologue. Prosite analysis identified a CARD at the NH_2-terminus and both p20 and p10 domains in the C-terminus, characteristic of caspase-9 molecules. The presence of the CARD has great importance. Indeed, upon induction of apoptosis, pro-apoptotic proteins can trigger mitochondrial outer membrane permeabilization (Narita et al., 1998; Shimizu et al., 1999) and release of cytochrome *c* into the cytosol (Liu et al., 1996). Cytochrome *c* then binds to apoptotic protease-activating factor-1 (Apaf-1) increasing Apaf-1 affinity for dATP or ATP and inducing nucleotide binding to the Apaf-1/cytochrome *c* complex (Jiang and Wang, 2000). This results in Apaf-1 oligomerisation and exposure of its caspase recruitment domain (CARD), forming the apoptosome (Saleh et al., 1999; Zou et al., 1997, 1999). Interactions of CARD from Apaf-1 and that of procaspase-9 leads to the recruitment

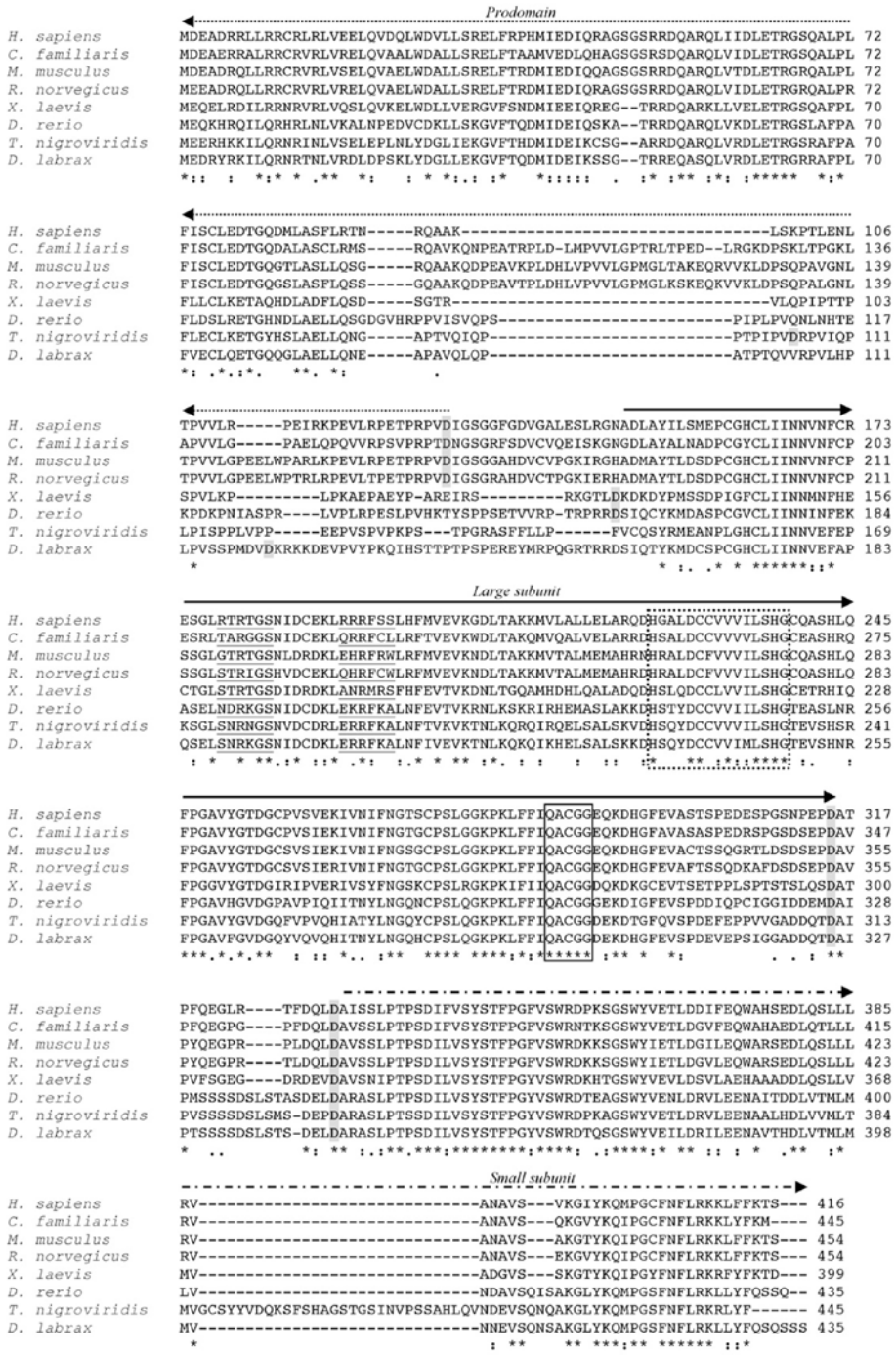

Fig. 2. Multiple alignment of the caspase-9 amino acid sequence of sea bass and other vertebrate. The pentapeptide active-site motif (QACRG) and the caspase family signature are boxed in a continuous and discontinuous line, respectively. The putative cleavage site at aspartic acid residues, which separates the prodomain (◄┈┈) from the large subunit (⟶) and this one from the small subunit (·-►), are shaded in gray. Underlined are the consensus and putative Akt phosphorylation motifs. The numbers indicate the amino acid positions and dashes indicate gaps introduced to optimize similarity between sequences. Asterisks and ": and ." denote residues identical in all sequences and chemical similarity between amino acids, respectively.

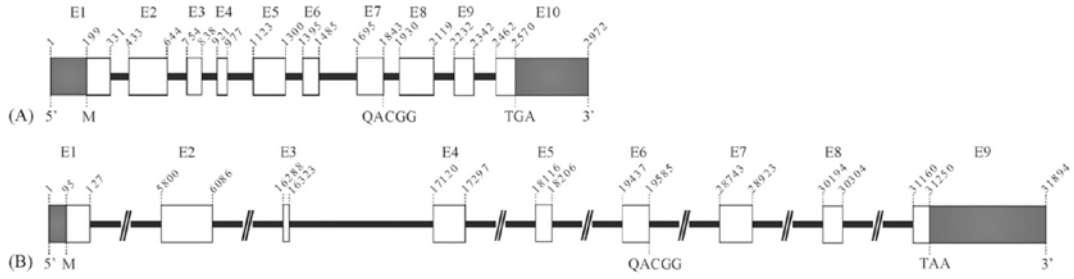

Fig. 3. Schematic representation of genomic organisation of caspase-9 from sea bass (A), and human (B). The genomic organisation of human caspase-9 was obtained from the Ensembl Genome site (http://www.ensembl.org) and the transcript ID is ENST00000333868. The exons are indicated by E above the boxes and introns by the solid line (introns larger than 800 bp are represented by a solid line interrupted by two lines). Coding nucleotides and untranslated regions are differentiated by black and white boxes, respectively. Values above boxes represent the nucleotide number.

of multiple procaspase-9 molecules into the proximity of one another and their dimerization and subsequent autoactivation (Li et al., 1997; Saleh et al., 1999; Zou et al., 1999). The characteristic caspase-9 pentapeptide active-site QACGG (Duan et al., 1996) is conserved in the sea bass sequence and also located in the large subunit (Nishiyama et al., 2001; Srinivasula et al., 1996) (Fig. 2). In humans, the cleavage site between the large and small caspase-9 subunits occurs in $PEPD_{315}$ and $DQLD_{330}$,

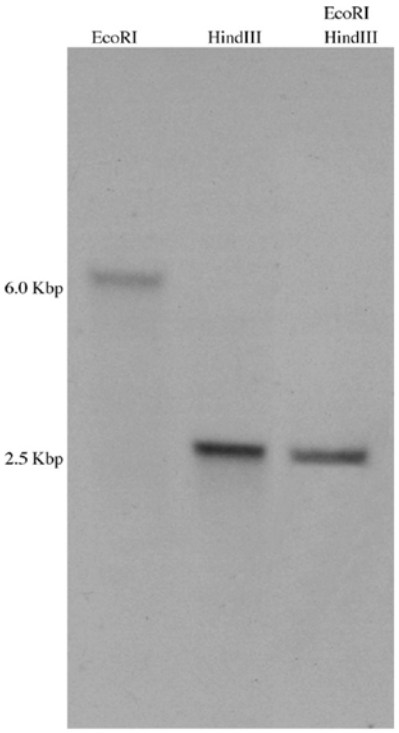

Fig. 4. Southern blot of genomic organisation of sea bass caspase-9. Genomic DNA was digested with *Eco*RI and *Hin*dIII (individually and combined) and hybridized with a probe, which was constructed from the cDNA. The numbers indicate the size in kilo bases from the Hipper ladder I marker (Bioline).

which are also described as granzyme B and caspase-3 cleavage sites (Srinivasula et al., 1996). Thus, by matching this cleavage sites in the sequences of caspase-9 from *H. sapiens* with the sequence obtained for sea bass it is possible to determine the putative cleavage sites at Asp_{325} and Asp_{343}, and the two possible locations of the large and small subunits. These two potential cleavage sites are conserved in all species analysed. The putative cleavage site that separates the prodomain and the large subunit is not so evident in any of the fish sequences since, due to the much higher variability of the prodomains, none of the Asp present in the fish sequences clearly aligns with the ones reported for human Asp_{130} (Duan et al., 1996) and rat Asp_{168} (Angelastro et al., 2001; Nishiyama et al., 2001). However, in sea bass caspase-3 the putative cleavage aspartic acid that separates its prodomain has been predicted in the context of the motif MDVD (Reis et al., 2006), which is also present in the caspase-9 sequence. Therefore, it is likely that the putative sea bass prodomain cleavage site is located at Asp_{121} (Fig. 2). Further support for this interpretation comes from the fact that all the aspartic acid residues mentioned above as putative cleavage sites are presented in the context of tetrapeptide motifs that have been shown to fulfil the requirement for caspase substrate motifs (Thornberry et al., 1997). Based on the X-ray crystal structure of ICE/caspase-1, it has been shown (Duan et al., 1996) that the amino acids residues involved in catalysis and the ones that form a binding pocket for the carboxylate side chain of P1 aspartic acid are conserved in all ICE/Ced-3 family members. These residues are also conserved in the sea bass caspase-9 sequence as well as in the two putative caspase-9 fish genes here analysed.

It has been reported that human caspase-9 can be inactivated by Akt phosphorylation, a form of post-translational modification employed to modulate caspase activity. Human caspase-9 contains sites that are conforming to the consensus Akt phosphorylation motif RXRXX(S/T) at Ser_{183} (RTRTGS) and Ser_{196} (RRRFSS). The latter site is recognized and phosporylated by Akt (Chang and Yang, 2000). However, this regulation does not seem to be conserved in all species, because mouse, rat and monkey caspase-9 do not contain the Akt motif found in human caspase-9 (Chang and Yang, 2000; Fujita et al., 1999) and in *Xenopus* caspase-9 the corresponding sequences (STRTGS and ANRMRS) do not fulfil the requirements of the consensus

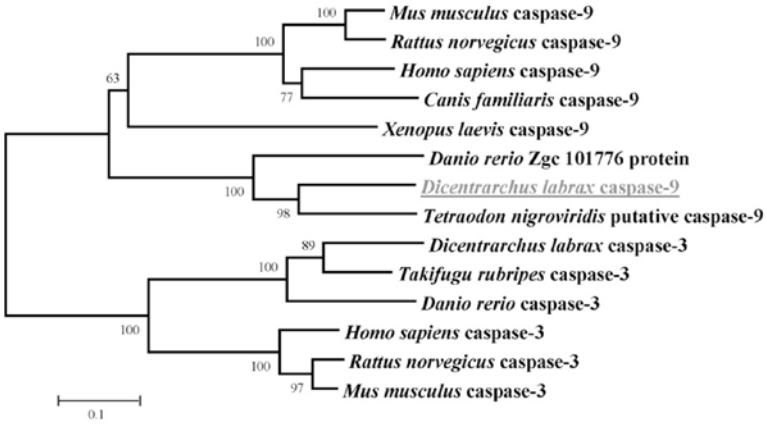

Fig. 5. Neighbour-joining tree (MEGA 2) with p-distance and complete deletion of gaps of caspase-9 and -3 amino acid sequences. The sequences were aligned using CLUSTALW according to default parameters. The numbers in branches nodes denote the bootstrap percentages for 1000 replicates. The GenBank accession numbers of the sequences used are: for caspase-3 – DQ345773 (*Dicentrarchus labrax*), AAM43816 (*Takifugu rubripes*), BAB32409 (*Danio rerio*), CAC88866 (*Homo sapiens*), AAH81854 (*Rattus norvegicus*), AAH81854 (*Mus musculus*); for caspase-9 – see Table 2. The sequence of *Tetraodon nigroviridis* putative caspase-9 was obtained by blast search with the sequence of sea bass caspase-9 as query.

motif (Nakajima et al., 2000). In sea bass, the corresponding sequences (S_{188}NRKGS and E_{201}RRFKA) do not match the consensus motifs, although as hypothesized for *Xenopus* (Nakajima et al., 2000), it cannot be disclosed that sea bass may have a different recognition sequence compared with the human protein.

The human caspase-9 gene has been characterised and was found to contain 9 exons and 8 introns spanning approximately 35 kbp of the genomic DNA mapped to human chromosome 1p36.1–p36.3 (Hadano et al., 1999). In sea bass genome, caspase-9 gene has 2972 bp and is organised in 9 exons and 10 introns. The extra exon in sea bass caspase-9 gene is located in

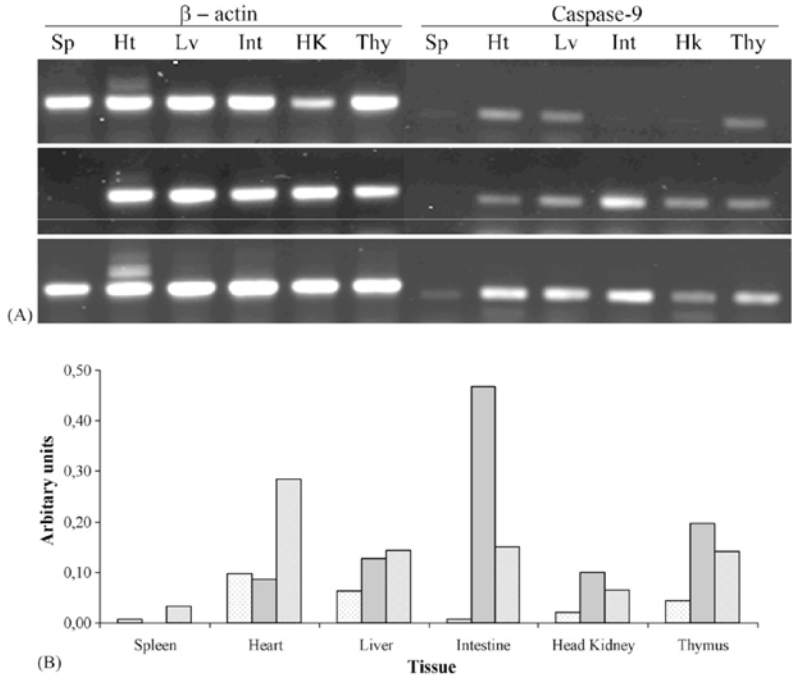

Fig. 6. (A) RT-PCR analysis of sea bass caspase-9 and β-actin gene expression in different tissues from three non-stimulated fish. SP: spleen; Ht: heart; Lv: liver; Int: intestine; HK: head kidney; Thy: thymus. (B) Ratios between the densitometry values of caspase-9 and β-actin expression of for each fish (□, ■ and ▨).

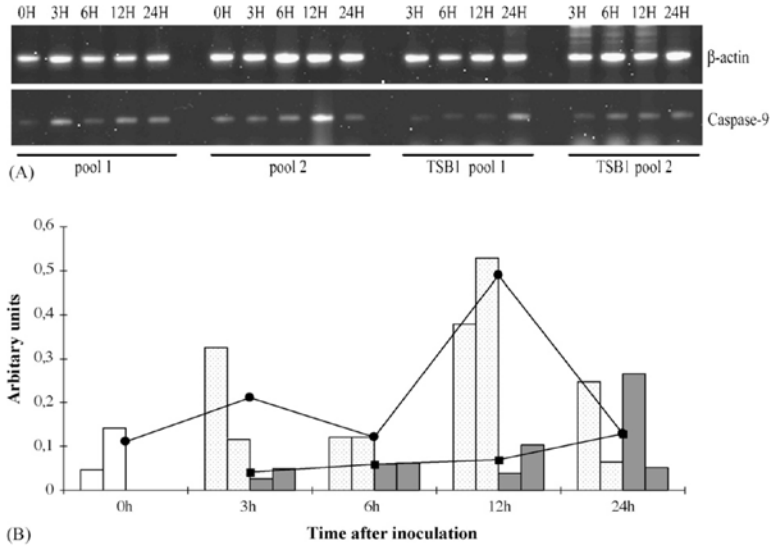

Fig. 7. (A) RT-PCR analysis of sea bass caspase-9 and β-actin gene expression in head kidneys from infected fish. (B) Ratios between the densitometry values of caspase-9 gene and β-actin gene from: non-infected fish (□), fish infected with *Phdp* (▨) and fish injected with TSB1 (▩). The average between the infected and TSB1 inoculated fish is represented by (●) and (■), respectively.

the predicted prodomain. The intron lengths vary from 83 to 210 nucleotides, which are significantly smaller than the ones from the human caspase-9 gene (797–10202 nucleotides) rendering an approximately 10 times smaller gene than the human caspase-9 gene. The results from the Southern blotting hybridization suggest that the caspase-9 gene exists as a single copy in sea bass genome as reported for rat caspase-9 (Angelastro et al., 2001).

The phylogenetic analysis shows that sea bass caspase-9 has a great homology with that of other vertebrate species, particularly with the putative caspase-9 sequences from the other two fish analysed (Table 2). The higher degree of similarity between the caspase-9 genes of the fish species analysed is supported by the multiple alignment and the BLAST search, as well as the neighbour-joining tree, where fish are clearly clustered in a group apart from the non-fish vertebrates. The high similarity observed between sea bass caspase-9 and those from other animals may imply a functional homology which is expectable, since processes involving caspase-9 are evolutionarily conserved (Hu et al., 1998).

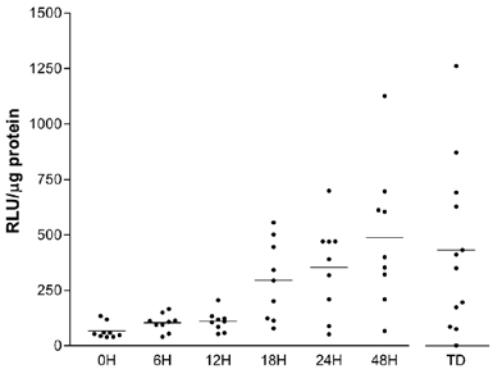

Fig. 8. Caspase-9 activity in head-kidney lysates. Caspase-9 activity of each individual (dots) and average (horizontal line) at each time point, expressed as relative luminescent units (RLU) per microgram of protein in the supernatants of head-kidney extracts.

In humans, caspase-9 is distributed in a wide range of tissues, being the heart the one with higher levels, followed by liver, skeletal muscle and pancreas; the lowest levels were detected in spleen, thymus, small intestine and kidney (Srinivasula et al., 1996). Testis and ovary have also been shown to have high expression levels (Duan et al., 1996). In rat, the expression levels are not much different from those in humans; heart, liver and embryo showed the higher levels of caspase-9 expression while in brain, spleen, testicle and kidney the caspase-9 expression is moderated (Angelastro et al., 2001). The sea bass caspase-9 expression in several tissues was analysed by semi-quantitative RT-PCR. As it was detected for human and rat, heart and liver showed the higher expression levels while spleen had the lowest expression level. Sea bass thymus and intestine showed similar expression levels as the heart and liver, and head kidney (mammalian bone marrow equivalent) slightly lower but higher than spleen.

It was recently shown by our group that *Phdp* secretes the apoptogenic exotoxin AIP56 that triggers apoptosis in sea bass phagocytes involving induction of caspase-3 activity (do Vale et al., 2003, 2005) (Reis et al., 2006). Here it is shown that the expression level of caspase-9 in an experimental sea bass septicaemia infection by *Phdp* followed the same kinetics as the caspase-3 expression (Reis et al., 2006), increasing from 0 to 12 h post-infection in infected head kidneys and declining to basal levels at 24 h. The decrease of expression at the later stages of

the infection likely reflects the occurrence of progressive breakdown of DNA (Nagata et al., 2003) and RNA (Bushell et al., 2004; Mondino and Jenkins, 1995) molecules during the degradation phase of apoptosis. Furthermore, caspase-9-like activity could be measured from 18 to 48 h post-infection as well as in fish with terminal disease. This data suggests that the mitochondrial pathway is involved in the *Phdp* septicaemic fish disorder. Whether this involvement is directly triggered by the AIP56 exotoxin or the mitochondrial/intrinsic pathway is functioning as a machinery for amplification of caspase activity (Marsden and Strasser, 2003) remains to be elucidated.

In conclusion, this work reports for the first time the sequencing and characterisation of caspase-9 in fish. It is shown that a counterpart of mammal caspase-9 gene is present in sea bass and that caspase-9 activity is apparently involved in the apoptosis of macrophages and neutrophils induced by *Phdp* in sea bass pasteurellosis.

Acknowledgements

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CHAPTER V

Molecular cloning and characterisation of sea bass (*Dicentrarchus labrax* L.) caspase-3 gene.

Marta I.R. Reis, Diana S. Nascimento, Ana do Vale, Manuel T. Silva and Nuno M.S. dos Santos

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Molecular cloning and characterisation of sea bass (*Dicentrarchus labrax* L.) caspase-3 gene

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Abstract

Caspase-3 is one of the major caspases operating in apoptosis, cleaving and inactivating a number of molecules and largely contributing to the apoptotic phenotype and the dismantling of the apoptoting cell.

The opening reading frame of sea bass (*Dicentrarchus labrax* L.) caspase-3 has 281 amino acids. The complete sequence of caspase-3 shows a very close homology to the correspondent sequence from other vertebrates, in particularly with that of *Takifugu rubripes* and *Oryzias latipes*, with 87.7 and 87.9% of similarity, respectively. Furthermore, the sea bass caspase-3 sequence retains the motifs that are functionally important, such as the pentapeptide active-site motif (QACRG) and the putative cleavage sites at the aspartic acids. In the sea bass genome, the caspase-3 gene exists as a single copy gene and is organised in six exons and five introns.

A very low expression of caspase-3 was detected by RT-PCR in various organs of non-stimulated sea bass, with slightly higher levels in thymus and heart and was increased in head kidneys of *Photobacterium damselae* ssp. *piscicida* infected sea bass. This increased expression was accompanied by the occurrence of high numbers of apoptoting cells with activated caspase-3.
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Keywords: Sea bass; Apoptosis; Caspase-3; *Photobacterium damselae* ssp. *piscicida*

1. Introduction

Caspases are a family of cysteine-dependent aspartate specific proteases sharing similarities in amino acid (aa) sequence and structure and can be broadly divided into two groups: cytokine processors and apoptosis initiators or effectors (Fink and Cookson, 2005; Ho and Hawkins, 2005; Wolf and Green, 1999).

Caspase-3 is a caspase that normally exists in the cytosol and in the mitochondrial intermembrane space of resting cells as a 32-kDa inactive zymogen that is proteolytically activated in many cases of apoptosis (Fink and Cookson, 2005; Kroemer et al., 1995; Wolf and Green, 1999) giving rise to the active form (Nicholson et al., 1995). Activated caspase-3 is a central effector of apoptosis that cleaves and inactivates a number of molecules contributing to the typical morphology of apoptosis and leading to the dismantling of the apoptoting cell (Fischer et al., 2003; Wolf and Green, 1999). Besides activation of pre-formed caspase-3 zymogen, increased gene expression of caspase-3 has been described in cells under apoptosis (Chiang et al., 2001; Miller et al., 1997; Yakovlev et al., 2001).

It has been recently described that a key virulence mechanism of the fish pathogen *Photobacterium damselae* ssp. *piscicida* (*Phdp*) is the induction of apoptosis of macrophages and neutrophils (do Vale et al., 2003). This pathogenic apoptosis was found to be mediated by the *Phdp* exotoxin AIP56 and to involve caspase-3-like activity (do Vale et al., 2003). Since *Phdp*-induced apoptosis plays an essential role in the virulence of that pathogen (do Vale et al., 2003, 2005) and the apoptotic cell death is still poorly understood in fish, specially in sea bass where no caspase genes have been reported so far, we have sequenced and characterised the sea bass caspase-3 and assessed its expression following an apoptotic stimulus.

2. Material and methods

2.1. Fish

Sea bass, *Dicentrarchus labrax*, were kept in a recirculation, UV-treated salt-water (30–35%) system at 20 ± 1 °C, and fed

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at a ratio of 2% body weight per day. For collection of organs fish were euthanised with a lethal dose of 2-phenoxyethanol (Panreac; >5 mL/10 L).

2.2. cDNA cloning and sequencing

Thymuses from two fish (500 g) were used for mRNA extraction according to MicroPoly(A)Pure™ (Ambion®). Superscript™ II First-Strand Synthesis System (Invitrogen–Life Technologies) was used to reverse-transcribe the first cDNA strand using 400 ng of mRNA and the adaptor/anchor primer APv (Table 1). Degenerate primers designed based on conserved regions from the multiple alignment (CLUSTAL W) of caspase-3 aa sequences from different vertebrates (*Felis catus*, *Rattus norvegicus*, *Gallus gallus*, *Takifugu rubripes* and *Danio rerio*) were used to first amplify the cDNA (2 μL) with primers CASP3FW1 and CASP3RV1 (Table 1) in a 50 μL reaction (0.2 mM dNTPs mix, 1.5 mM $MgCl_2$, 5 μL 10× buffer, 0.4 μM of each primer and 1.25 units of Taq polymerase) with the following conditions: 94 °C for 2 min; 30 cycles of amplification (94 °C for 45 s; 50 °C for 1 min; 72 °C for 25 s); 72 °C for 5 min. A semi-nested PCR was performed using the same conditions as described above and primers CASP3FW1 and CASP3RV2 (Table 1). The PCR product (359 bp) was purified (Gel extraction Kit, Qiagen), ligated overnight at 4 °C and used to transform *E. coli* XL1 competent cells following the pGEM-T easy Vector System (Promega). Transformants were plated on LB agar medium containing ampicilin (200 μg/mL) and IPTG/X-GAL (50 μg/mL each) and incubated at 37 °C overnight. The plasmid DNA of one positive colony was extracted (QIAprep Spin Miniprep kit, Qiagen) and used (200–300 ng) with vector primers T7 and SP6 (Table 1) in sequencing reactions performed according with Big Dye terminator 2.0 (Applied Biosystems), and analysed in an automated sequencer. The sequence was used to design the primers DLCASP3RV2 and DLCASP3RV1 (Table 1), used for obtaining the 5′ untranslated region (UTR) following the 5′ RACE System Kit (Gibco). The obtained PCR product was purified, cloned and sequenced as previously described. In order to obtain the 3′-UTR, a first amplification was performed on the cDNA with the primer DLCASP3FW1 and the Abridged Universal Amplification Primer (AUAP) (Table 1) in a 50 μL reaction (as previously) with the following conditions: 94 °C for 2 min; 30 cycles of amplification (94 °C for 45 s; 58 °C for 30 s; 72 °C for 1 min); 72 °C for 5 min. A semi-nested amplification on the PCR product with the primers DLCASP3FW2 (Table 1) and AUAP was done as before except for annealing temperature (60 °C). After reamplification a band with 480 bp was cloned and sequenced as describe before. Since the primer AUAP was annealing unspecifically upstream the poly A tail, the primer DLCASP3RV5 (Table 1) was designed in the obtained 3′-UTR stretch and used with DLCASP3FW8 (Table 1) for amplifying the complete sea bass caspase-3 fragment in a 50 μL reaction (0.2 mM dNTPs mix, 1.5 mM $MgCl_2$, 5 μL 10× buffer, 0.4 μM of each primer, 1.25 units of Taq polymerase and

Table 1
Degenerate and specific primers

Primer	Sequence
APv	5′-GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT TT (AGC)-3′
AUAP	5′-GGC CAC GCG TCG ACT AGT AC-3′
APv2	5′-GAC TCA GGA CTT CAG GAC TTA GTT TTT TTT TTT TTT TTT (AGC)-3′
AUAP2	5′-GAC TCA GGA CTT CAG GAC TTA G-3′
T7	5′-TAA TAC GAC TCA CTA TAG GGC GA-3′
SP6	5′-CTA TTT AGG TGA CAC TAT AGA ATA C-3′
CASP3FW1	5′-AAY GGN ACN GAY GTN GAY GC-3′
CASP3RV1	5′-CAI ARN SWY TGN ATR AAC CA-3′
CASP3RV2	5′-ARR AAR TCN GCY TCN ACN GGD AT-3′
DLCASP3FW1	5′-CCA GGC ATT GAA ACA GAC AGT G-3′
DLCASP3FW2	5′-GAT CCC TGT AGA GGC TGA CTT C-3′
DLCASP3FW4	5′-CAA TGC TGA CCA AGG AGA TG-3′
DLCASP3FW6	5′-ACA AGT GCA TCT GCG CAT GA-3′
DLCASP3FW7	5′-GAC AGG AGA ACA GGC ATG AA-3′
DLCASP3FW8	5′-ACG GTA GGC GTG GGA GTG AA-3′
DLCASP3FW9	5′-GTC GTG CAT TCC GTA CAT TG-3′
DLCASP3FW10	5′-TGG TGC GAG TAC GTT CCT GT-3′
DLCASP3FW11	5′-CGA CGG ACA AGA GTC GGA-3′
DLCASP3RV1	5′-TTC ATC TGC TCG ACT GTC TG-3′
DLCASP3RV2	5′-GGC GTT GCA GCT GTG ATC T-3′
DLCASP3RV3	5′-ACA GGA AGT CAG CCT CTA CA-3′
DLCASP3RV5	5′-CCC CCA CTA AAG GAA CCC TA-3′
DLCASP3RV6	5′-GGC AGT AGG TGT GAC GTT GA-3′
DLCASP3RV7	5′-CGT GGG AGT CTA AGT GTG AA-3′
DLCASP3RV8	5′-CCT CCA TGA GTA GTA GCC TGG G-3′
DLCASP3FWXhoI	5′-CCG CTC GAG CGT GGG AGT GAA AAA CAA GTG-3′
DLCASP3RVXhoI	5′-GGC CTC GAG CCC ACT AAA GGA ACC CTA AAA TG-3′
DLBACTINFW	5′-ATC GTG GGG CGC CCC AGG-3′
DLBACTINRV	5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′

Y: C/T; N: A/C/T/G; I: inosine; R: A/G; S: C/G; W: A/T; D: G/T/A.

0.15 units of Pfu DNA Polymerase) with the following conditions: 94 °C for 2 min; 30 cycles of amplification (94 °C for 45 s; 62 °C for 1 min; 72 °C for 1 min); 72 °C for 5 min. A semi-nested amplification was performed using DLCASP3FW6 and DLCASP3RV5 (Table 1) as above except for the annealing temperature (58 °C). The amplified product (1235 bp) was cloned as previously and three positive colonies sequenced with the primers T7, SP6 and DLCASP3FW7 (Table 1) at MWG (https://ecom.mdwdna.com).

To obtain the complete 3′-UTR, total RNA was extracted according to Versagene™ RNA tissue kit (Gentra Systems) from head kidney collected 3 h after injection of *Phdp*. The RNA was reverse transcribed into cDNA according to BioScript RNase H Minus kit (Bioline) using APv2 and amplified using primers DLCASP3FW4 and APv2 (Table 1) in a 50 μL reaction (0.2 mM dNTPs mix, 1.5 mM $MgCl_2$, 5 μL 10× buffer, 0.4 μM of each primer and 1.25 units of Taq polymerase) with the following conditions: 94 °C for 2 min; 30 cycles of amplification (94 °C for 45 s; 58 °C for 1 min; 72 °C for 1 min 30 s); 72 °C for 5 min. A semi-nested amplification was performed using DLCASP3FW4 and AUAP2 (Table 1) following the conditions as the first amplification. The amplified product (1378 bp) was cloned and three positive colonies sequenced as above.

2.3. Genomic DNA cloning and sequencing

Genomic DNA was isolated from sea bass erythrocytes as described (Stet et al., 1993). To obtain the caspase-3 gene sequence, the genomic DNA was used as template with primers DLCASP3FWXhoI and DLCASP3RVXhoI (Table 1), designed in the 5′- and 3′-UTR and containing a restriction site for *Xho*I, in a two step 50 μL PCR reaction (0.2 mM dNTP mix, 2 mM $MgCl_2$, 5 μL 10× buffer, 0.4 μM of each primer, 2.5 units of Taq polymerase and 0.15 units of Pfu DNA Polymerase) with the following conditions: 94 °C for 2 min; 2 cycles of 94 °C for 45 s, 64 °C for 1 min and 72 °C for 3 min followed by 30 cycles of amplification (94 °C for 45 s; 70 °C for 1 min; 72 °C for 3 min); 72 °C for 5 min. The obtained PCR product was purified using the GFX™ PCR DNA and Gel Band Purification kit (Amersham Biosciences) and, after digesting with *Xho*I, cloned into pGEX-4T-3 or pET-32b vectors. Minipreps (2 μg) from 2 pGEX-4T-3 and 1 pET-32b positive clones were sequenced (https://ecom.mdwdna.com), with the following primers: DLCASP3FW6, DLCASP3FW9, DLCASP3FW10, DLCASP3RV2, DLCASP3RV5, DLCASP3RV6 and DLCASP3RV7 (Table 1).

2.4. Southern blotting

Restriction enzymes *Eco*RI, *Hin*dIII and *Spe*I isolated and combined were used to digest genomic DNA in 40 μL reactions (10 μg of DNA, 4 μL 10× buffer B, and 20 units of each enzyme) overnight at 37 °C. Electrophoresis of the digested DNA was made in 0.8% agarose gel in TAE buffer (40 mM Tris, 0.11% Acid Acetic Glacial, 0.2% 0.5 M EDTA pH 8.0) and subjected to Southern blotting (Ausubel et al., 1999).

A fragment of the sea bass caspase-3 cDNA, including exons 1 (partially), 2, 3, 4 and 5 (partially), was amplified twice with DLCASP3FW6 and DLCASP3RV3 (Table 1) with the following conditions: 94 °C for 2 min; 30 cycles of amplification (94 °C for 45 s; 57 °C for 1 min; 72 °C for 30 s); 72 °C for 5 min. The PCR product (754 bp) was extracted (Gel Extraction Kit, Qiagen) and used as a probe. Labelling of the probe, hibridization and post-hibridization stringency washes were performed accordingly to Gene Images™ AlhPhos Direct™ Labelling and Detection System kit (Amersham Biosciences). For signal generation and detection the Chemiluminescent Signal Generation and Detection with CDP-*Star*™ protocol from the same kit was followed.

2.5. Analysis of caspase-3 expression by RT-PCR

Total RNA was extracted from organs of three non-stimulated fish (10 g) following the instructions of the RNAqueous™—4PCR kit (Ambion). For the first strand cDNA reverse transcription, Superscript III™ protocol (Invitrogen) was followed. The cDNAs were normalised by diluting the cDNAs using a β-actin PCR product as control gene, amplified (2 μL) with the primers DLBACTINFW and DLBACTINRV (Table 1) in 50 μL reactions (0.2 mM dNTPs mix, 2 mM $MgCl_2$, 5 μL 10× buffer, 0.4 μM of each primer and 1.25 units Taq polymerase) with the following conditions: 94 °C for 2 min, 35 cycles of amplification (94 °C for 45 s; 65 °C for 1 min; 72 °C for 30 s); 72 °C for 5 min. Amplifications of caspase-3 transcripts were performed with the same conditions and 12 μL of the cDNA dilutions using the primers DLCASP3FW11 and DLCASP3RV8 (Table 1).

Sea bass (10 g) were infected by intraperitoneal injection of 4.5×10^3 colony forming units/fish of the *Phdp* virulent strain PP3 in 100 μL of tryptic soy broth containing 1% NaCl (TSB1). At 24 h post-infection most fish were moribund or dead, with 100% mortality at 48 h. Control fish received 100 μL of TSB1. Two pools of three head kidneys per time point were used to extract total RNA according to the NucleoSpin® RNA II Kit (Macherey-Nagel) and the Versagene™ RNA tissue kit (Gentra Systems) protocol. RNA was reverse transcribed following the instructions of the BioScript RNase H Minus kit (Bioline) protocol. The same procedure as above was followed to normalise the cDNAs, except that 30 cycles of amplification were used. The caspase-3 transcripts were amplified as above but using 6 μL of cDNA and 30 cycles of amplification.

The gels were stained by soaking (30 min) with ethidium bromide (5 μg/mL TAE) and analysed by densitometry using the ImageQuant® Molecular Dynamics®.

2.6. Detection of activated caspase-3

Detection of activated caspase-3 in the 6 non-stimulated and 12 *Phdp*-infected head kidneys was carried out by immunocytochemistry using a 1:500 dilution of an affinity-purified rabbit anti human caspase-3 active (catalogue no. AF835, R&D Systems, Abingdon, UK). Detection of primary antibody was done using the Histostain®-Plus Bulk Kit (Zymed Laboratories Inc,

San Francisco, USA), following the manufacturer's instructions and using 3-amino-9-ethylcarbazole (AEC) as substrate.

2.7. Sequence analysis

The sea bass caspase-3 aa sequence was deduced using expasy translate tool (www.expasy.org). Full nucleotide and protein sequences from sea bass caspase-3 were compared to several caspase-3 sequences currently available in the GeneBank database retrieved using the BLAST program (www.ncbi.nlm.nih.gov). The multiple alignment was made using CLUSTAL W program (www.ebi.ac.uk/clustalw/index.html). The caspase-3 domains and possible *N*-glycosylation sites were based on PROSITE predictions (www.expasy.org/prosite).

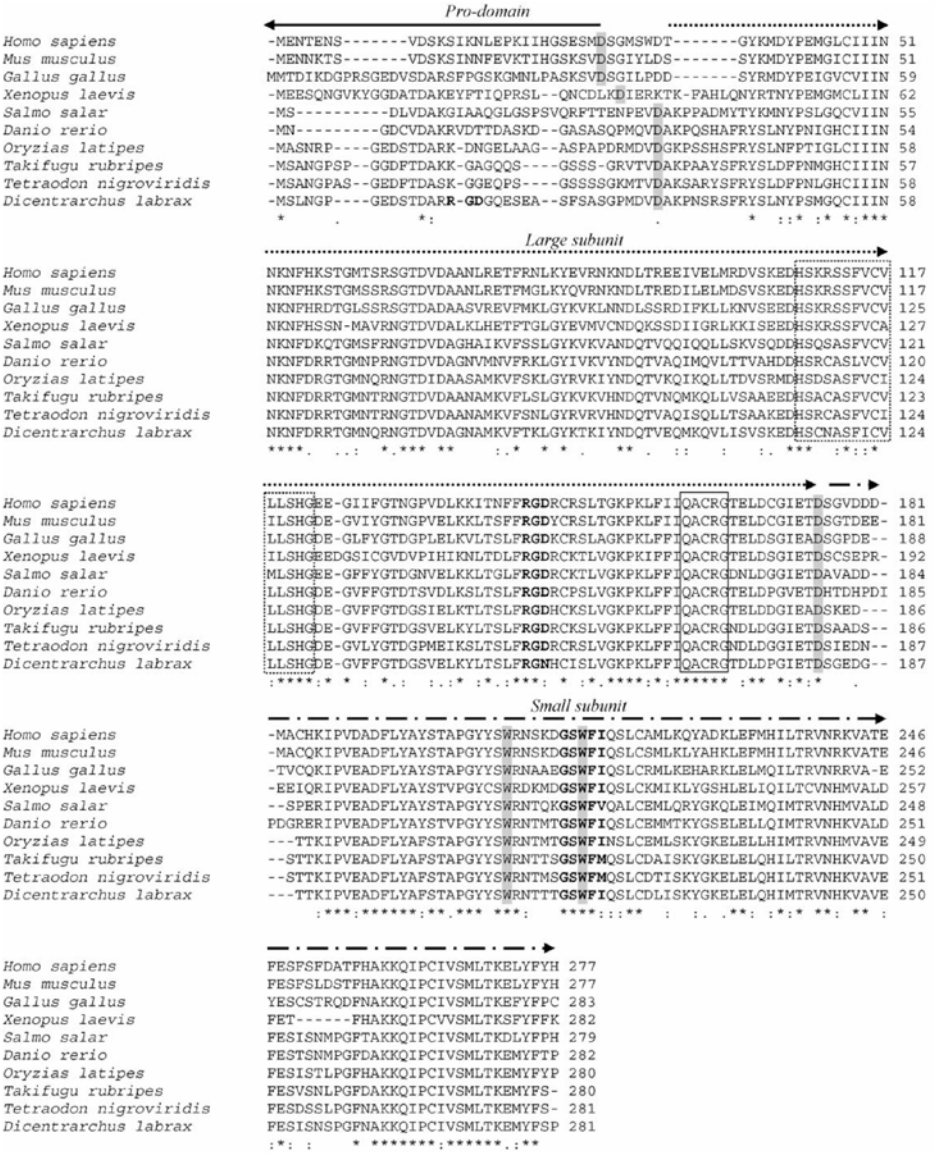

Fig. 1. Multiple alignment of the caspase-3 amino acid sequence of sea bass and other vertebrate caspases-3. The pentapeptide active-site motif (QACRG) and the caspase family signature are boxed in a continuous and discontinuous line, respectively. The protein binding domain (GSWFI) and RGD motifs are in bold. The putative cleavage site at aspartic acid residues, which separates the prodomain (◄——) from the large subunit (·····►) and this one from the small subunit (— · ►), are shaded in gray. The two tryptophan residues that undergo rearrangement when caspase-3 is activated are shaded in gray. The numbers indicate the amino acid positions and dashes indicate gaps introduced to optimize similarity between sequences. Asterisks and ": and ." denote residues identical in all sequences and chemical similarity between amino acids, respectively.

Molecular weight was calculated by expasy compute p*I*/M_w tool (www.expasy.org). The Neighbour-joining phylogenetic tree was constructed using MEGA2 program (Kumar et al., 2001), using *p*-distance parameter and complete deletion of gaps, and tested for reliability using 1000 bootstrap replications. The percentages of similarity and identity were calculated by pair-wise alignments by the program needle at www.ebi.ac.uk, with first and extending gap of 10 and 0.5, respectively.

3. Results

3.1. cDNA sequence

The full-length sea bass caspase-3 cDNA (accession number DQ345773) was obtained by RT-PCR using mRNA extracted from thymus. The sea bass caspase-3 sequence obtained has an open reading frame (ORF) of 846 bp translated into a 281 aa sequence with a predicted molecular weight of 31.1 kDa, a 5′-UTR of 183 bp, and a 3′-UTR of 1321 bp including a polyadenylation site and tail. A BLAST search at www.ncbi.nlm.nih.gov revealed a high degree of homology with many caspases-3 with the first 30 retrievals being caspase-3 with an *E*-value between $7e^{-127}$ and $8e^{-84}$.

Analysis in the PROSITE database predicted that sea bass caspase-3 contains both caspase family p20 (S^{50} to D^{174}) and p10 (G^{187} to P^{281}) domain profiles and a caspase family histidine active site (caspase family signature; H^{155}SCNASFICVLLSHG^{129}) and a caspase family cysteine active site (K^{161}PKLFFIQACRG^{172}). Three *N*-glycosyilation sites (S^{41}FR, T^{188}LK and S^{209}WR) were also predicted.

The pentapeptide active-site motif (QACRG) and the putative aspartic acid (Asp^{182}), which splits the large and small subunits, is conserved among all the sequences from the different species analysed (Fig. 1). The putative aspartic acid (Asp^{34}), which in mammals separates the prodomain from the large subunit, is conserved, although in the alignment in the fish species considered (Fig. 1) is placed 6 nucleotides ahead. Although the prodomain is well conserved among mammals (14 identical and 10 chemically similar aa out of 28), it is less conserved among the fish species analysed (6 identical and 1 chemically similar aa out of 34), and poorly conserved when all the different classes are analysed. This contrasts with the two subunits, which are conserved among the analysed species.

The protein binding domain GSWFI (Johnson and Bridgham, 2000) is also well conserved among the species analysed with only a conservative substitution in the last aa of the motif for *S. salar*, *T. rubripes* and *T. nigroviridis* (Fig. 1). The two tryptophan residues that had been shown to undergo rearrangement during activation of caspase-3, resulting in the reorganisation of the active site (Park et al., 2004) are also conserved among the species analysed (Fig. 1), suggesting that in fish the same process of reorganisation can occur. Furthermore, an integrin-recognition motif (RGD) (Pierschbacher and Ruoslahti, 1984; Ruoslahti, 1996) near the active site was found to be conserved in all species analysed with the exception of sea bass, where the aspartate residue is replaced by an asparagine (N^{153}). On the other hand, an RGD motif with unknown function is present exclusively in the prodomain region of the sea bass caspase-3 sequence (R^{15}GD).

3.2. Genomic organisation

The sea bass caspase-3 gene (accession number DQ345774), although different from *D. rerio* and *H. sapiens*, has the same genomic organisation as the caspase-3 gene from *T. rubripes* with six exons and five introns (Fig. 2). There is a high variabil-

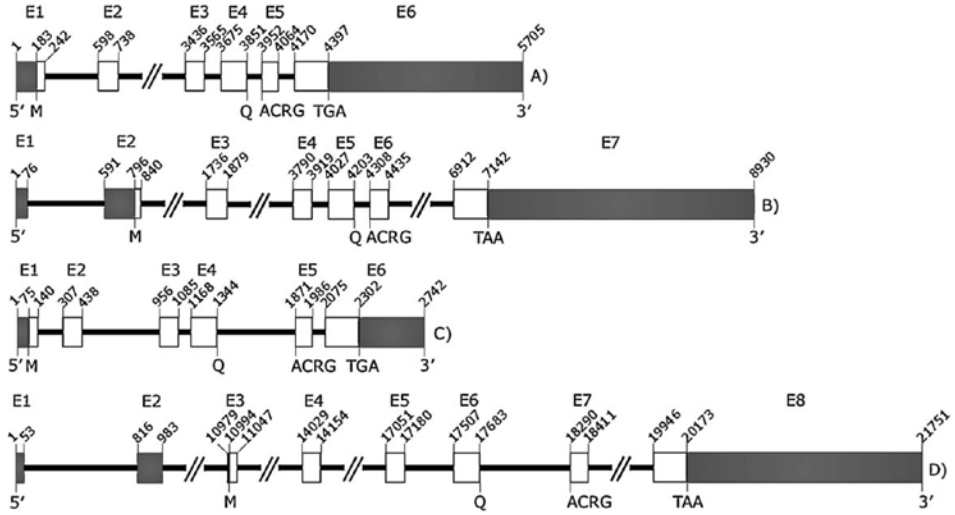

Fig. 2. Schematic representation of genomic organisation of caspase-3 from sea bass (A), zebrafish (B), puffer fish (C) and human (D). The genomic organisations of human, puffer fish and zebrafish caspase-3 were obtained from the Ensembl Genome site (www.ensembl.org) and the transcripts ID are ENST00000308394, SINFRUT00000160403 and ENSDART00000005593, respectively. The exons are indicated by *E*(*x*) above the boxes and introns by the solid line (introns larger than 800 bp are represented by a solid line interrupted by two lines).

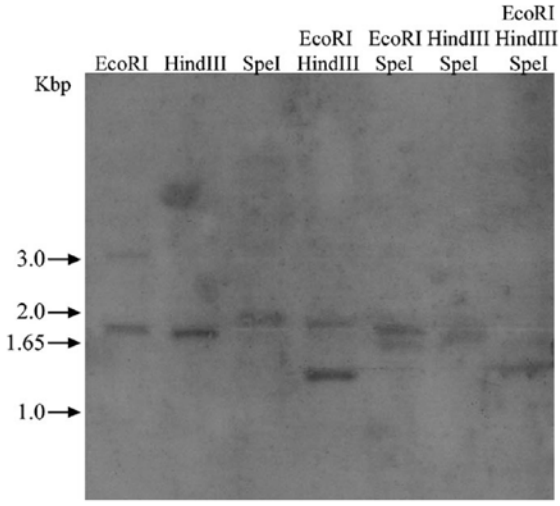

Fig. 3. Southern blot of genomic organisation of sea bass caspase-3. Genomic DNA was digested with *Eco*RI, *Hin*dIII and *Spe*I (individually and combined) and hybridized with a probe, which was constructed from the cDNA. The numbers indicate the size in kilo bases from the Hipper ladder I marker (Bioline).

ity in the length of the introns, which are significantly bigger in the human gene. The donor and acceptor exon/intron splice junctions is conform to the GT/AG rule (Breathnach and Chambon, 1981). Within all caspase-3 genes analysed the pentapeptide active-site motif (QACRG) is interrupted after its first aa (Q) by an intron.

Southern blot analysis (Fig. 3) revealed that the caspase-3 gene in sea bass genome is a single copy gene.

3.3. Phylogenetic analysis

Pair-wise alignments reveal the higher percentage of similarity (>87%) and identity (>79%) of sea bass caspase-3 with that of other fishes (Table 2), including the unnamed protein product from *T. nigroviridis*, retrieved using blast analysis at the ensemble data base, suggesting its probable identification as caspase-3. In relation to the non-fish vertebrates, the degree of identity ranges from 70.4% (*S. scrofa*) to 50.2% (*X. laevis*). Concerning the similarity, caspase-3 sequence from *G. gallus* shows the highest percentage (73%), while that of *C. familiaris* has the lowest percentage of similarity (57.5%). The results from the pair-wise alignments are supported by the neighbour-joining tree data (Fig. 4), which cluster all the caspase-3 and -9 sequences in their respective group. Moreover, it groups together all the fish caspases-3, with closer relation with the one from *X. laevis*, while grouping all the mammalian sequences with closer relation with the one from *G. gallus*. These data also places sea bass caspase-3 more closely related to that of *T. rubripes* and *T. nigroviridis* than to the other fish species analysed.

3.4. Caspase-3 gene expression and detection of activated caspase-3

A very low expression of caspase-3 was detected in spleen, head kidney, liver and intestine of non-stimulated sea bass, and with slightly higher levels in thymus and heart (Fig. 5). An increase in caspase-3 expression was found in the head kidneys of *Phdp*-infected fish at 3, 6 and 12 h after infection, decreasing to initial values at 24 h (Fig. 6) when the fish were with very advanced septicemia.

Apoptotic cells, identified by chromatin condensation, nuclear fragmentation and formation of apoptotic bodies (Wyllie et al., 1980) were seen in infected head kidneys. Activated caspase-3 was present in most of these apoptoting cells and apoptotic bodies, as revealed by immunocytochemistry; activated caspase-3 was already present at 17 h post-infection (Fig. 7A) and abundant at 39 h (Fig. 7B). Head kidneys of non-infected fish showed very rare apoptotic cells (not shown).

Table 2
Percentage of identity, similarity and gaps of the complete sequences of caspase-3 from other species and sea bass

Species	*Dicentrarchus labrax*			Accession numbers
	Similarity (%)	Identity (%)	Gaps (%)	
Takifugu rubripes	87.7	79.6	2.5	AAM43816
Oryzias latipes	87.9	77.9	0.4	BAC00948
Tetraodon nigroviridis	84.9	76.1	2.1	CAF96609
Danio rerio	81.9	72.1	3.8	BAB32409
Salmo salar	63.3	79.7	4.2	AAY28972
Sus scrofa	59.2	70.4	3.5	NP 999296
Felis catus	59.0	69.4	6.2	NP_001009338
Canis familiaris	57.5	69.3	5.6	Q8MKI5
Gallus gallus	73.0	59.2	2.8	AF083029
Oryctolagus cuniculus	71.2	58.3	6.2	Q8MJC3
Rattus norvegicus	69.7	58.1	3.5	AAH81854
Mus musculus	69.4	57.7	3.5	CAA73528
Pan troglodytes	69.4	56.7	3.5	NP 001012435
Homo sapiens	68.3	56.0	3.5	CAC88866
Xenopus laevis	61.7	50.2	3.2	P55866

The results were based on pair alignments of all species sequences with sea bass using the programme needle in the server www.ebi.ac.uk.

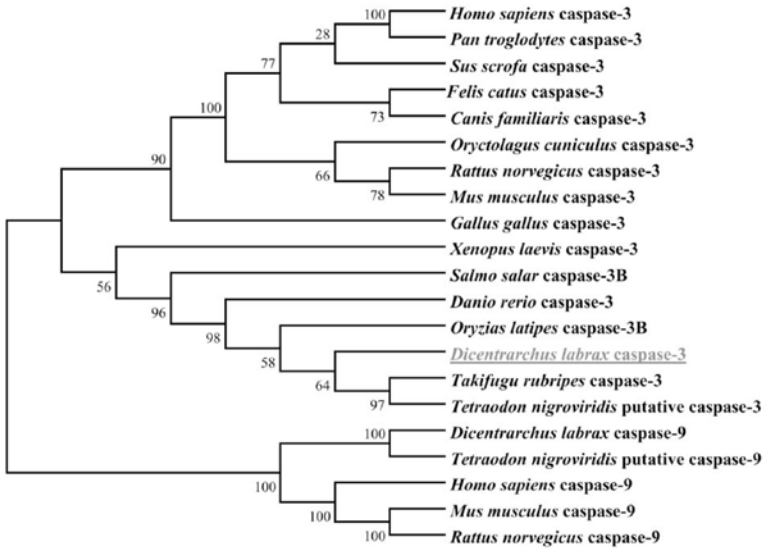

Fig. 4. Neighbour-joining tree based on the sequence of caspase-3 and -9 from sea bass and other species. The numbers on the internal branches denote the bootstrap percentages for 1000 replicates. The GenBank accession numbers of the sequences used are: DQ345775, for *Dicentrarchus labrax* caspase-9; CAG01765, *Tetraodon nigroviridis* putative caspase-9; AY214168, *Homo sapiens* caspase-9; NM_031632, *Rattus norvegicus* caspase-9, NM_015733, *Mus musculus* caspase-9. The sequence of *Tetraodon nigroviridis* putative caspase-3 and -9 were obtained by blast search with the sequence of sea bass caspase-3 and -9, respectively.

4. Discussion

In fish, information on the apoptotic process is relatively scarce and caspase genes only recently started to be sequenced. In sea bass, one of the most important fish species in aquaculture in Southern Europe, no caspase genes have been sequenced so far. Caspase-3 has been sequenced and characterised only in zebrafish (Yabu et al., 2001), although sequences are available

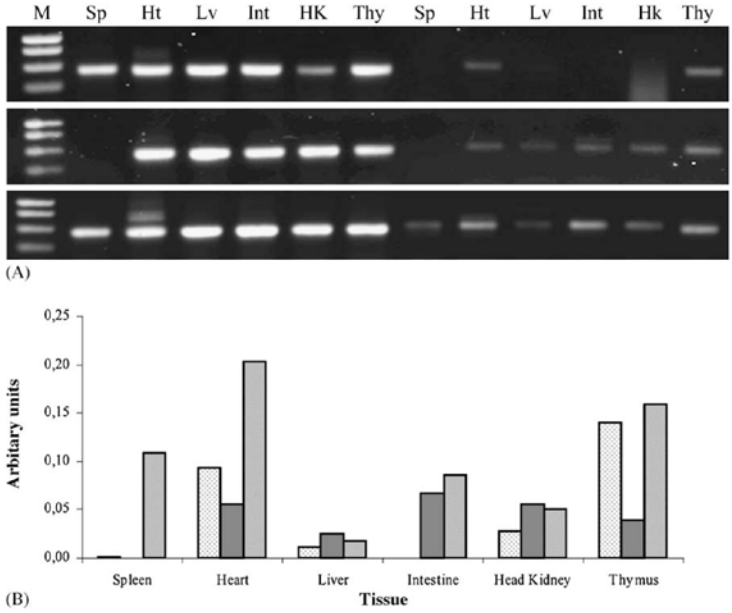

Fig. 5. RT-PCR analysis of sea bass caspase-3 gene expression in different tissues from three non-stimulated fish. (A) M, molecular marker; first six lanes, β-actin amplification; following lanes, caspase-3 amplification. SP, spleen; Ht, heart; Lv, liver; Int, intestine; HK, head kidney; Thy, thymus. (B) Ratios between the densitometry values of caspase-3 and β-actin expression of for each fish (▨, ■ and ▩).

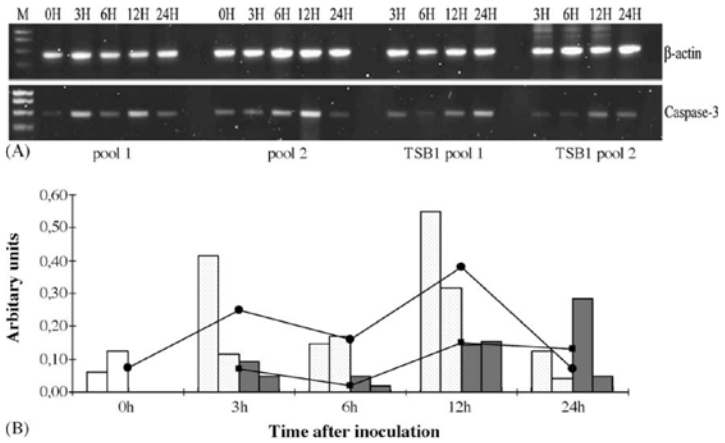

Fig. 6. RT-PCR analysis of sea bass caspase-3 gene expression in head kidneys from infected fish. (A) M, molecular marker; upper lanes, β-actin amplification; lower lanes, caspase-3 amplification. (B) Ratios between the densitometry values of caspase-3 gene and β-actin gene from: non-infected fish (□), fish infected with *Phdp* (▨) and fish injected with TSB1 (■). The average between the infected fish is represented by —●— and between the TSB1 injected fish by —■—.

in the databases for puffer fish, salmon, Japanese medaka and Tetraodon (putative/unknown gene).

The cDNA encoding sea bass caspase-3 was identified with the identification supported by several analyses, as detailed in the results, and further confirmed by Blast analysis. The sea bass caspase-3 sequence shows an ORF (281 aa) and respective predicted molecular weight (31.1 kDa) similar to those of other caspase-3 molecules (Fernandes-Alnemri et al., 1994; Johnson and Bridgham, 2000; Wang and Keiser, 1998; Yabu et al., 2001). Both the caspase family p20 and p10 domain profiles as well as the caspase family signature and the caspase family cysteine active site have been identified by PROSITE and are well conserved in the sea bass sequence. The pentapeptide active-site motif QACRG is also conserved and, as in all species here analysed, is interrupted after its first aa (Q) by an intron.

The active form of caspase-3 is generated after proteolytic cleavage of the proenzyme at aspartic acid residues. In humans, these cleavage sites were predicted to be between Asp^{28}-Ser^{29} (ESMD ↓ S), which separates the prodomain and the large subunit, and Asp^{175}-Ser^{176} (IETD ↓ S), separating the large and small subunits (Nicholson et al., 1995). In the presented alignment (Fig. 1) the cleavage sites of sea bass caspase-3 are predicted at Asp^{34} and Asp^{182}. However, it has been proposed that caspase-3 can be cleaved at several alternative sites to become

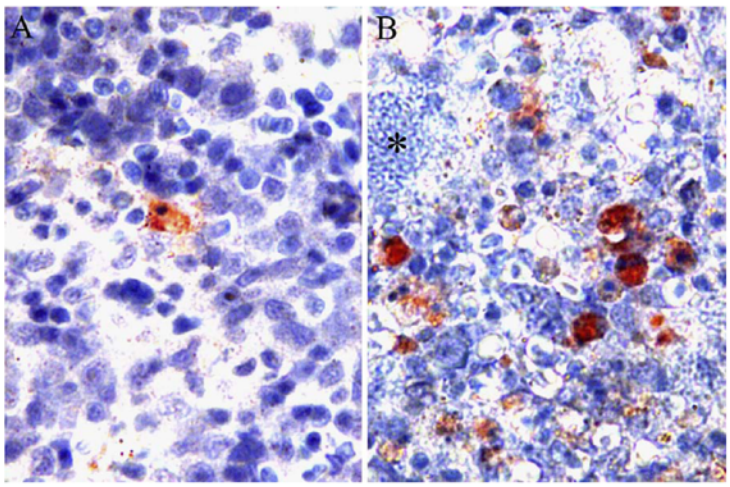

Fig. 7. Immunocytochemistry for activated caspase-3 (red staining) in head kidneys of sea bass infected with strain PP3 of *Photobacterium damselae* ssp. *piscicida* (*Phdp*). A) Sample collected at 17 h post-infection. One apoptosing cell shows a nucleus with condensed chromatin and cytoplasm with activated caspase-3. (B) Sample collected at 39 h post-infection. Several apoptosing cells with fragmented nuclei and/or condensed chromatin and cytoplasmic activated caspase-3 are present. Also visible are several apoptotic bodies with activated caspase-3. At the left-hand side, part of a microcolony of *Phdp* is visible (*). Micrographs recorded with a 100× objective.

activated (Wang et al., 1996), and therefore the indicated putative cleavage sites may not be the only ones because more conserved and non-conserved aspartic acids are present in the sea bass caspase-3 sequence.

An RGD motif before the active site is conserved in all species analysed with the exception of the sea bass sequence, where the aspartate residue is replaced by an asparagine (N^{153}). Since the 11 sea bass caspase-3 clones sequenced, resulting from 7 different PCR, showed the same nucleotides resulting in the N residue from the RGD motif, it is unlikely that the N residue is due to PCR or sequencing errors. The functionality of this RGD motif in caspase-3 could not be found in the literature. In addition to an RGD motif, human pro-caspase-3 also contains a protein RGD-binding motif (Pasqualini et al., 1995), aspartate-aspartate-X (DDX), near the site of processing (D^{180}DM) to produce the p12 and p17 subunits. This motif has been suggested to interact with RGD peptides leading to direct triggering of conformational changes that promote pro-caspase-3 autoprocessing and activation (Buckley et al., 1999). DDX motifs near possessing sites are only present in pro-caspase-3 sequences from *G. gallus* (D^{42}DS), *S. salar* (D^{183}DS) and *O. latipes* (D^{175}DG) suggesting that direct activation of caspase-3 through RGD motifs may not occur in all species.

The anti-active caspase-3 antibody used in this study has been raised against the peptide CRGTELDCGIETD of human caspase-3 (R&D Systems) and recognizes the large sub-unit of activated human caspase-3, but does not detect, at the used dilution, the precursor form. The observation that this antibody recognized apoptoting cells in head kidneys of *Phdp*-infected sea bass is explained by the present finding in the sea bass caspase-3 sequence of a very similar peptide (CRGTDLDPGIETD), and shows that sea bass caspase-3 is processed in a similar way as the human one. The occurrence of activated caspase-3 in apoptoting cells of *Phdp*-infected head kidneys is in accordance with our previous observation in organs of *Phdp*-infected sea bass of proteolytic activity against Ac-DEVD-AMC (unpublished data) which is a substrate of caspase-3 (Thornberry et al., 1997).

The genomic organisation of caspase-3 gene is different among the different species analysed here. However, the extra exons observed in zebrafish and human caspase-3 are due to the introduction of 1 or 2 extra introns in the 5′-UTR, respectively. In fact, considering the gene only from the starting to the stop codon, all the genes would present the same exon/intron organisation. The results from the Southern blotting hybridization suggest that the caspase-3 gene exists as a single copy in sea bass genome.

The semi-quantitative RT-PCR analysis of caspase-3 in different tissues from non-stimulated sea bass shows a very low expression in spleen, liver, intestine and head kidney with slightly higher levels in thymus and heart. In non-stimulated fish, the expression levels observed in thymus were higher than in the other organs, which probably is related to the process of negative selection that thymocytes undergo in thymus (Alam et al., 1997; Jiang et al., 1999; Palmer, 2003).

It was recently shown by our group that *Phdp* secretes the apoptogenic exotoxin AIP56 that triggers apoptosis in sea bass phagocytes involving induction of caspase-3-like activity (do Vale et al., 2003, 2005). Here, it is shown that, in an experimental sea bass septicemic infection by *Phdp*, the expression level of caspase-3 increased from 0 to 12 h post-infection in infected head kidneys. Importantly, the increase in expression was accompanied by the occurrence in that organ of high numbers of cells with activated caspase-3. The level of caspase-3 expression declined to basal levels at 24 h, which likely reflects the occurrence of progressive breakdown of DNA (Nagata et al., 2003) and RNA (Bushell et al., 2004; Mondino and Jenkins, 1995) molecules during the degradation phase of apoptosis. The occurrence of increased expression of caspase-3 gene and of active caspase-3 in sea bass head kidneys infected with *Phdp* show that caspase-3 is involved in the key pathogenicity mechanism of *Phdp* dependent on the apoptotic death of phagocytes.

In conclusion, we show that a counterpart of mammal caspase-3 gene is present in sea bass encoding a protease with a role in bacteria-induced pathogenic apoptosis.

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CHAPTER VI

Caspase-1 and IL-1β processing in sea bass.

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Abstract

IL-1β is the most studied pro-inflammatory cytokine, playing a central role in the generation of systemic and local responses to infection, injury, and immunological challenges. In mammals, IL-1β is synthesized as an inactive 31 kDa precursor that is cleaved by caspase-1 generating a 17.5 kDa secreted active mature form. The caspase-1 cleavage site strictly conserved in all mammalian IL-1β sequences is absent in those reported so far in non-mammalian vertebrates. Recently, fish caspase-1 orthologues have been identified in sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) but very little is known regarding its processing and activity.

In this work it is shown that sea bass caspase-1 auto-processing is similar to that of the human enzyme, resulting in active p24/p10 and p20/p10 heterodimers. Moreover, the presence of alternatively spliced variants of caspase-1 in sea bass is reported. The existence of caspase-1 isoforms in fish and in mammals suggests that they have been evolutionarily maintained and therefore are likely to play a regulatory role in the inflammatory response, as shown for other caspases. Finally, it is shown that sea bass and avian IL-1β are specifically cleaved by caspase-1 at different but phylogenetically conserved aspartates, which in turn are distinct from the cleavage site of mammalian IL-1β.

Introduction

IL-1β is the most studied pro-inflammatory cytokine, much due to its role in mediating auto-inflammatory diseases (see e.g., [1-4]). It is mainly produced by activated macrophages, monocytes, and dendritic cells and affects almost every cell type, playing a central role in the generation of systemic and local responses to infection, injury, and immunological challenges, although it can also have detrimental effects (see e.g., [1-6]). IL-1β exerts its activity by binding to IL-1 type I receptor (IL-1RI), which then recruits IL-1 receptor accessory protein (IL-1RAP) forming a complex that triggers a series of phosphorylation events leading to the activation of IκB kinase (IKK) and MAPK pathways (see e.g., [4, 7, 8]). These signalling pathways activate the transcription factors NF-κB and AP-1, resulting in the induction of genes encoding chemokines, cytokines, acute-phase proteins, cell adhesion molecules, and enzymes involved in the production of small pro-inflammatory substances [5]. In mammals, IL-1β is synthesized as an inactive 31 kDa signal peptide-less precursor molecule (proIL-1β) [9, 10]. In humans and mice, caspase-1, also known as ICE (interleukin-1β converting enzyme) [11-13], specifically cleaves proIL-1β after aspartate 116 and 117, respectively, yielding a C-terminal 17.5 kDa secreted active form [11, 12, 14-18]. Non-caspase-1-mediated cleavage mechanisms generating different active forms of IL-1β have also been described (reviewed in [2, 4, 5, 19]).

IL-1β has been identified in several fish species [20-30]. However, although it was reported that processing of IL-1β by a gilthead sea bream fibroblast cell line is abrogated by a specific caspase-1 inhibitor [31], neither direct evidence for the participation of caspase-1 in this processing event was provided nor the cleavage site in proIL-1β was defined. This last issue is particularly important because the caspase-1 cleavage site reported for mammalian proIL-1β is absent in the proIL-1β sequences of non-mammalian vertebrates. Putative trout [32], sea bass [33], and chicken [34-36] recombinant mature IL-1β starting respectively at Ala^{95}, Ala^{86}, and Ala^{106}, equivalent sequence-wise to the N-terminal residue of mature mammalian IL-1β, were shown to be biologically active. However, in none of these is the putative mature N-terminal preceded by an aspartate residue, making those sites rather improbable caspase-1 cleavage residues. In trout, a 29 kDa IL-1β precursor expressed in RTS-11 cells was processed to a 24 kDa peptide [37] and a 15 kDa band has been detected by Western blotting in PHA activated carp leucocyte culture supernatant [38]. However, the mechanism and exact cleavage site of processing were not clarified [39-41].

Caspase-1 is the prototype of a family of inflammatory caspases that contain a caspase recruitment domain (CARD) within their amino-terminal pro-domains (see e.g., [5, 42]). Normally, it exists in the cytoplasm as a 45 kDa inactive precursor [43], being

activated by CARD oligomerization in a multiprotein complex known as the inflammasome (see e.g., [44-51]). Once activated, caspase-1 processes the pro-inflammatory cytokines, starting an inflammatory response. Caspase-1 was recently found to be also involved in pyroptosis, a rapid active cell death programme characterised by early plasma-membrane rupture and release of pro-inflammatory intracellular contents ([52]; reviewed in [53, 54]). In humans, caspase-1 is activated by cleavage after Asp^{103}, D^{119}, D^{297} and D^{316}, releasing the 11.5 kDa pro-domain and a 2 kDa linker peptide and originating the active heterodimer p20/p10 [17, 18, 55-57].

Fish inflammatory caspases have been primarily sequenced in zebrafish [58] and sea bass (GenBank accession no.: DQ198377), the later being a caspase-1 homologue that has recently also been reported for sea bream [59] but there is still no functional data from these enzymes. Here, the characterisation of the sea bass caspase-1 homologue, its processing and activity are reported. It is shown that sea bass caspase-1 auto-processing follows the mechanism described for the mammalian enzyme, yielding p24/p10 and p20/10 active heterodimers. In addition, three other caspase-1 isoforms have been identified. Finally, analysis of sea bass IL-1β processing by active sea bass caspase-1 revealed that the cytokine is cleaved at a phylogenetically conserved aspartate residue, distinct from the cleavage site in birds.

Materials and Methods

Fish

Sea bass (*Dicentrarchus labrax*) were kept in a recirculating, ozone-treated salt-water (25-30‰) system at 20 ± 1 ºC, and fed at a ratio of 2 % body weight per day. For organ collection fish were euthanized with 2-phenoxyethanol (Panreac; >5 ml/10 L).

This study was carried out in accordance with European and Portuguese legislation for the use of animals for scientific purposes (Directive 86/609/EEC; Decreto-Lei 129/92; Portaria 1005/92). The work was approved by Direcção Geral de Veterinária, the Portuguese authority for animal protection.

Complementary and genomic DNA cloning and sequencing, southern blotting, sequence analysis of caspase-1 and expression analysis of caspase-1 isoforms

Standard techniques for molecular biology have been used, as described in supporting information.

Production of recombinant sea bass caspase-1 isoforms

The coding region of sea bass caspase-1 isoform 1 was amplified from the pGEM-T Easy plasmid DNA carrying the full length cDNA using specific primers DLCASP1FWNdeI and DLCASP1RVXhoI (Table SII). The PCR product was cloned into pGEM-T Easy, the plasmid DNA was digested with NdeI and XhoI (Fermentas), and the insert cloned into pET-28a (Novagen) in frame with N- and C-terminal His-tags. Recombinant sea bass caspase-1 was expressed in *E. coli* BL21 Rosetta (DE3) overnight at 37 ºC with 1 mM IPTG. The recombinant protein was extracted from bacterial cells as inclusion bodies and solubilized with 8 M Urea, 50 mM Tris-HCl, 0.2 M NaCl, 2 mM EDTA pH 8.0. The protein was refolded by dilution in 50 volumes of refolding buffer (50 mM Taps pH 8.5, 1.5 M Sorbitol, 1 mM TCEP, 24 mM NaCl and 1 mM KCl) overnight at 22 ºC. As a control, the refolding process was also performed in the presence of 100 µM of a specific caspase-1 inhibitor (Ac-YVAD-CHO, Caspase-1 inhibitor I, Calbiochem). The refolded protein was bound to an IMAC column (HisTrap HP, GE Healthcare) in refolding buffer. The column was washed with the same buffer supplemented with 10 mM imidazole and the protein was then eluted in 4 steps with increasing concentrations of imidazole (50, 100, 250 and 500 mM). The purified protein was analysed by SDS-PAGE and, after blotted onto polyvinylidine difluoride membrane (PVDF), fragments of 24, 20 and 10 kDa were subjected to N-terminal Edman sequencing (Proteome Factory AG, Germany), in

order to determine the cleavage sites between the large and small subunits of sea bass caspase-1.

The cDNAs encoding caspase-1 isoforms 2, 3 and 4 were obtained with the forward primer DLCASP1FWNdeI together with DLISO2/3RVXhoI for isoform 2 and 3 and with DLISO4RVXhoI for isoform 4 (Table SII). The cloning strategy, expression, purification and refolding protocols were those described for isoform 1, except that isoform 2 was cloned in pET-30a (Novagen).

In vitro processing of caspase-1 isoforms

Two polyclonal antibodies directed against sea bass caspase-1 were produced (Davids Biotechnologie GmbH, Germany). An anti-p10 polyclonal antibody was raised against the peptide VHKEKDFISLLSST, and detected all caspase-1 forms containing the p10 domain. The antibody produced against the peptide QACRGNAGGAVLVSD corresponding to the carboxyl-terminal residues adjacent to the proteolytic cleavage site, detected processed forms that were cleaved at the p20 cleavage site (therefore, lacking the linker and the small subunit), but did not recognise unprocessed caspase-1.

Auto-processing of the caspase-1 isoforms was analysed by SDS-PAGE and Western blotting. Samples of the isoforms collected after urea solubilisation or after an overnight refolding step in the presence or absence of 50-100 µM caspase-1 inhibitor were subjected to SDS-PAGE. Proteins in the gels were stained with Coomassie brilliant blue or were transferred to nitrocellulose membranes and probed with the rabbit anti-p10 or anti-p20 antibodies (both at a 1/10000 dilution) for 1 h at room temperature. Goat anti-rabbit Ig conjugated with alkaline phosphatase (Sigma) was used as the secondary antibody and the detection performed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT).

The ability of active sea bass caspase-1 isoform 1 to process isoforms 2, 3 and 4 was tested by incubating 2 µg of the purified isoforms with recombinant active caspase-1 (p24/p10) at a ratio of 2:1 (w/w) in a caspase-1 substrate buffer (0.1% v/w CHAPS, 0.1 M HEPES, 10 % v/w sucrose, 10 mM TCEP, pH 7.5) overnight at 22 ºC. As control, similar incubations were performed in the presence of 100 µM caspase-1 inhibitor. The proteins in the samples were precipitated with TCA and analysed by SDS-PAGE and Western blotting using the anti-p10 and anti-p20 antibodies, as described above, or transferred to a PVDF membrane for N-terminal Edman sequencing.

Activity of recombinant sea bass caspase-1 isoforms

The activities of the refolded sea bass caspase-1 isoforms were tested by a fluorimetric assay using 0.1-2 µg of enzyme and a specific substrate for caspase-1 (Z-YVAD-AFC, Caspase-1 substrate VI, Calbiochem). To control cleavage specificity, 100 µM caspase-1 inhibitor was used. All assays were performed in duplicate. In each reaction, 71.5 µl of reaction buffer (0.2% CHAPS, 0.2 M HEPES, 20% sucrose, 29 mM DTT, pH 7.5) and 16 µM of caspase-1 substrate were used. The mixtures were incubated at 25 ºC and the fluorescence recorded for up to 3 h with 20 min intervals on a Spectra Max Gemini XS fluorimeter (Molecular Devices) at an excitation wavelength of 405 nm and an emission wavelength of 492 nm. The results were expressed as relative fluorescence units (RFU).

In vitro production of sea bass, chicken and human IL-1β by rabbit reticulocytes lysates

Sea bass

Plasmids harboring different IL-1β forms have been produced as detailed below.

pET23proIL1β[His]: the coding region of sea bass proIL-1β was first amplified by PCR with primers DLIL1βFWNde3 and DLIL1βRVXho4 (Table SII) using a pGEX-4T-3 plasmid (GE Healthcare) carrying the proIL-1β cDNA (previously amplified from sea bass head kidney) as template [23]. The PCR product was purified and cloned into the pGEM-T Easy vector. After digestion with NdeI and XhoI, the sea bass proIL-1β was cloned into the expression vector pET-23a (Novagen) in frame with a C-terminal His-tag.

pET-23proIL1β, for producing proIL-1β: the histidine tag from pET-23proIL1β[His] was removed by site-directed mutagenesis (QuickChange® Site-Directed Mutagenesis Kit, Stratagene) using primers listed in Table SII.

pET-23proIL1β[D^{60}A], pET-23proIL1β[D^{100}A], pET-23proIL1β[D^{252}A], and pET-23proIL1β[D^{100}A/D^{252}A], for producing mutated forms of proIL-1β in D^{60}, D^{100}, D^{252}, or D^{100}/D^{252}: each aspartate was mutated to alanine by site-directed mutagenesis using pET-23proIL1β as template and primers listed in Table SII.

pET-23matureIL1β, for producing mature sea bass IL-1β (from S^{101} to Q^{261} and non-tagged): produced using pET-23proIL1β[His] as template and primers listed in Table SII.

pET-23matureIL1β[D^{252}A], for producing mature sea bass IL-1β mutated at D^{252}: aspartate 252 was mutated to alanine by site-directed mutagenesis using pET-23matureIL1β as template and primers listed in Table SII.

Chicken

The plasmids harboring the coding region of proIL-1β from chicken (GGpGEX-6P-1proIL1β), duck (APpET28proIL1β), goose (AApET28proIL1β and turkey

(MGpET28proIL1β) were kindly provided by Dr. Hsien-Sheng Yin and Dr. Long Huw Lee. The coding region of chicken proIL-1β was first amplified by PCR with primers listed in Table SII using the plasmid GGpGEX-6P-1proIL1β as template. The PCR product was purified and cloned into the pGEM-T Easy vector. After digestion with NdeI and XhoI, the chicken proIL-1β was cloned into the expression vector pET-23a (Novagen) rendering GGpET23proIL1β.

GGpET23proIL1β[D^{77}A], GGpET23proIL1β[D^{80}A] and GGpET23proIL1β[D^{82}A] for producing mutated forms of chicken proIL-1β in D^{77}, D^{80} or D^{82}: each aspartate was mutated to alanine by site-directed mutagenesis using GGpET23proIL1β as template and primers listed in Table SII.

The cDNAs encoding mature IL-1β (starting at S^{101}) with or without a C-terminal His-tag were amplified from pET23proIL1β[His] with the forward primer DLIL1BFWNde4 and the reverse primers DLIL1BRVXho4 or DLIL1BRVXho1, respectively (Table SII) and cloned in pET-23a.

Human

HSpCMV-XL5proIL1β[D^{116}A], HSpCMV-XL5proIL1β[D^{128}A] and HSpCMV-XL5proIL1β[D^{116}A/D^{128}A], for producing mutated forms of human proIL-1β in D^{116}, D^{128} or D^{116}/D^{128}: each aspartate was mutated to alanine by site-directed mutagenesis using an IL-1β Human cDNA clone (Origene #SC122566) as template and the primers listed in Table SII.

Regions coding for mature human IL-1β from A^{117} or from S^{129} were amplified by PCR using the IL-1β Human cDNA clone as template and primers described in Table SII and cloned into the NcoI/XhoI restriction sites of pET28a (Novagen), yielding plasmids HSpET28mature1IL1β and HSpET28mature2IL1β, respectively.

The above mentioned plasmids, harboring different IL-1β forms, were used to synthesize the respective proteins using the TNT® T7 Quick Coupled Transcription/Translation kit (Promega) in the presence of [^{35}S] methionine (specific activity >1000 Ci/mmol; PerkinElmer Life Sciences) following the manufacturer's instructions.

Production of recombinant sea bass IL-1β

His-tagged proIL-1β (proIL-1β[His]) was expressed in *E. coli* BL21 (DE3) CodonPlus at 37 ºC with 1 mM IPTG for 4 h, extracted from bacterial cells as inclusion bodies, solubilised as described for recombinant caspase-1 and purified under denaturing conditions using His-select™ Nickel Affinity Gel (Sigma). The protein was then refolded by dialysis in three steps against 50 volumes of decreasing concentrations of urea (4 M for

12 h, 2 M for 12h, 0 M overnight) in 10 mM sodium phosphate buffer pH 7.2 containing 184 mM NaCl at 4 ºC. The dialyzed protein was centrifuged at 12000 g and the supernatant analysed by SDS- and Native-PAGE.

The cDNAs encoding mature IL-1β (starting at S^{101}) with or without a C-terminal His-tag were amplified from pET23proIL1β[His] with the forward primer DLIL1BFWNde4 and the reverse primers DLIL1BRVXho4 or DLIL1BRVXho1, respectively (Table SII), cloned in pET-23a and expressed in *E. coli* BL21 (DE3) CodonPlus using the approach described for proIL-1β.

In vitro processing of proIL-1β by caspase-1

The sea bass ^{35}S-labeled IL-1β forms produced using rabbit reticulocyte lysates were incubated at 22 ºC for different periods with recombinant sea bass or human (Sigma) active caspase-1, with or without a caspase-1 substrate buffer (0.2% CHAPS, 0.2 M HEPES, 20% sucrose, 20 mM TCEP, pH 7.5) and in the presence or absence of 100 µM of caspase-1 inhibitor. The same procedure was done for the human and chicken ^{35}S-labeled IL-1β forms, but incubating overnight at 37 ºC and with or without 250 µM of the caspase-1 inhibitor. The duck, turkey and goose ^{35}S-labeled IL-1β forms were also incubated with recombinant active sea bass caspase-1 as described for chicken IL-1β. Samples were subjected to SDS-PAGE, and transferred to nitrocellulose membrane for autoradiography.

The ability of caspase-1 to process recombinant proIL-1β was tested at 22 ºC by mixing recombinant active caspase-1 with recombinant proIL-1β[His] at a ratio of 1:2 (v/v) in the caspase-1 substrate buffer, in the presence or absence of 20 µM caspase-1 inhibitor. The samples were precipitated with TCA and subjected to SDS-PAGE. Proteins in the gels were stained with Coomassie brilliant blue for mass spectrometry (MS) analysis, or blotted onto a PVDF membrane for N-terminal Edman sequencing, or analysed by Western blotting using rabbit anti-sea bass IL-1β or anti-His (Santa Cruz) antibodies. For antibody production, dialyzed recombinant proIL-1β[His] was further purified on a UNO^{TM} Q column (Bio-Rad) using 20 mM Tris pH 8.0 and 20 mM Tris pH 8.0 1.0 M NaCl as buffer system. The fractions containing proIL-1β[His] were pooled and subjected to SDS-PAGE. The band corresponding to proIL-1β[His] was excised from the gel and used to immunize rabbits (Davids Biotechnologie GmbH).

Sea bass IL-1β processing in vivo

Over 20 fish were injected intraperitoneally with 200 µl of *Photobacterium damselae* ssp. *piscicida* concentrated heat-inactivated extracellular products, prepared as described elsewhere [60]. After 3 h, cells from the peritoneal cavity were collected by washing with 10 ml of HBSS, centrifuged and resuspended in fresh HBSS [61, 62]. Cells were counted, diluted to a final concentration of $1x10^6$ cells/ml and incubated for 3 h at 22 ºC in the presence or absence of 100 µM caspase-1 inhibitor. One millilitre of cell suspension was centrifuged at 400 g, the cell pellet resuspended in gel loading buffer and the proteins in the supernatant precipitated with TCA. After SDS-PAGE, samples were analysed by Western blotting, using the rabbit anti-IL1β antibody, as described above.

Results

The sea bass caspase-1 transcript is alternatively spliced

The sea bass caspase-1 gene (GenBank accession number: DQ198377) was found to consist of 8 exons and 7 introns (Fig. 1), while the human caspase-1 gene has 10 exons and 9 introns (Ensembl transcript ID ENST00000260309) and presents much longer intronic regions (8266 bp *vs.* 2318 bp).

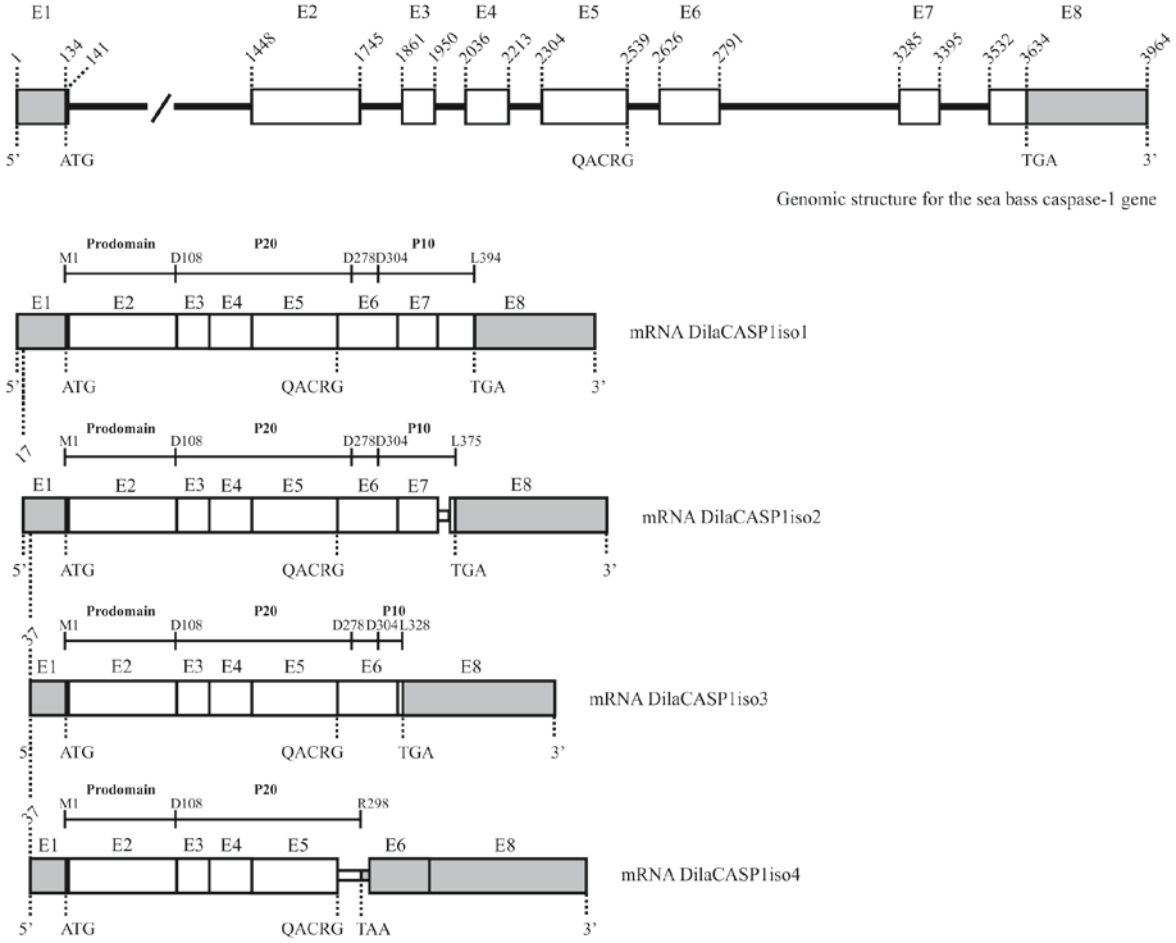

FIGURE 1. Schematic representation of the genomic organisation and alternative splicing of sea bass caspase-1. The exons are represented by boxes (E1-E8) and introns by a solid line (introns larger than 800 bp are represented by an interrupted solid line). Coding and untranslated regions are indicated by white and shaded boxes, respectively. Nucleotide numbers are given above the boxes. Caspase-1 domains and cleavage sites are schematically represented on top of each transcript. ATG: starting codon; QACRG: caspase-1 pentapeptide active-site motif; TGA/TAA: stop codon. Genomic DNA (gDNA) was isolated from erythrocytes of a single fish, as described by Stet and co-workers [63]. To obtain the complete caspase-1 gene, gDNA was amplified using the 5'-UTR primer DLCASP1FWEcoRI and the 3'-UTR primer DLCASP1RVEcoRI (Table SII), each containing a restriction site for EcoRI. The obtained PCR product was purified, digested with EcoRI (Fermentas) and cloned into the pET-32b vector (Novagen). Plasmid DNAs from three independent clones were purified and sequenced.

Southern blot analysis suggested that, similar to the mouse and human caspase-1 genes [64-66], sea bass caspase-1 is a single copy gene (Fig. S1A), although four different transcripts encoding sea bass caspase-1 have been detected by RT-PCR using specific primers (Fig. 1, S1B and S1C). The corresponding deduced proteins are referred to as isoform 1-4 (DilaCASP1iso1-DilaCASP1iso4; GenBank accession numbers DQ198376, HQ398873, HQ398874 and HQ398875, respectively). DilaCASP1iso1 contains the typical caspase family p20 and p10 domain profiles and a caspase family active site signature (K^{257}PKIIIIQACRG), as well as a characteristic CARD-containing domain. DilaCASP1iso2 and DilaCASP1iso3 have a shorter p10 domain, whereas DilaCASP1iso4 contains a longer p20 domain and no p10 (Fig. S1B). All isoforms comprise the active center pentapeptide QACRG.

Pair-wise alignments confirmed the homology of DilaCASP1iso1 with caspase-1 sequences from other species (Table SI). The phylogenetic tree (Fig. S2) clustered the inflammatory and apoptogenic caspases into two different clades. Within the inflammatory caspase clade, the fish caspases cluster into a single distinct branch with two subgroups: one for sea bass and sea bream caspase-1, and another one for the two caspy sequences (caspase-a and -b) from zebrafish.

Sea bass caspase-1 is activated to p24/p10 and p20/p10 heterodimers in vitro.

In order to characterise caspase-1 auto-processing and IL-1β activation in fish, a recombinant form of sea bass caspase-1 (DilaCASP1iso1) was produced.

Solubilisation and refolding of recombinant DilaCASP1iso1 from inclusion bodies originated three major polypeptides, two corresponding to putative large subunits (p24 and p20), and one corresponding to a putative small subunit (p10; Fig. 2A). N-terminal sequencing of the three polypeptides revealed that they were generated by proteolytic cleavage of sea bass caspase-1 after E^{86}, D^{108} and D^{304} originating a p24, a p20, and a p10 polypeptide, respectively. Both p24/p10 and p20/p10 heterodimers (elutions #2 and #3, respectively, in Fig. 2A) displayed activity in a caspase-1 specific fluorimetric assay, which was abolished by the caspase-1 inhibitor (Fig. 2B). The more abundant p24/p10 heterodimer was selected for subsequent experiments. A kinetic analysis of the caspase-1 refolding process revealed a time-dependent decrease of the higher molecular weight forms and an increase of lower molecular weight forms (Fig. 3). The polypeptides detected in these experiments as well as the non-linear kinetics of processing, typical of an autocatalytic event, are remarkably similar to those described for the human protein [56, 57].

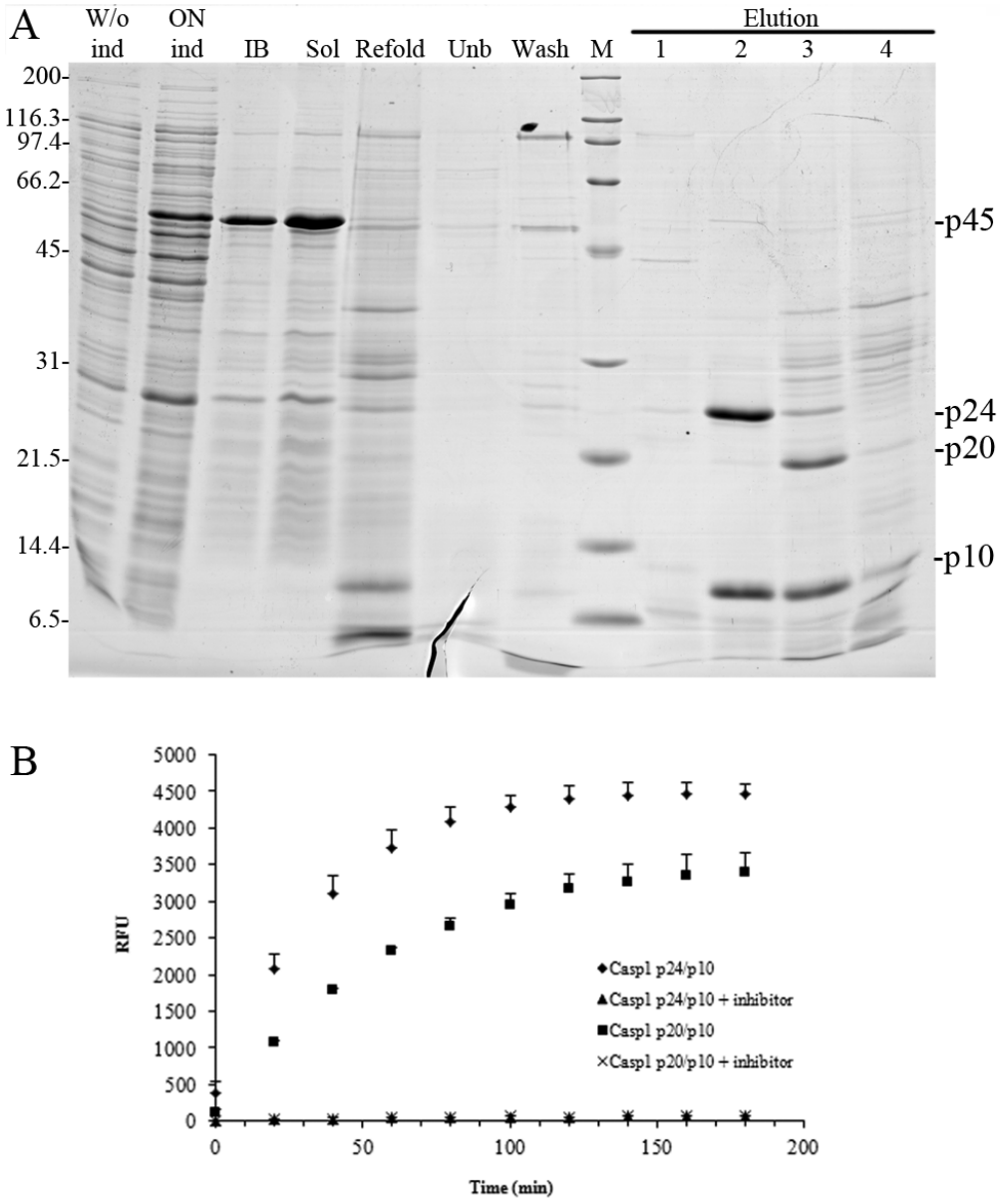

FIGURE 2. Recombinant sea bass caspase-1 displays enzymatic activity. **(A)** Production and purification of recombinant sea bass caspase-1. W/o ind: non induced; ON ind: overnight induction; IB: inclusion bodies' fraction; Sol: soluble fraction; Refold: overnight refolding; Unb, Wash and Elution: non-bond, washed and eluted (1-4) fractions, respectively, of Ni-NTA chromatography purified refolded caspase-1. The positions of the p45, p24, p20 and p10 fragments are indicated. Numbers on the left indicate the position of the molecular weight markers in kDa. **(B)** Enzymatic activity of recombinant p24/p10 and p20/p10 sea bass caspase-1 heterodimers. p24/p10 and p20/p10 were standardized for 1 µg of p10 and were incubated with a specific fluorogenic substrate for caspase-1 in the presence or absence of the caspase-1 inhibitor Ac-YVAD-CHO. Hydrolysis of the substrate was followed by fluorimetry and results were expressed as relative fluorescent units (RFU).

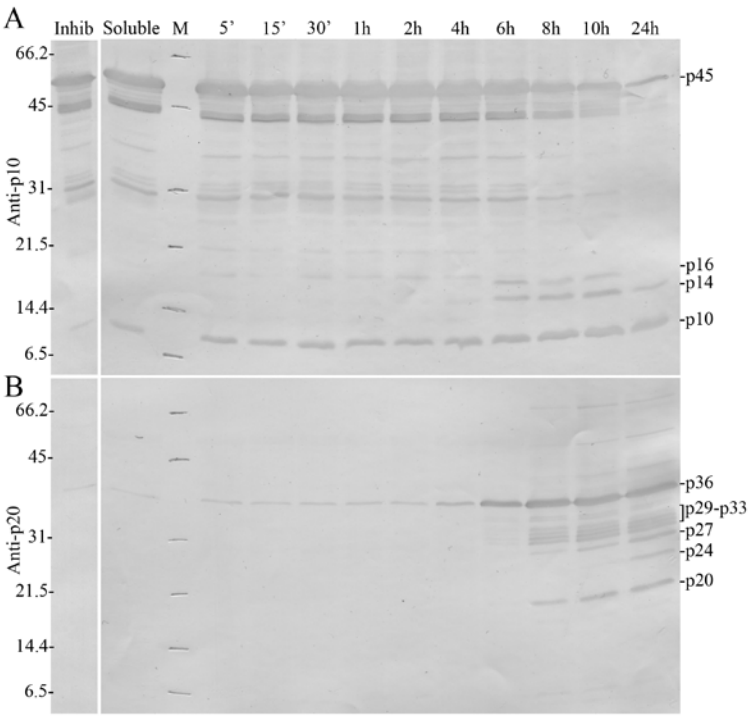

FIGURE 3. *In vitro* auto-processing of sea bass caspase-1. **(A)** Western blotting analysis of *in vitro* auto-processing of sea bass caspase-1 using a polyclonal antibody that detects all polypeptides containing the p10 domain. **(B)** Western blotting analysis of *in vitro* auto-processing of sea bass caspase-1 using a polyclonal antibody that detects only caspase-1 fragments that have been processed at the p20 cleavage site, i.e. that have released the linker plus the p10 domain. Soluble: solubilised DilaCASP1iso1. Inhib: sample incubated for 24 h in the presence of the specific caspase-1 inhibitor Ac-YVAD-CHO. The position of the different polypeptide fragments is indicated. Numbers on the left indicate the mass of the molecular weight (M) markers in kDa.

DilaCASP1iso2 and DilaCASP1iso3 have little auto-processing activity but are processed by DilaCASP1iso1.

Considering that all spliced sea bass caspase-1 isoforms contain intact pro- and p20 domains (which include the active site), recombinant forms of sea bass caspase-1 isoforms 2, 3 and 4 were produced in order to test their auto-processing activity and/or processability by active DilaCASP1iso1 (Fig. 4). In contrast to DilaCASP1iso1, isoforms 2 and 3 showed auto-processing activity, which was totally absent in isoform 4 (Fig. 4), and do not become active by auto-proteolysis during the refolding process, as assessed by the fluorimetric assay. Nevertheless, isoforms 2 and 3, but not isoform 4, are readily processed by DilaCASP1iso1 (Fig. 4). Additionally, a fragment corresponding to the shorter p10 of isoform 2 was also detectable (Fig. 4*B*). For isoform 3, the shorter p10 fragment could not be detected probably because it is too small to be detected in these gels (Fig. 4*A* and *B*). The identity of the fragments was confirmed by N-terminal sequencing of p36 (GSSHHHHHHM^{1}ADK; band 1 in Fig. 4*A*) and p10 (T^{305}LHFVH; band 2 in Fig. 4*A*). Fragment 3 was a short fragment from the N-terminus (GSSHHHHHHM^{1}ADK; band 3 in Fig. 4*A*). These results suggest that both isoforms 2 and 3 are processed by isoform 1, releasing the pro-domain and the p10 plus linker.

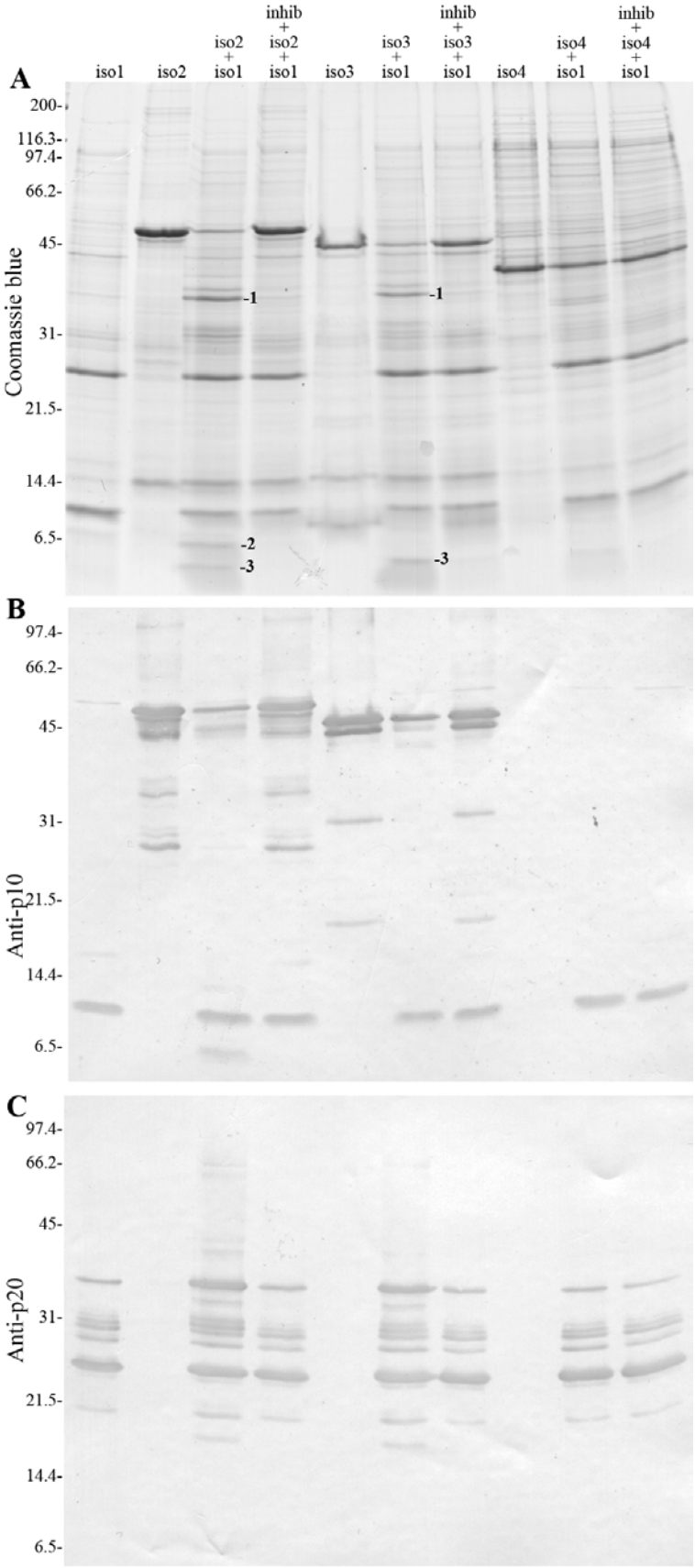

FIGURE 4. *In vitro* auto-processing and processing of sea bass caspase-1 isoforms 2, 3 and 4 by isoform 1.

(A) Coomassie brilliant blue stained SDS-PAGE of sea bass DilaCASP1iso2 (iso2), DilaCASP1iso3 (iso3) or DilaCASP1iso4 (iso4) incubated alone or in combination with active (p24/p10) DilaCASP1iso1 (iso1), in the presence or absence of caspase-1 inhibitor Ac-YVAD-CHO (inhib). N-terminal sequencing revealed that the indicated fragments 1 and 2 correspond to p36 (GSSHHHHHHM^{1}ADK) and p10 (T^{305}LHFVH), respectively. Fragment 3 corresponds to an N-terminal fragment of p45 (GSSHHHHHHM^{1}ADK).

(B) Western blotting of the *in vitro* processing of caspase-1 isoforms as in A, using the anti-p10 polyclonal antibody that detects all polypeptides containing the p10 domain.

(C) Western blotting of the *in vitro* processing of caspase-1 isoforms as in A, using the anti-p20 polyclonal antibody that detects only caspase-1 fragments that have been processed at the p20 cleavage site, i.e. that have released the linker plus the p10 domain. Numbers on the left indicate the mass of the molecular weight markers in kDa.

In vivo-generated IL-1β is an 18 kDa polypeptide

Peritoneal leukocytes harvested from stimulated sea bass were incubated *ex vivo* in the presence or absence of a caspase-1 inhibitor. Western blotting analysis with an anti-sea bass IL-1β antibody showed a specific band with an apparent molecular mass of 18 kDa in culture supernatants and total extracts of cells incubated *ex vivo* without caspase-1 inhibitor (Fig. 5). This band co-migrates with a recombinant putative mature sea bass IL-1β staring after aspartate D^{100} (MS^{101}-Q^{261}), an aspartate phylogenetically conserved in all available IL-1βs, from sharks to humans (Fig S3).

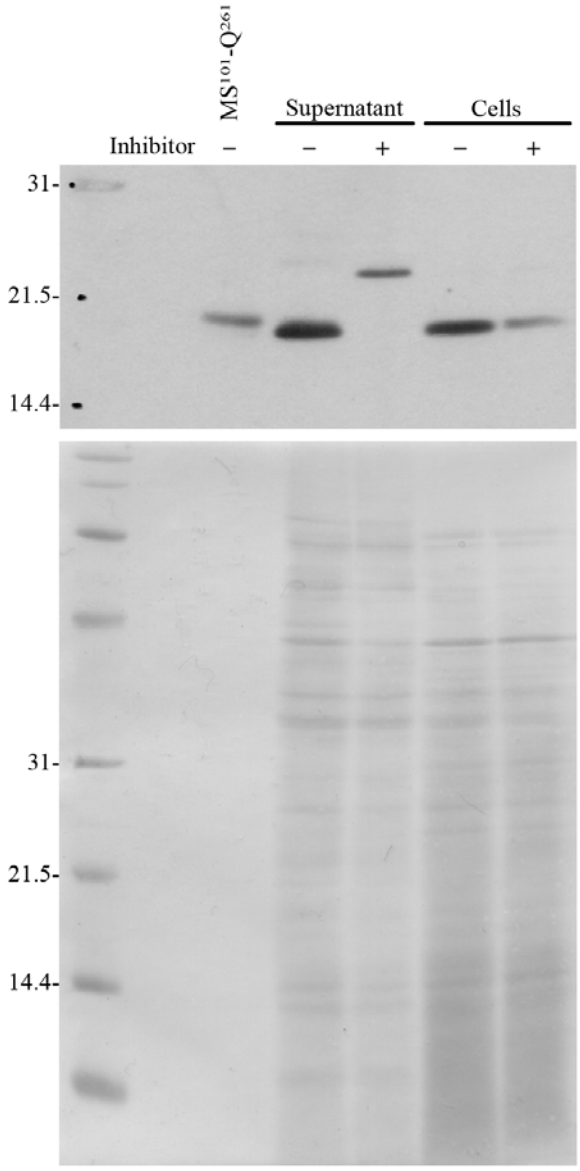

FIGURE 5. Sea bass IL-1β processing *in vivo*.
(A) Western blotting using the anti-sea bass IL-1β polyclonal antibody.
(B) Ponceau staining showing the protein loading. Inhibitor: specific caspase-1 inhibitor Ac-YVAD-CHO. Recombinant putative mature sea bass IL-1β (MS^{101}-Q^{261}) was loaded as control. Supernatant and Cells denote TCA precipitates of the supernatants and cell pellet corresponding to 1 ml of cell suspension after incubation and centrifuging, respectively. Numbers on the left indicate the mass of the molecular weight markers in kDa.

Evolutionary divergence of the caspase-1 cleavage site in proIL-1β

As several attempts to identify the caspase-1 cleavage site in native mature IL-1β have failed, *in vitro* approaches were used.

As shown in figure 6, *in vitro* synthesized sea bass proIL-1β was processed by sea bass active caspase-1 in a time-dependent manner giving rise to a band with an apparent molecular mass of 18 kDa that, as the *in vivo* obtained band, co-migrates with the putative mature sea bass IL-1β MS^{101}-Q^{261}. Mutation of D^{100} abolished the generation of the 18 kDa fragment, with accumulation of higher molecular mass species (Fig. 6), whereas mutating D^{60}, an aspartate residue previously suggested as a possible caspase-1 cleavage site in fish [31], did not affect proIL-1β processing even when incubated with a caspase-1 buffer that promotes IL-1β cleavage (Fig. S4A). This suggests that cleavage of sea bass proIL-1β by caspase-1 occurs C-terminal to D^{100}.

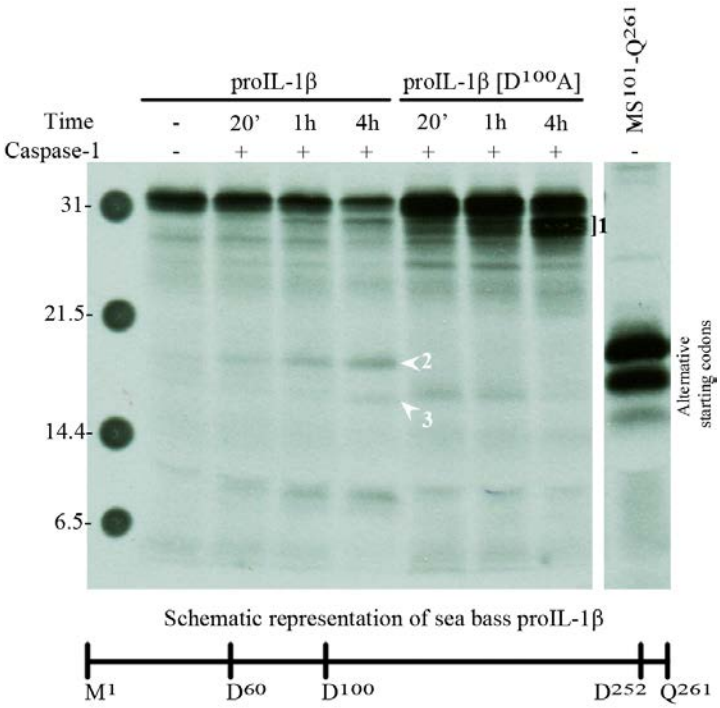

FIGURE 6. Time-course analysis of *in vitro* synthesized bass proIL-1β and proIL-1β[D^{100}A] by sea bass caspase-1. The same volume of *in vitro* synthesized proIL1β and proIL-1β[D^{100}A] were used and loaded on the gel. Fragments 1, 2 and 3 are labelled. Putative mature *in vitro* synthesized sea bass IL-1β (MS^{101}-Q^{261}) has been loaded as control. Numbers on the left indicate the mass of the molecular weight markers in kDa. The duple bands corresponding to the highest molecular weight form (fragment 1) suggested cleavage at the C-terminal end of proIL-1β. This interpretation was supported by N-terminal sequencing of fragment 1' (Fig. S4A), which revealed that this fragment has the N-terminal sequence of proIL-1β (M^1ESEMKC). Furthermore, mutation of D^{252} results in a proIL-1β protein that no longer yields fragment 1 upon incubation with caspase-1 (Fig S4C).

An 18 kDa fragment was also generated from recombinant proIL-1β (Fig. S4B), together with a shorter fragment (fragment 3') which intensity markedly increased with time. N-terminal sequencing of fragments 2' and 3' (Fig. S4B) revealed that they start at S^{101}EKRSLVLVP, confirming that they originated from a cleavage after D^{100}. Fragment 3' was also processed after D^{252}, as mutating D^{252} abolished its formation (Fig. S4C). Using *in vitro* synthesized proIL-1β it was found that the appearance of fragment 3' was time-, buffer- and caspase-1 concentration-dependent (Fig. S4A,C,D). Furthermore, only few fish species present a slightly longer C-terminal region including potential cleavable aspartates (Fig. S3). Together with the lack of detection of fragment 3´ *in vivo*, these data suggest that its appearance may be a consequence of the *in vitro* conditions, which do not completely mimic the *in vivo* environment.

As the caspase-1 cleavage site described for mammalian proIL-1β is only present in mammals (Fig. S3), it is tempting to speculate that the phylogenetically conserved aspartate identified as the caspase-1 cleavage site in sea bass proIL-1β is also the residue targeted by caspase-1 in the proIL-1β of non-mammalian vertebrates. To address this issue, the processing of *in vitro* synthesized avian pro-IL1β was investigated. Since avian caspase-1 is not available, sea bass or human caspase-1 were used. Both sea bass and human caspase-1 processed chicken proIL-1β into a mature IL-1β running in SDS-PAGE above the 21 kDa marker (Fig. 7A and S5A). A similar fragment was obtained when sea bass caspase-1 was incubated with duck, goose or turkey proIL-1β (Fig. S5B). Due to its apparent size, this suggested that avian proIL-1β could have been cleaved at D^{77}/D^{80}/D^{82} (sea bass D^{60}), which also align with other phylogenetically conserved aspartates (Fig. S3). Site-directed mutagenesis confirmed that D^{80} is the preferential aspartate cleavage site (Fig. 7A). Despite the concerns associated with the use of heterologous caspases, both human and sea bass caspase-1 showed cleavage specificity when incubated with heterologous proIL-1βs, as supported by the fact that sea bass caspase-1 processed human proIL-1β at the specific mammalian caspase-1 cleavage site, originating the same mature form obtained by incubation with the human enzyme (Fig. 7B). Moreover, despite the presence of several aspartates in the sea bass proIL-1β including D^{60} (putative cleavage site in chicken), D^{100} and D^{252} (both targeted by sea bass caspase-1 *in vitro*), the human caspase-1 was unable to cleave sea bass proIL-1β (Fig. S5C), suggesting a higher specificity acquired during phylogeny.

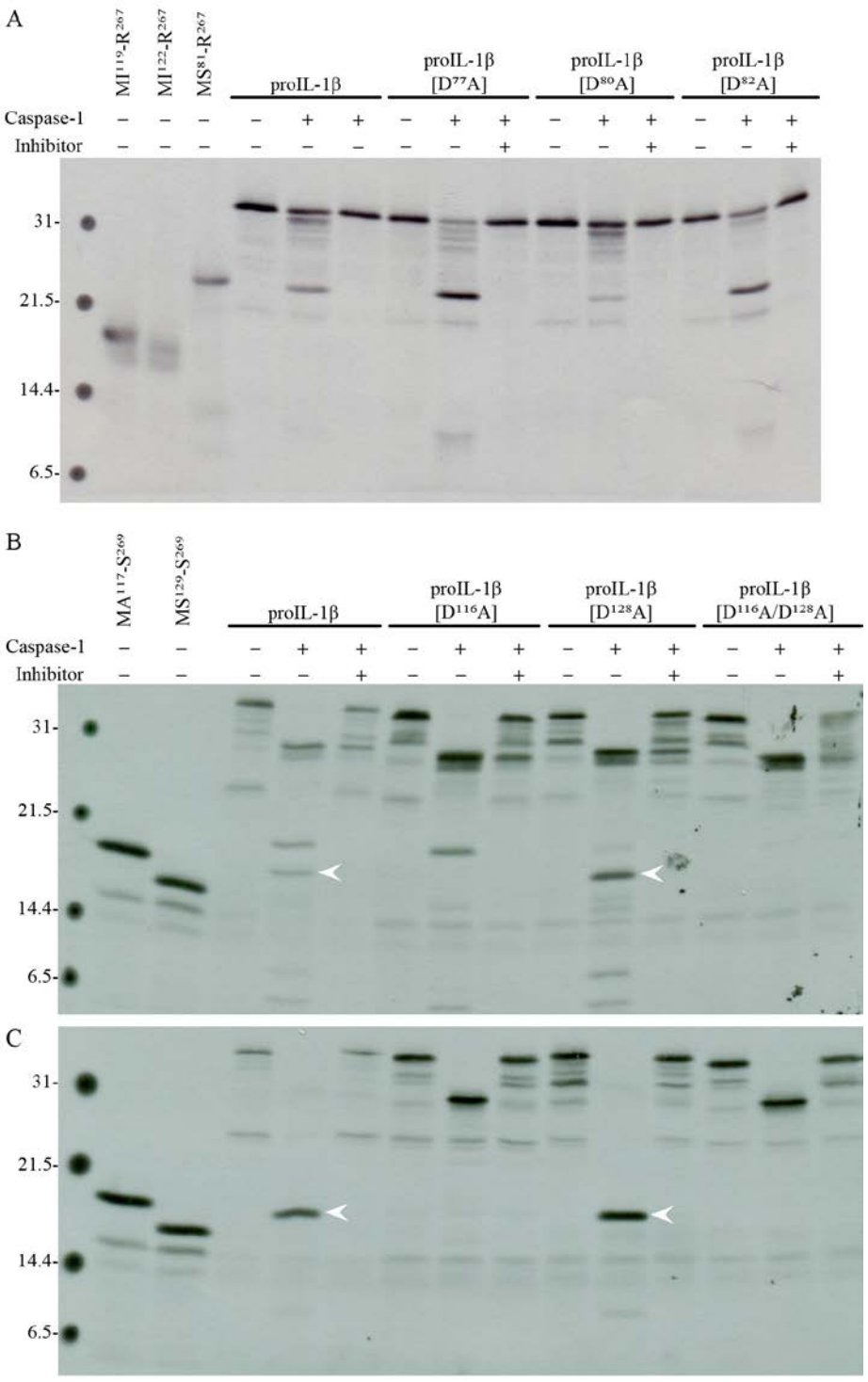

FIGURE 7.

Processing of *in vitro* synthesized chicken and human proIL-1β by sea bass caspase-1.

(A) Processing of *in vitro* synthesized chicken proIL-1β and mutants by sea bass caspase-1. The same volume of *in vitro* translated chicken proIL-1β, proIL-1β[D^{77}A], proIL-1β[D^{80}A] and proIL-1β[D^{82}A] were loaded on the gel. *In vitro* translated putative mature chicken IL-1β forms (MI^{119}-R^{267}, MI^{122}-R^{267} and MS^{81}-R^{267}) were loaded as controls.

(B) Processing of *in vitro* synthesized human proIL-1β and mutants by sea bass caspase-1.

(C) Processing of *in vitro* synthesized human proIL-1β and mutants by human caspase-1. The same volume of *in vitro* synthesized proIL-1β, proIL-1β[D^{116}A], proIL-1β[D^{128}A] and proIL-1β[D^{116}A/D^{128}A] was loaded on the gel. *In vitro* translated mature human IL-1β (MA^{117}-S^{269}) and human IL-1β form (MS^{129}-S^{269}) starting at S^{129}, homologue to sea bass S^{101}, were loaded as controls. Mature forms are highlighted by arrow heads. Numbers on the left indicate the mass of the molecular weight markers in kDa.

Discussion

Caspase-1 primary structure has since long been known in humans [17, 18, 66] and mice [55, 64], but was only recently determined for two fish, sea bass (GenBank: DQ198376 and DQ198377) and sea bream [59]. Comparative analyses of the gene and of the primary structure of sea bass caspase-1 indicate that a strikingly conserved structure has been kept throughout evolution, suggesting that its function, specificity and processing mechanisms are highly conserved from at least teleost fish to mammals.

In humans, in addition to the caspase-1 isoform α, four mRNA isoforms (β, γ, δ and ε) resulting from one or more alternative splicing events have been described [67]. In the present work, besides sea bass caspase-1 isoform 1 (DilaCASP1iso1), three alternatively spliced mRNAs encoding DilaCASP1iso2, DilaCASP1iso3, and DilaCASP1iso4 were identified (Figs. 1 and S1B). Contrary to some of the human isoforms, where the pro-domain (isoform γ) or both the pro-domain and the region containing the active site (isoforms δ and ε) have been deleted [67], all sea bass isoforms contain the pro- and p20 domains, including the fully conserved active site QACRG sequence. The differences introduced by the alternative splicing events in sea bass caspase-1 isoforms are all located downstream from the active site, with DilaCASP1iso2 and DilaCASP1iso3 displaying a shorter p10 domain with a different amino acid sequence at the C-terminal end. DilaCASP1iso4 has a longer p20 domain but no p10 (Fig. S1B). The presence of alternative transcript variants of caspase-1 in sea bass and humans [68, 69] suggests that their existence has been conserved throughout evolution and therefore they are likely to play a functional role, as described for other caspase isoforms (see below).

Similarly to what has been described for human caspase-1 [17, 56, 57, 70], DilaCASP1iso1 is autocatalytically converted *in vitro* into two active heterodimeric complexes consisting of p10 paired either with p24 or p20 (Figs. 2A and 3). Strikingly, as for *Drosophila melanogaster* DRONC [71], sea bass caspase-1 p24 is generated by cleavage after a glutamate residue (P1 position) instead of the aspartate described in mammalian caspases as an absolute requirement for enzyme specificity [13, 17, 72]. Conversion of sea bass recombinant p45 to p20/p10 and p24/p10 heterodimers occurs in a time-dependent manner through a series of intermediates resembling the auto-processing of the human caspase-1 precursor, in which the first step is the cleavage of p10 and linker from the p45 form, followed by proteolytic conversion of the resulting p36 into smaller and more active p20 and p24 forms [56, 57]. Thus, these results suggest that processing of pro-caspase-1 through several intermediates has been evolutionarily maintained at least from teleost fish to humans.

Sea bass caspase-1 isoforms 2 and 3 display some auto-processing activity, although in a much lesser degree than isoform 1. Of notice is that none of the fragments resulting from auto-processing of isoforms 2 and 3 correspond to p24, p20 or p10 (Fig. 4*B* and *C*), explaining why no enzymatic activity could be detected. However, contrary to the human isoforms [67], both sea bass isoforms 2 and 3 are processed into their p36, p20 and shorter p10 subunits by isoform 1. These data do not allow to conclude whether isoforms 2 and 3 participate in IL-1β processing or isoform 1 activation *in vivo*. On the other hand, being largely inactive, they may participate in a direct inhibitory mechanism of caspase-1, together with DilaCASP1iso4, by competing through CARD-CARD interactions for the inflammasome complex that activates caspase-1, as has been proposed for other CARD-containing proteins such as COP, INCA, ICEBERG and caspase-12 (reviewed in [73]). Alternative isoforms have been implicated in the inhibition of caspase-9 [74-76], and more recently also of caspase-3, where a short isoform (caspase-3S) antagonizes its apoptotic activity [77], thereby playing a physiological role in regulating cell death. Therefore, sea bass caspase-1 isoforms could also play a role in the regulation of the inflammatory response. Moreover, processing of isoforms 2 and 3 by isoform 1 released shorter p10 fragments, which could compete with normal p10 to generate inhibitory p20/short p10 heterodimers, as suggested for human ICEε [67].

In mammals, IL-1β is a pro-inflammatory cytokine produced as a 31 kDa inactive precursor [9, 10] that is processed by caspase-1 cleavage after D^{116} in humans and D^{117} in mice, yielding a C-terminal secreted active form of 17.5 kDa [11, 12, 14-18]. The primary structure of fish proIL-1β is similar to that of the mammalian cytokine [20-30]. However, the caspase-1 cleavage site present in all mammalian proIL-1β molecules is absent in the proIL-1β sequences reported so far for non-mammalian vertebrates (Fig. S3 and [39-41]), and the actual cleavage site has been greatly debated since the identification of the first non-mammalian IL-1β [22]. Cleavage of *in vitro* synthesized and recombinant sea bass proIL-1β by caspase-1 suggests that mature sea bass IL-1β results from processing of its pro-form at D^{100} giving rise to an 18 kDa polypeptide, in accordance with the form detected *in vivo* (Fig. 5).

In view of the above, it would be tempting to speculate that mammalian IL-1β is the exception, rather than the norm, in what concerns proteolytic activation, since an aspartate residue corresponding to sea bass D^{100} is strictly conserved in all other vertebrate IL-1β sequences (Fig. S3). Particularly in chicken, this position could correspond to the activation site, rather than the alanine residue that was considered for the putative mature protein that was structurally characterised [36], as that constitutes an improbable caspase-1 cleavage site. However, the results presented here suggest that

avian proIL-1βs may not be cleaved at the equivalent sea bass D^{100}, but instead preferentially cleaved after D^{80} (Fig. 7 and S5B), present in another proIL-1β stretch where aspartates are phylogenetically well conserved (Fig. S3). In other fish species, fragments with different molecular masses have been proposed as putative active IL-1β [31, 37, 38, 78, 79], and aspartate D^{60} in sea bream, equivalent to chicken D^{80}, has even been suggested as a possible cleavage site, because processing at D^{60} would generate a fragment with a molecular mass consistent with the apparent size (22 kDa) of the IL-1β fragment detected by Western blotting in that species [31]. However, not only none of the reported studies included amino acid sequencing of the processed IL-1β fragments, but mutation of D^{60} did not affect at all sea bass IL-1β processing (Fig. S4A), in opposition to mutation of D^{100} where IL-1β processing to an 18 kDa form was abrogated (Fig. 6, S4A,C). To which extent processing of proIL-1β at this position is the norm among fish remains to be elucidated. However, cleavage at D^{100}, here reported for sea bass proIL-1β, will most certainly have a significant structural impact in the mature molecule. Homology models of mature sea bass IL-1β produced using the available experimental models as templates (PDB entries 1IOB (human), 8I1B (mouse) or 2WRY (chicken)) indicate that cleavage at D^{100} would result in the ablation of the N-terminal beta strand, with concomitant destabilization of the other beta sheet-forming segment at the C-terminus of the molecule (Fig. 8). Therefore, sea bass proIL-1β is certainly structurally distinct in this region, although the existing differences cannot be inferred by homology modelling with the available templates.

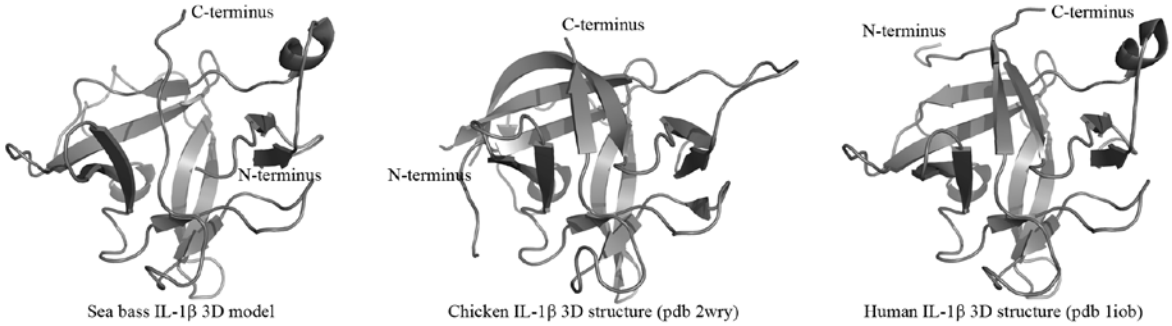

FIGURE 8. Three-dimensional homology model of sea bass Il-1β (left), and chicken (pdb entry: 2wry; center) and human (pdb entry: 1iol; right) 3D structures. All molecules are displayed in the same relative orientation. The last β-strand is absent in the sea bass model, destabilizing the pairing C-terminus of the molecule. The sea bass molecule was modelled with the Swiss Model server (http://swissmodel.expasy.org/) using the experimental coordinates of human IL-1β as template and default parameters. The resulting model had good quality indicators. Figure prepared with PyMol (http://www.pymol.org/).

Notably, in the mammalian proIL-1β -1 cleavage site the P1' position is almost always occupied by a small hydrophobic amino acid, which has been described as one of the preferential features of the peptide substrates of mammalian caspase-1 [13, 17]. On the contrary, most of the aspartates in the putative caspase-1 cleavage sites of non-mammalian vertebrates, sequence-wise to sea bass D^{100} or chicken D^{80}, are not followed by a small hydrophobic amino acid. Also of notice is the fact that a small hydrophobic amino acid is present in the P1' position after the phylogenetically conserved sea bass caspase-1 cleavage sites in monkeys (A^{129} in P1'), raising questions about the evolutionary driving force that resulted in the appearance of the mammalian cleavage site, as the mammalian aspartate at position P1 is only conserved in mammalian IL-1β sequences.

In conclusion, the present work shows that in a teleost fish, pro-caspase-1 auto-processing occurs through a series of intermediates, yielding active p24/p10 and p20/p10 heterodimers in a similar way to that described previously for human caspase-1. Moreover, the existence of alternative spliced variants of caspase-1 in sea bass is reported, suggesting that caspase-1 isoforms have been evolutionarily maintained and therefore likely play a regulatory role in the inflammatory response, as shown for isoforms of other caspases. Finally, it is shown that sea bass IL-1β is cleaved by caspase-1 at a phylogenetically conserved aspartic acid present in all known IL-1β sequences. However, in avian proIL-1βs another well phylogenetically conserved aspartate, only absent in humans, chimpanzees and zebrafish, may correspond to the cleavage site, suggesting that IL-1β processing may be class and/or species specific. Taken together, these results confirm that proIL-1β from non-mammalian vertebrates possess a specific caspase-1 cleavage site.

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SUPPORTING INFORMATION

Cloning and sequencing of caspase-1

Messenger RNA was purified with the MicroPoly(A)Pure™ kit (Ambion®) and transcribed to cDNA using the Superscript™ II First-Strand Synthesis System (Invitrogen). PCRs were done according to standard procedures. For amplifying full length sequences Pfu DNA polymerase was used in the reactions. DNA sequencing was performed using primers detailed in Table SII. Complementary DNAs used for 5' Rapid Amplification of cDNA Ends (5'RACE) experiments were purified using High Pure PCR Product Purification Kit reagents (Roche), and used with the 5´RACE System from Invitrogen (Version 2.0). The purified cDNA was dATP tailed using Recombinant Terminal Transferase (Roche).

Messenger RNA (~340 ng) extracted from the head kidney of a fish 1 h after stimulation with LPS was reverse transcribed using primer APv (Table SII). Degenerate primers were designed based on conserved regions of caspase-1 amino acid sequences from different vertebrates. The cDNA was first PCR amplified using primers CASP1FW1/CASP1RV1 followed by a semi-nested amplification using primers CASP1FW1/CASP1RV2 (Table SII). A third amplification with primers CASP1FW1/CASP1RV2 was carried out and a PCR product with the expected size (168 bp) was purified, cloned and sequenced. This sequence was used to design the specific reverse primers DLCASP1RV1, DLCASP1RV2 and DLCASP1RV3 (Table SII), in order to obtain the 5' untranslated region (5´-UTR) using the 5'RACE strategy. The obtained PCR product was purified, cloned and sequenced and, because it did not include the start codon, the 5'RACE strategy was repeated using again the specific reverse primers DLCASP1RV1 and DLCASP1RV2, plus the reverse primer DLCASP1RV5, designed based on the sequence obtained from the previous 5'RACE (Table SII). The fragment obtained was cloned, sequenced and used to design the specific primer DLCASP1FW7 (Table SII) in the 5'-UTR. This primer was combined with primer AUAP (Table SII), in a PCR aiming to obtain the complete sequence of sea bass caspase-1. Four PCR products ranging from 1500 to 1680 bp were purified, cloned into the pGEM®-T Easy Vector (Promega) and sequenced.

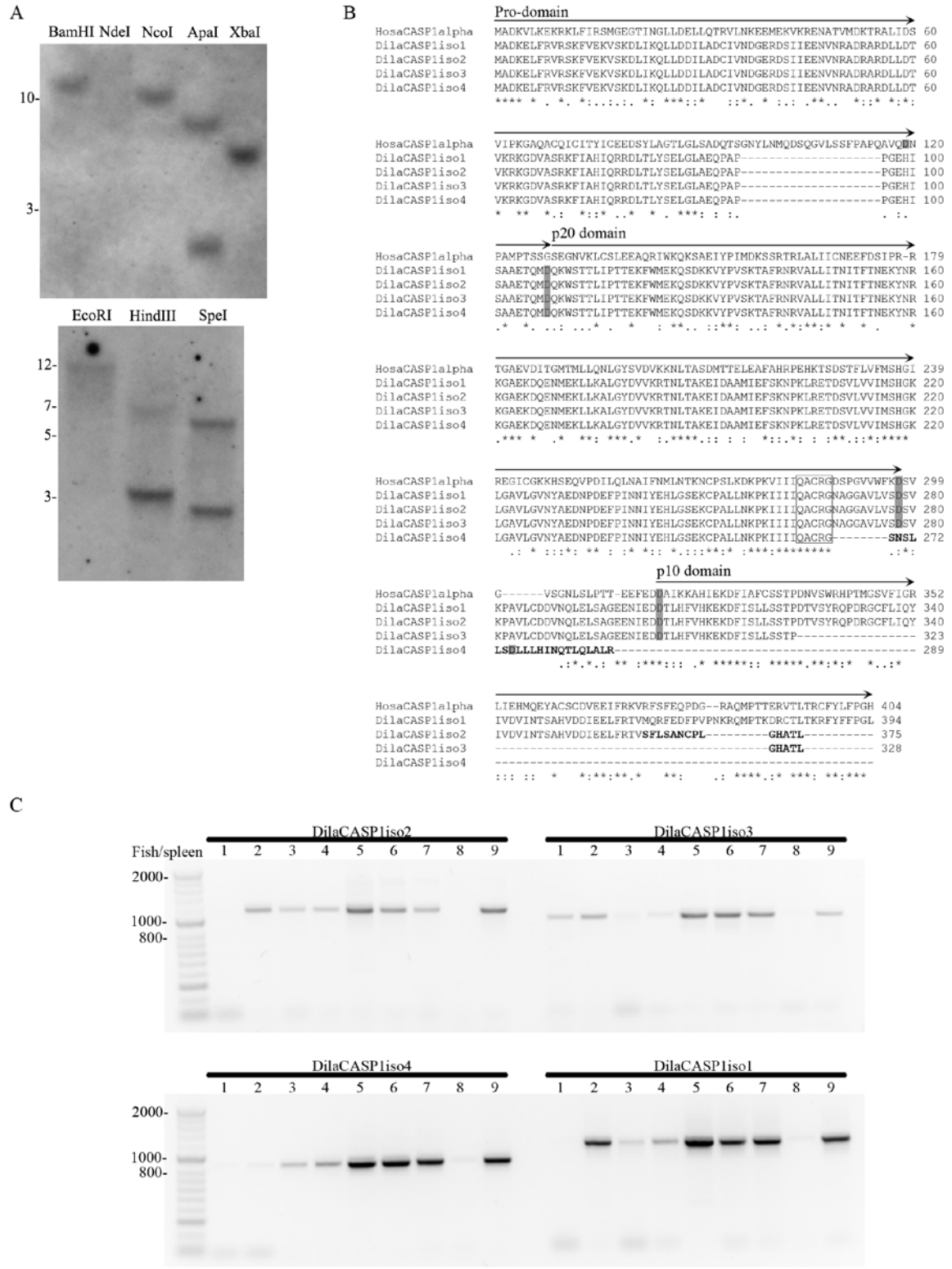

FIGURE S1. Caspase-1 is encoded by a single gene in sea bass and encodes at least four different transcripts. **(A)** Southern blotting analysis. Restriction enzymes EcoRI, Spe I, BamHI, NcoI, NdeI, ApaLI (zero cutters in sea bass caspase-1 gene), and HindIII and XbaI (single and double cutter in intron 1 of sea bass caspase-1 gene, respectively) were used to digest sea bass gDNA from 4 fish, according to the manufacturer's instructions (Fermentas). Digestion products were subjected to 0.8% agarose gel

electrophoresis and Southern blotting [2]. A fragment of the sea bass caspase-1 coding region was amplified with primers DLCASP1FW4 and DLCASP1RV6 (Table SII). The PCR product (367 bp) was purified as previously described, labelled using the Gene Images^{TM} AlhPhos Direct^{TM} Labelling and Detection System (Amersham Biosciences), and used as probe to detect the caspase-1 gene. For signal generation and detection the Chemiluminescent Signal Generation and Detection with CDP-*Star*^{TM} protocol from the same kit was followed. Numbers on the left indicate kbp. **(B)** Multiple sequence alignment of the predicted primary structures of sea bass caspase-1 isoforms and human capase-1α (HosaCASP1alpha). The sequences were aligned with CLUSTAL W [1] using default parameters. Domain boundaries were determined with PROSITE (www.expasy.org/prosite) and comparison with human capase-1α. Sea bass caspase-1 comprises a pro-domain (Met^{1}-Asp^{108}), followed by a p20 domain (Gln^{109}-Asp^{278}), a short connecting peptide (Ser^{279}-Asp^{304}), and a p10 domain (Thr^{305}-Leu^{394}), and signaled on top of the alignment. The active-site pentapeptide QACRG is boxed. The aspartic acid cleavage sites are shaded in gray. Asterisks and ":" and "." denote identity and chemical similarity between amino acids according to the default CLUSTAL W scoring matrix. The amino acid residues in the sea bass caspase-1 isoforms 2, 3 and 4 that differ from the isoform 1 are in bold. DilaCASP1iso1 (GenBank accession number: DQ198376) has 1663 bp including, as in human and mouse caspase-1 mRNA [3, 4], two polyadenylation signals within the 3'-UTR. The ORF encodes a 394 aa long protein that, as in the human and mouse sequences, has no hydrophobic signal sequence. Sequence analysis revealed that the sea bass protein contains both caspase family p20 and p10 domain profiles and a caspase family active site signature (K^{257}PKIIIIQACRG). A pro-domain containing a caspase recruitment domain (CARD), typical of pro-inflammatory caspases, is also present. DilaCASP1iso2 (GenBank accession number: HQ398873) results from an alternative splicing event involving the utilization of an alternative splicing site located within intron 7, which leads to the retention of 31 nucleotides from this intron (Fig. 1). This creates a putative caspase-1 isoform containing the complete pro- and p20 domains but having a shorter p10 domain (72 aa compared to 90 aa of the full length p10). DilaCASP1iso3 (GenBank accession numbers: HQ398874) is generated by an exon skipping event, in which exon 7 is lost (Fig. 1). This creates a putative caspase-1 isoform containing the complete pro- and p20 domains but having a much shorter p10 domain (25 aa compared to 90 aa of the full length p10). DilaCASP1iso4 (GenBank accession number: HQ398875) results from two alternative splicing events. One results in the retention of intron 5, generating a stop codon located 62 nucleotides downstream the beginning of this intron. The other is generated by an exon skipping event, in which exon 7 is spliced out of the transcript, as in DilaCASP1iso3 (Fig. 1). This creates a putative caspase-1 isoform containing a complete pro-domain; however, the splicing originates a shift in the reading frame that generates a different coding region after the active center. Although there is an aspartate residue within this alternative coding region, it is apparently not used as cleavage site, as this isoform is not auto-processed nor processed by active DilaCASP1iso1 (see below), therefore rendering a longer p20 domain (11 aa longer and 21 aa different) and no p10. **(C)** Expression analysis of caspase-1 isoforms in sea bass spleen. Complementary DNA was synthesized from total RNA extracted from spleens of 9 non-stimulated fish as described in supporting information section *"cloning and sequencing of caspase-1"*. The cDNAs were amplified using the forward primer DLCASP1FW11 (designed in an exon/intron boundary) for all isoforms and a reverse primer specific for different isoforms (Table SII): isoform1, DLCASP1RV18 (between exon 7 e 8); isoform 2, DLCASP1RV16 (intron 7); isoform 3/4m, DLCASP1RV19 (between exon 6 and 8); and isoform 4, DLCASP1RV17 (intron 5). The reverse primer DLCASP1RV19 amplifies isoforms 3 and 4 but, in combination with the primer DLCASP1FW11, different size products are obtained (989 bp for isoform 3 and 1076 bp for isoform 4). The PCR products were purified and sequenced.

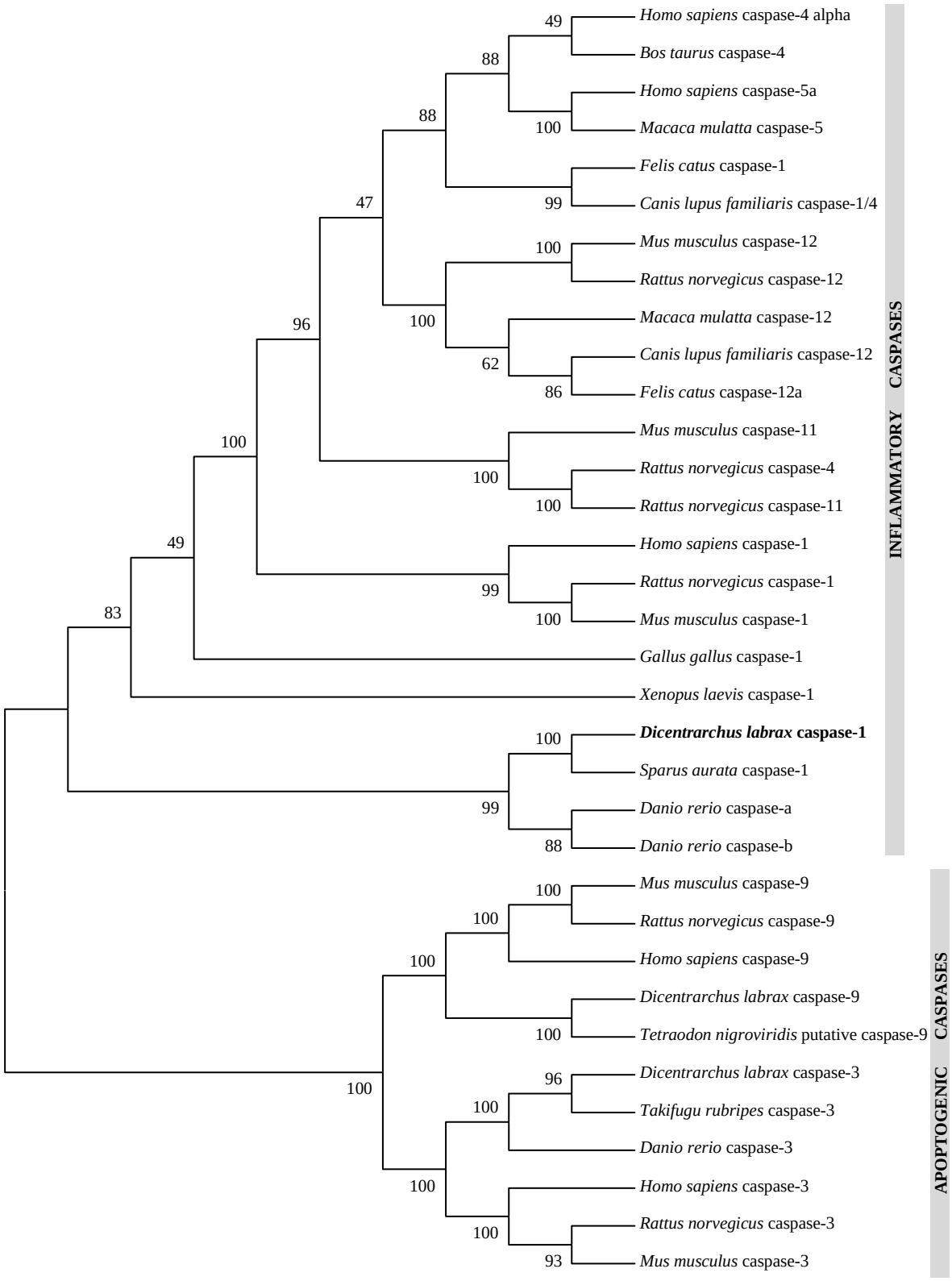

FIGURE S2. Neighbour-joining tree (*MEGA* version 3.1) [5] with p-distance and complete deletion of gaps of inflammatory (caspase-1, -4, -5, -11 and -12) and apoptogenic (caspase-3 and -9) caspases. The amino acid sequences were aligned with CLUSTAL W [1] using the default parameters. The numbers in branching nodes

denote the bootstrap percentages for 1000 replicates. The different branches are supported by high bootstrap values. GenBank accession numbers: for caspase-1 see Table SI; for caspase1/4: ABX79372 (*Canis lupus familiaris*); for caspase-3: ABC70996 (*Dicentrarchus labrax*), AAM43816 (*Takifugu rubripes*), BAB32409 (*Danio rerio*), CAC88866 (*Homo sapiens*), AAH81854 (*Rattus norvegicus*), AAH38825 (*Mus musculus*); for caspase-4: NP_001216 (*H. sapiens*), NP_788811 (*Bos taurus*), NP_446188 (*R. norvegicus*); for caspase-5: ABB58698 (*H. sapiens*), XP_001100375 (*Macaca mulatta*); for caspase-9: BAA87905 (*H. sapiens*), AAK26235 (*R. norvegicus*), AAH56447 (*M. musculus*), ABC70998 (*D. labrax*); the sequence of *Tetraodon nigroviridis* putative caspase-9 (CAG01765) was obtained by Blast search of the Genbank database with the sequence of sea bass caspase-9 as query; for caspase-11: CAA73531 (*M. musculus*), AAK38735 (*R. norvegicus*); for caspase-12: AAT91067 (*M. musculus*), EDL78548 (*R. norvegicus*), ABG21363 (*M. mulatta*), NP001070704 (*C. lupus familiaris*), ABX79369 (*Felis catus*).

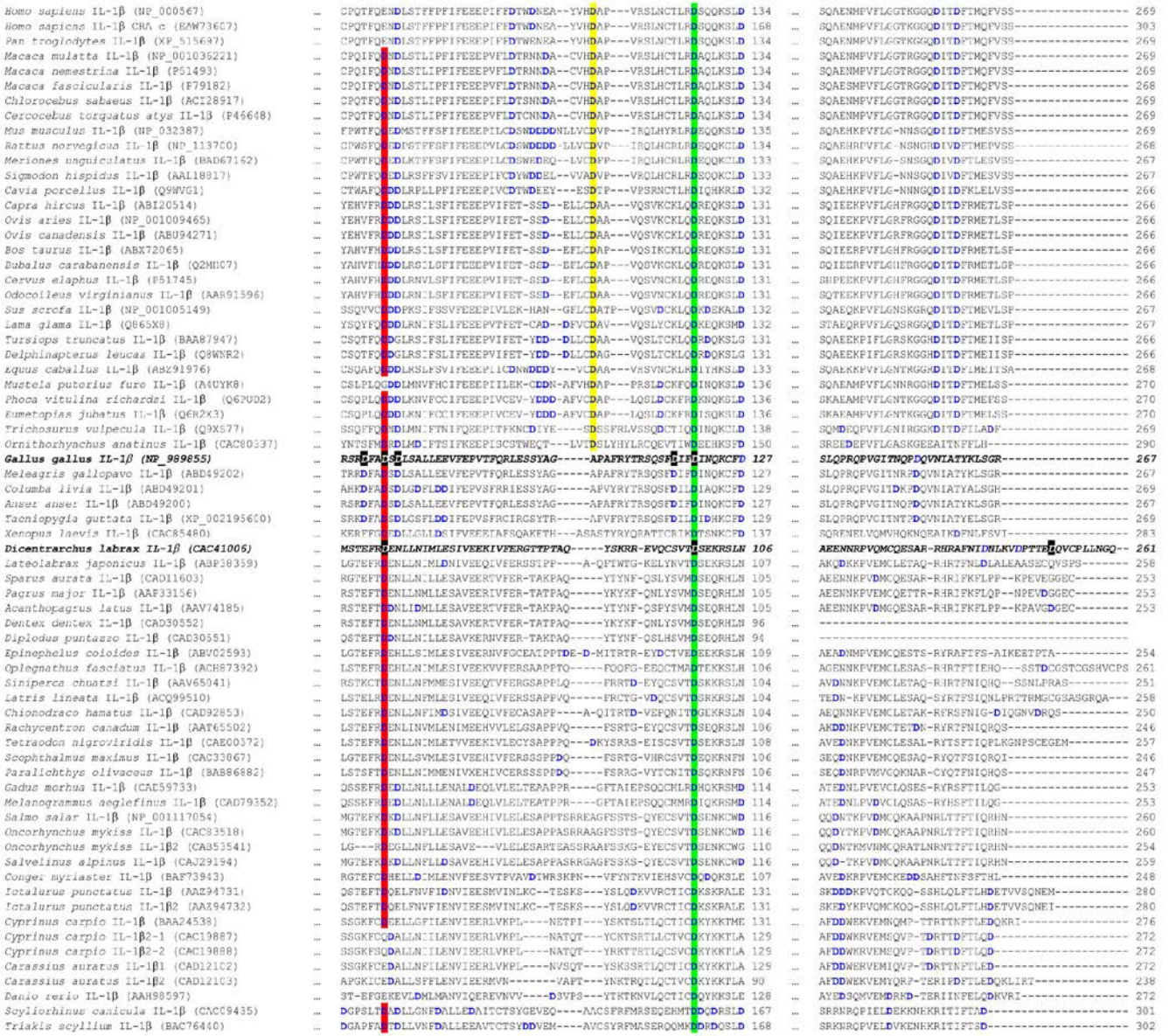

FIGURE S3. Alignment of IL-1β amino acid sequences from vertebrates belonging to different Classes. The regions having aspartate cleavage sites are shown (aspartates are shaded black). The sequences were aligned with CLUSTAL W [1] using the default parameters. GenBank accession numbers are shown within brackets. Aspartate residues are blue colour and bold type. The caspase-1 cleavage site in mammalian IL-1β sequences is shaded yellow. The conserved aspartic acids homologous to the caspase-1 cleavage site in sea bass IL-1β sequence are shaded green. The conserved aspartic acids homologous to the putative caspase-1 cleavage site in avian IL-1β sequence are shaded red. Mutated aspartate residue in the sea bass and chicken IL-1β sequences are shaded black.

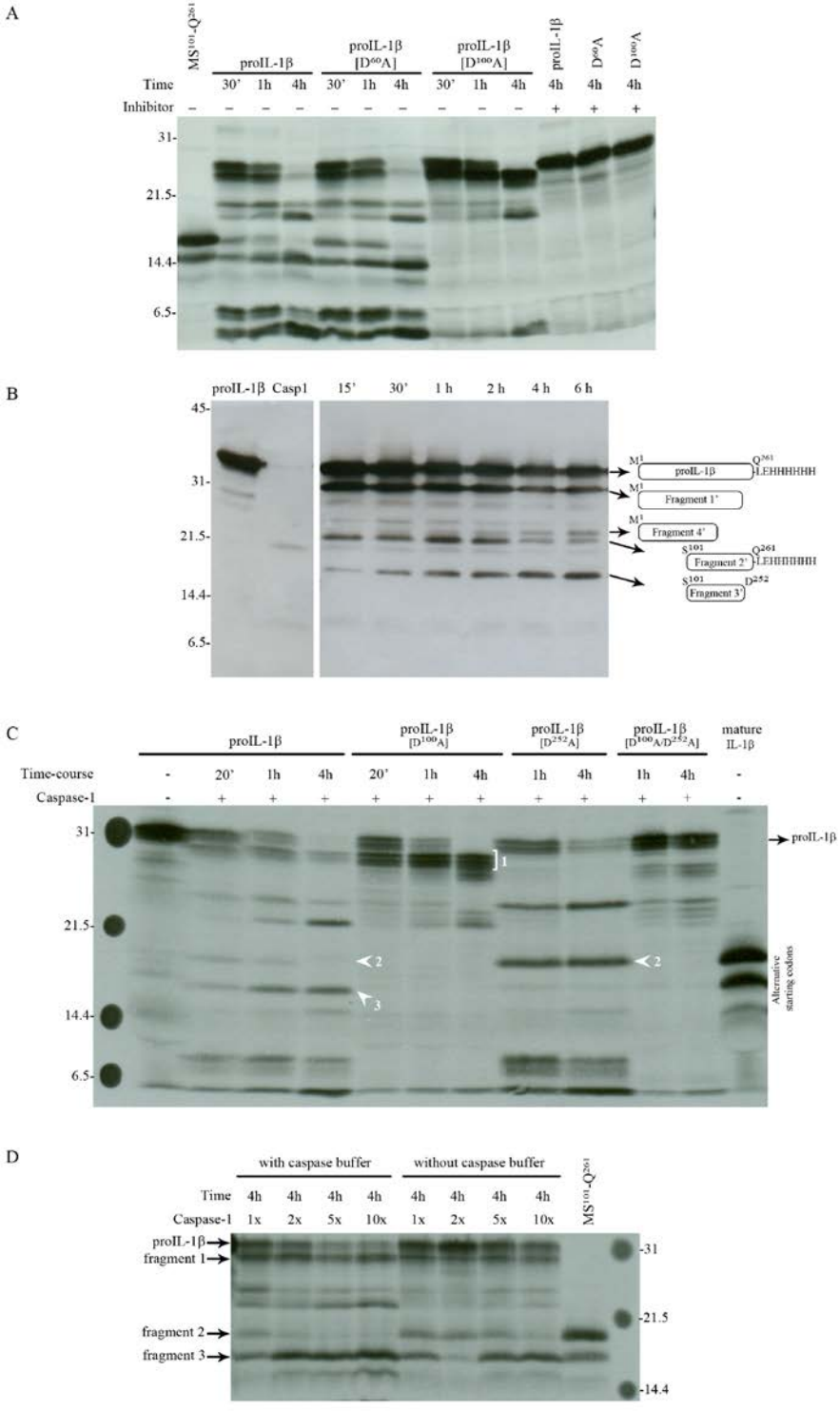

FIGURE S4. Processing of i*n vitro* synthesized and recombinant sea bass proIL-1β by caspase-1. **(A)** Time-course processing *in vitro* synthesized sea bass proIL-1β proIL1β[D^{60}A] and proIL1β[D^{100}A] by sea bass caspase-1, in the presence or absence of caspase-1 inhibitor Ac-YVAD-CHO, using caspase-1 buffer. *In vitro* synthesized putative mature sea bass IL-1β (MS^{101}-Q^{261}) was loaded as control. **(B)** Time-course processing of recombinant sea bass proIL-1β by caspase-1 using caspase-1 buffer. proIL-1β[His] (proIL-1β) and DilaCASP1iso1 (Casp1) were loaded as controls. On the right, a schematic illustration of the fragments obtained, as concluded from N-terminal sequencing, is shown; capital letters denote amino acid residues and the superscript numbers the amino acid position within the sea bass IL-1β sequence. **(C)** Time-course processing of *in vitro* synthesized sea bass proIL-1β proIL1β[D^{60}A] and proIL1β[D^{100}A] using caspase-1 buffer. *In vitro* synthesized mature sea bass IL-1β (MS^{101}-Q^{261}) was loaded as control. **(D)** Processing of *in vitro* synthesized sea bass proIL-1β with or without caspase-1 buffer and with different amounts of caspase-1 (relative amount of caspase-1 added to the reaction). *In vitro* synthesized mature sea bass IL-1β (MS^{101}-Q^{261}) was loaded as control. ProIL-1β fragment 1, 2 and 3 are indicated in (C) and (D). Numbers on the left (right in D) indicate the mass of the molecular weight markers in kDa. The same volume of each *in vitro* synthesized proIL-1β forms were used and loaded on the gel.

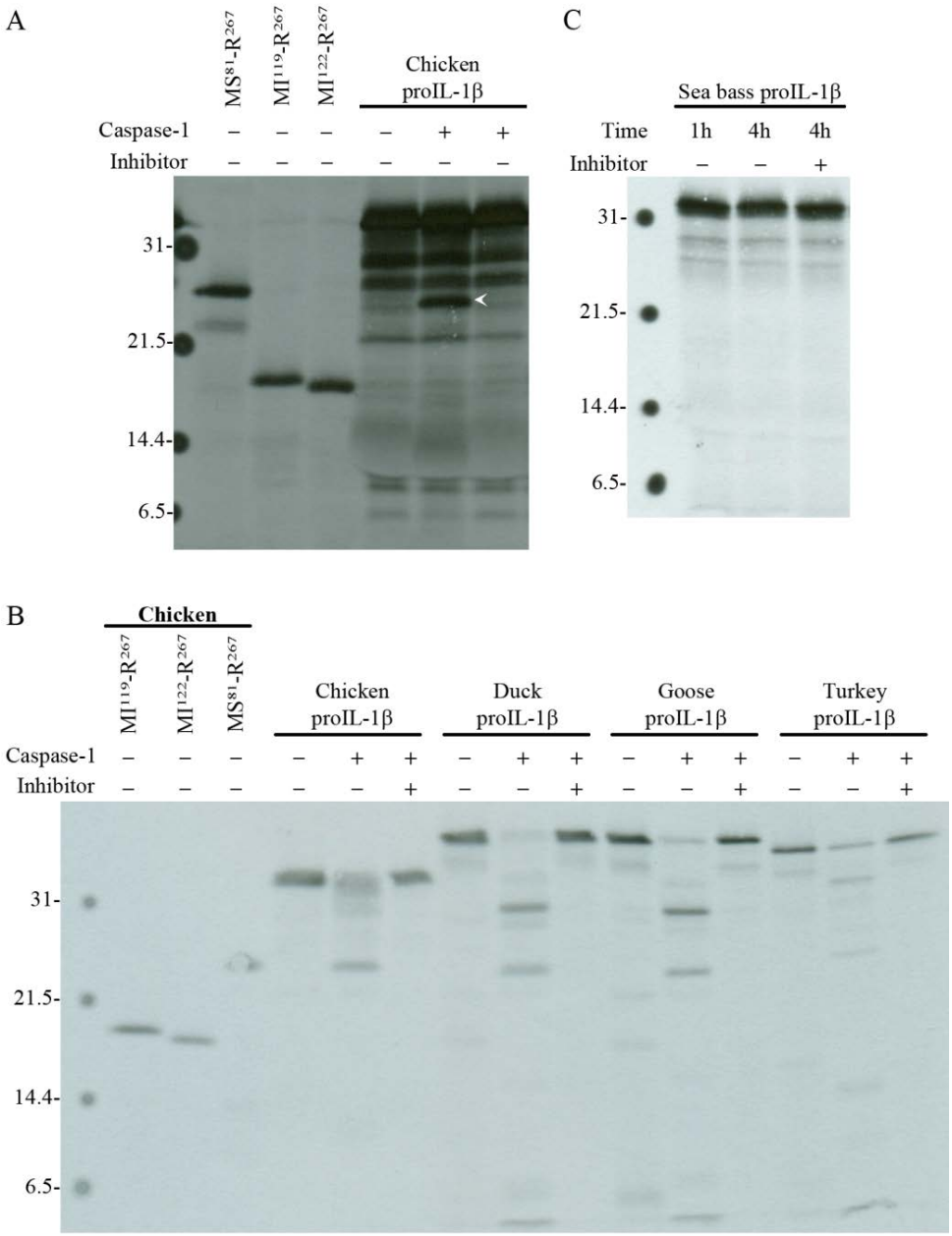

FIGURE S5. Processing of *in vitro* synthesized sea bass and chicken proIL-1β by human and sea bass caspase-1, respectively. **(A)** Chicken proIL-1β is processed by human caspase-1. Putative mature chicken IL-1β forms ($MS^{81}R^{267}$, $MI^{119}R^{267}$ and $MI^{122}R^{267}$) were loaded as controls. **(B)** Processing of *in vitro* synthesized chicken, duck, goose and turkey proIL-1β by sea bass caspase-1. Putative mature chicken IL-1β forms ($MS^{81}R^{267}$, $MI^{119}R^{267}$ and $MI^{122}R^{267}$) were loaded as controls. **(C)** Sea bass proIL-1β is not processed by human caspase-1. Numbers on the left indicate the mass of the molecular weight markers in kDa. The same volume of *in vitro* synthesized proIL-1β forms for each species was used and loaded on the gel.

Table SI. Amino acid sequence conservation for caspase-1 of different species. The percentages of similarity and identity were calculated by pair-wise alignments with the program needle [6] as implemented at EBI web site (www.ebi.ac.uk) with first and extending gap penalties of 10 and 0.5, respectively.

Species	***Dicentrarchus labrax***			Accession number
	Identity	Similarity	Gaps	
Sparus aurata	62.7%	76.7%	4.7%	CAM32183
Danio rerio caspy	38.5%	53.4%	19.7%	NP_571580
Danio rerio caspy2	30.7%	45.0%	23.8%	NP_690840
Rattus norvegicus	37.0%	52.3%	10.0%	NP_036894
Mus musculus	37.0%	51.2%	12.1%	NP_033937
Xenopus laevis XICE-a	36.9%	51.1%	9.3%	BAA14017
Homo sapiens isoform alpha percursor	36.0%	51.1%	8.6%	NP_150634
Felis catus	35.4%	51.6%	11.3%	NP_001009365
Gallus gallus	26.4%	39.1%	31.6%	AAC69917

Table SII. Primer sequences and application. [a] R= A/G; Y= C/T; N= A/C/T/G; K= G/T; D=A/G/T; W=A/T; S=G/C ; V=A/C/G

Primer	Sequence[a]	Application
APv	5' – GGCCACGCGTCGACTAGTAC(T_{17})V – 3'	cDNA synthesis
AUAP	5' – GGCCACGCGTCGACTAGTAC – 3'	cDNA amplification
CASP1FW1	5' – GARAARGAYGARGARAAYATG – 3'	cDNA amplification
CASP1RV1	5' – GACYTTNCKRAANARYTCYTC – 3'	cDNA amplification
CASP1RV2	5' – GCCRTGNSWCATDATNACNAC – 3'	cDNA amplification
DLCASP1FW7	5' – TCTGAACCAGAGCGCATTAC – 3'	5'- Racing
DLCASP1RV1	5' – CATATCCCAGAGCTTTGAGAAGT – 3'	5'- Racing
DLCASP1RV2	5' – CTCAATCATGGCAGCGTCAA – 3'	5'- Racing
DLCASP1RV3	5' – TGTCAGTCTCCCTGAGTTTC – 3'	5'- Racing
DLCASP1RV5	5' – CTTTCAGGAATCGTGTGGCC – 3'	5'- Racing
DLCASP1FWEcoRI	5' – CGGAATTCTCTGAACCAGAGCGCATTA – 3'	Genomic amplification
DLCASP1RVEcoRI	5' – CCGAATTCCGACAGATGGAGCAAACAGTGTG – 3'	Genomic amplification
DLCASP1FW4	5' – TGTGGCCCTTCTCATCACTA – 3'	Probe amplification
DLCASP1RV6	5' – GGCCTGGATGATGATGATCT – 3'	Probe amplification
DLCASP1FW11	5' – AGAAATGGCAGATAAGGAG – 3'	Expression
DLCASP1RV16	5' – GAGGACAGTTTGCCCTTAGA – 3'	Expression
DLCASP1RV17	5' – CGGAGTGCAAGTTGCAATGT – 3'	Expression
DLCASP1RV18	5' – AGCGTTGCATGACTGTTCTG – 3'	Expression
DLCASP1RV19	5' – AAAGCGTTGCATGACCGGGG – 3'	Expression
DLCASP1FWNdeI	5' – GGAATTCCATATGGCAGGTAAGGAGCTTTTCAG – 3'	Recombinant production
DLCASP1RVXhoI	5' – CCGCTCGAGGCCTGGGAAGAAGTAGAA – 3'	Recombinant production
DLISO2/3RVXhoI	5' – CTCGAGAAGCGTTGCATGAC – 3'	Recombinant production
DLISO4RVXhoI	5' – ACTCGAGACGGAGTGCAAGT – 3'	Recombinant production
DLIL1βFWNde3	5' – AGCAGCATATGGAATCTGAGATGAA – 3'	Recombinant/*in vitro* translation
DLIL1βRVXho4	5' – CCGCTCGAGCTGTCCATTCAAAAGGGG – 3'	Recombinant/*in vitro* translation
DLIL1βFWNde4	5' – CATATGAGCGAAAAGAGGAGCTTAGTTCTG – 3'	Recombinant/*in vitro* translation
DLIL1βRVXho1	5' – GCGCTCGAGTTACTGTCCATTCAAAAGGGGACAA– 3'	Recombinant/*in vitro* translation
DLIL1βMUT0FW1	5' – CCCCTTTTGAATGGACAGTGAGAGCACCACCACCACC – 3'	Site direct mutagenesis (Remove LE6His)
DLIL1βMUT0RV1	5' – GGTGGTGGTGGTGCTCTCACTGTCCATTCAAAAGGGG – 3'	Site direct mutagenesis (Remove LE6His)
DLIL1βD^{60}FW1	5' – GATGAGCACTGAGTTCAGAGCGGAAAACCTGCTA – 3'	Site direct mutagenesis (D^{60}A)
DLIL1βD^{60}RV1	5' – TAGCAGGTTTTCCGCTCTGAACTCAGTGCTCATC – 3'	Site direct mutagenesis (D^{60}A)
DLIL1βD^{100}FW1	5' – AATGCAGCGTGACCGCGAGCGAAAAGAGGA – 3'	Site direct mutagenesis (D^{100}A)
DLIL1βD^{100}RV1	5' – TCCTCTTTTCGCTCGCGGTCACGCTGCATT – 3'	Site direct mutagenesis (D^{100}A)
DLIL1βD^{252}FW1	5' – TCCAACAACTGAGGCGCAAGTTTGTCCC – 3'	Site direct mutagenesis (D^{252}A)
DLIL1βD^{252}RV1	5' – GGGACAAACTTGCGCCTCAGTTGTTGGA – 3'	Site direct mutagenesis (D^{252}A)
HSIL1βD^{116}FW1	5' – GAGGCTTATGTGCACGCGGCACCTGTACGA – 3'	Site direct mutagenesis (D^{116}A)
HSIL1βD^{116}RV1	5' – TCGTACAGGTGCCGCGTGCACATAAGCCTC – 3'	Site direct mutagenesis (D^{116}A)
HSIL1βD^{128}FW1	5' – ACTGCACGCTCCGGGCTTCACAGCAAAAAA – 3'	Site direct mutagenesis (D^{128}A)
HSIL1βD^{128}RV1	5' – TTTTTTGCTGTGAAGCCCGGAGCGTGCAGT – 3'	Site direct mutagenesis (D^{128}A)
HSIL1βmat1FWNcoI	5' – CCCCATGGCACCTGTACGATCACTGAACT – 3'	*In vitro* translation
HSIL1βmat1_2RVXhoI	5' – CGGCTCGAGTCAGGAAGACACAAATTGCAT – 3'	*In vitro* translation
HSIL1βmat2FWNcoI	5' – GACCATGGCGTCACAGCAAAAAAGC – 3'	*In vitro* translation
GGIL1βFWNdeI	5' – GGCATATGGCGTTCGTTCCCGACCTGGAC – 3'	*In vitro* translation
GGIL1βRVXhoI	5' – TTCTCGAGTCAGCGCCCACTTAGCTTGTAGGT – 3'	*In vitro* translation
GGIL1βD^{77}FW	5' – GAGGCCGAGGAGCAGGGCATTTGCTGACAGCGA – 3'	Site direct mutagenesis (D^{77}A)
GGIL1βD^{77}RV	5' – TCGCTGTCAGCAAATGCCCTGCTCCTCGGCCTC – 3'	Site direct mutagenesis (D^{77}A)
GGIL1βD^{80}FW	5' – GGGACTTTGCTGCGAGCGACCTGAG – 3'	Site direct mutagenesis (D^{80}A)
GGIL1βD^{80}RV	5' – CTCAGGTCGCTCGCAGCAAAGTCCC – 3'	Site direct mutagenesis (D^{80}A)
GGIL1βD^{82}FW	5' – AGGGACTTTGCTGACAGCGCACTGAG – 3'	Site direct mutagenesis (D^{82}A)
GGIL1βD^{82}RV	5' – CTCAGTGCGCTGTCAGCAAAGTCCCT – 3'	Site direct mutagenesis (D^{82}A)
GGIL1βmat1FWNdeI	5' – GGCATATGATCTTCGACATCAACCAGAAGTG – 3'	*In vitro* translation
GGIL1βmat2FWNdeI	5' – GGCATATGATCAACCAGAAGTGCT – 3'	*In vitro* translation
GGIL1βmat4FWNdeI	5' – GGCATATGAGCGACCTGAGCG – 3'	*In vitro* translation

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CHAPTER VII

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Concluding remarks and future perspectives

Two decades have passed since the discovery of the first caspase and meanwhile, emerging roles of mammalian caspases in different biologic processes have been disclosed. When this work started, the information on fish caspases was scarce, with few published papers, mainly in zebrafish, and some direct sequence entries available in the NCBI database. BLAST search of non-redundant, expressed sequence tags (EST) and genomic (Ensembl) databases enabled the identification of several apoptotic molecules in fish, including caspases from each different functional group (initiator, executioner and pro-inflammatory), as well as proteins involved in their regulation (Annex 1). Given the importance of fish research to support the much needed development of aquaculture and to study diverse mammalian normal and pathological issues, and given the participation of apoptosis [1, 2] and inflammation [3-6] in many biological processes and pathologies in vertebrates, research on fish caspases is of obvious relevance.

The work presented in this thesis was carried out at the Fish Immunology and Vaccinology (FIV) group at IBMC that studies host-pathogen interactions in fish. The group has been mainly involved in studying the sea bass (*Dicentrarchus labrax*) immune system as well as its interaction with *Photobacterium damselae* ssp. *piscicida* (*Phdp*), a Gram negative bacteria that causes massive mortalities with high economic impact in several cultured marine fish species [7]. In this context, it was found that the major virulent factor of *Phdp* is an apoptogenic AB toxin (manuscript in preparation), named apoptosis inducing protein of 56 kDa (AIP56) [8, 9] that induces post-apoptotic secondary necrosis of sea bass macrophages and neutrophils [8, 9]. It was also demonstrated that inoculation of AIP56 replicates the pathology of the *Phdp* infection. Due to the major role played by caspases in apoptosis, cloning and characterising sea bass apoptotic caspases became a priority, because it would allow to develop tools to study the involvement of those caspases in the *Phdp*-associated pathology. Indeed, the data presented in Chapters III, IV and V confirmed the involvement of caspase-8, -9 and -3 in the *Phdp* infection. Furthermore, it was shown that pro-inflammatory cytokines are involved in the *Phdp* infection and, in particular, increased levels of IL-1β secretion in the initial stages of the infection were demonstrated [10]. The mechanism for the processing of IL-1β in mammals is well characterised and known to be mediated by caspase-1, which cleaves proIL-1β at aspartate residue 116 in the context of the tetrapeptide YVHD originating its biologically active mature form of 17 kDa. Although several studies in fish have shown that IL-1β participates in inflammatory responses, the mechanism involved in the processing of pro-

IL-1β in fish has not been disclosed. Since the sequencing of the first non-mammalian IL-1β, 13 years ago [11], it became evident that non-mammalian IL-1βs do not have the mammalian caspase-1 cleavage site, therefore raising the question of how this cytokine is activated in non-mammalian species. Considering this, the sequence of sea bass caspase-1 and the study of its involvement in the processing of IL-1β appeared as an important issue to be addressed.

I. Sea bass apoptotic caspases

As mentioned in the introduction section, the initiator caspases have a critical role of transmitting the apoptotic signal through the activation of the executioner caspases and of generating the caspase cascade. The initiator caspases of the extrinsic and intrinsic pathways, caspase-8 and -9 respectively, as well as the central executioner of apoptosis, caspase-3, were identified in sea bass. Although some fish caspase genes have two copies (Annex 1), resulting from the early whole-genome duplication in the teleost lineage after the divergence from tetrapods about 450 million years ago [12-14], Southern blotting analysis suggest that the caspase genes identified in this thesis are present as single copies in the sea bass genome. At the molecular level, the obtained sequences of sea bass caspase-8, -9 and -3 retain the motifs that are functionally important, such as the pentapeptide active-site motif (QACXG) and the cleavage sites at the aspartic acids that give rise to the characteristic organisation of caspases in NH_2-terminal pro-domain, large and small subunits. Moreover, a short pro-domain typical of executioner caspases was identified in caspase-3, while long pro-domains characteristic from initiator caspases were present in caspases-8 and -9. In the case of sea bass caspase-8, two death effector domains (DED) were predicted by bioinformatics analysis. The same approach identified a caspase recruitment domain (CARD) in the NH_2-terminal pro-domain of sea bass caspase-9. As reviewed in the introduction, both DED and CARD are important domains involved in protein-protein interactions necessary to mediate dimerization and consequent activation of caspase-8 and -9, respectively. The sea bass caspases possess significant degrees of similarity to the corresponding sequences from several vertebrates of different taxonomic groups. Furthermore, it was shown that sea bass caspase-8, -9 and -3 were expressed at low levels in several organs from non-stimulated sea bass and that their expressions are up-regulated in the spleen in response to apoptotic stimuli. The data gathered from the molecular characterisation of sea bass apoptotic caspases allowed to demonstrate that caspase-8, -9 and -3 are activated in peritoneal macrophages and neutrophils treated *ex vivo* with AIP56 and that both the extrinsic and intrinsic pathways

are involved in the AIP56-induced apoptosis [15]. Thereby, this work provided information that contributed to a better understanding of the biology of fish apoptotic caspases and allowed the development of tools that may be used in the study of the mechanisms of apoptosis in fish.

II. Sea bass caspase-1 and its involvement in IL-1β processing

The work described in this thesis identified, in the sea bass genome, a caspase-1 gene which primary structure and gene organisation have been conserved through evolution. Sea bass caspase-1 pro-form is auto-processed through several intermediates in a similar way as its human homolog, resulting in active p24/p10 and p20/p10 heterodimers. However, similarly to what has been reported for *Drosophila melanogaster* DRONC [16], the exclusive preference for cleavage at an aspartic acid at P1 position described for mammalian caspases is not an absolute requirement for sea bass caspase-1. Also of notice, is the presence of alternative splice variants of sea bass caspase-1 that may have a role in the regulation of the inflammatory response as described for other caspase isoforms in mammals [17-22]. The important role of the pro-inflammatory cytokine IL-1β as a mediator of inflammatory responses has been documented in several species of fish [23-26]. In the present thesis (Chapter VI), significant advances in the understanding of fish IL-1β processing are presented, through the identification of a cleavage site for caspase-1 at an aspartic acid absolutely conserved in all the IL-1β sequences reported so far. However, the equivalent sequence-wise aspartate in avian IL-1βs is not used as cleavage site in birds. Instead, in birds, another phylogenetically conserved aspartate, only absent in humans, chimpanzees and zebrafish, is used, suggesting that IL-1β processing may be class and/or species specific. These results clearly show that proIL-1β from non-mammalian vertebrates have a specific caspase-1 cleavage site. Moreover, the cleavage at D^{100}, in sea bass proIL-1β, implies significant structural changes in the mature sea bass IL-1β when compared to that of mammals and chicken. Solving the 3D structure of sea bass IL-1β would help to define the functional implications, if any, of a different mature IL-1β structure.

In conclusion, the sea bass counterparts of mammalian caspases-3, -8 and -9 were sequenced and characterised, and shown to be expressed/activated upon an apoptotic stimulus. The data obtained allowed the production of monospecific polyclonal antibodies against peptides form each identified caspase that were shown to be valuable tools for monitoring the active forms of the caspases, as exemplified for caspase-1 in

Chapter VI. The genetic information as well as the antibodies produced in this work may be used to follow the involvement of sea bass caspases in biological processes at genetic and proteomic level, under physiological or pathological conditions. Among many questions that have been raised by the results obtained during this thesis, four main aspects must be highlighted: (i) the cleavage at D^{100} in sea bass proIL-1β leads to the question of whether other fish proIL-1β are also cleaved at an equivalent aspartate residue. Cleavage at this aspartate implies significant structural differences in the mature sea bass/fish IL-1β, when compared to that of mammals and chicken. Solving the 3D structure of sea bass IL-1β would help defining the functional implications of such structural differences and would give insights into the evolutionary driving force that has led to the appearance of different caspase-1 cleavage sites in proIL-1βs from different classes/species of vertebrates. (ii) The involvement of caspase-1 isoforms in the processing/regulation of caspase-1 and pro-IL-1β have been neglected. It is very likely that, as shown for other caspases, caspase-1 isoforms play an important role in the regulation of the inflammasome and consequent secretion of pro-inflammatory cytokines; these is an issue that is worth to investigate. (iii) The increasing significance of the inflammasomes in different pathologies, such as auto-inflammatory and infectious diseases, has been uncovered in recent years. In an attempt to limit the pathogen replication, the activation of inflammasomes is accompanied by the induction of pyroptosis during infection by several pathogens. However, in fish there are no studies regarding inflammasomes or pyroptosis. Thereby, dissecting the molecular mechanisms for caspase-1 activation at the inflammasomes as well as to study the interference of pathogens with inflammasome function in fish are research fields worth to be pursued. Since it is known that there is an increased expression of IL-1β, as well as other inflammatory cytokines, during the initial phase of the *Phdp* infection, it would be interesting to investigate the involvement of the inflammasome and the occurrence of pyroptosis in the *Phdp* infections. (iv) The emerging roles of caspases, including in embryonic development, monocyte differentiation and regulation of T cell activation as well as their participation in the balance between survival and death, show the importance of these molecules. Whether fish caspases possess non-apoptotic and non-inflammatory functions remains to be investigated.

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ANNEX I

Fish and Apoptosis: Molecules and Pathways.

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Fish and Apoptosis: Molecules and Pathways

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Abstract: Apoptosis is a genetically controlled and evolutionarily conserved form of active cell death, albeit with an increase in complexity with continuing development. A high conservation at the functional and molecular level has been described between the players of the apoptotic machinery in invertebrates (*Caenorhabditis elegans* and *Drosophila*) and mammals. However, fish represent an excellent and advantageous model for the study of vertebrate development and disease, bridging the gap between the *C. elegans/Drosophila* and mouse/human models. Moreover, contrary to *C. elegans* and *Drosophila*, fish can be used for studying the development and function of vertebrate-specific organs and have a fully developed immune system similar to that of mammals. Last but not less important, both the environment and human health will obviously gain by using the knowledge generated through the use of fish models, for developing better prophylactic and therapeutic measures with impact on the aquaculture industry. In the present article, structural and functional data on the most important apoptosis related molecules, namely death-receptor, Bcl-2 and caspase families, and mechanisms are reviewed. The data point to the existence in fish of apoptotic pathways equivalent to those of mammals, making fish useful animal models for studying apoptosis, which may have great applicability for the advance of the knowledge on the role of apoptotic cell death in human apoptosis-related disorders as well as in pharmaceutical design

Key Words: Fish, Apoptosis, Death receptors, Bcl-2 family, Caspases, Intrinsic pathway, Extrinsic pathway, Apoptosis models.

1. INTRODUCTION

Apoptosis [1] is a genetically controlled and evolutionarily conserved form of active cell death, albeit with an increase in complexity with continuing development. It is critical in the elimination of redundant, ectopic, damaged, mutated or infected cells, playing therefore an essential role in normal development, tissue homeostasis and immunity. Two main distinct apoptosis signalling pathways have been described in mammals: (i) the extrinsic/death-receptor pathway, initiated at the tumour necrosis factor (TNF) family receptors [2-4] or alternatively by the so-called dependency receptors [5] and (ii) the intrinsic/Bcl-2-regulated/mitochondrial pathway, regulated by the Bcl-2 family proteins and mainly characterized by mitochondrial membrane permeabilization (MMP) [6-8]. Both pathways can be connected [9, 10] through caspase-mediated activation of the pro-apoptotic Bcl-2 family member Bid [11-14], possibly as an amplification mechanism of the caspase activity (reviewed in [8, 15, 16]). While each of the pathways is differentially triggered and require different initiator caspases they both converge at the effector level, ultimately leading to cell shrinkage, chromatin condensation (pyknosis), nuclear fragmentation (karyorhexis), blebbing, and phosphatidylserine translocation to the outer layer of the plasma membrane (reviewed in [8]).

A high conservation at the functional and molecular level has been described between the players of the apoptotic machinery in invertebrates (*Caenorhabditis elegans* and *Drosophila*) and mammals (reviewed elsewhere in this issue and [17, 18]). However, fish represent an excellent and advantageous model for the study of vertebrate development and disease [19-22], bridging the gap between the *C. elegans/Drosophila* and mouse/human models [23]. *C. elegans* and *Drosophila* are established models that have been powerful systems for the study of many biological processes and are amenable to large scale screens; however, they cannot be utilized to address the development and function of vertebrate-specific organs [24]. Additionally, and in contrast with worms and flies, fish have a fully developed immune system similar to that of mammals [25]. Last but not less important, both the environment and human health will obviously gain by using the knowledge generated through the use of fish models of research, for developing better prophylactic and therapeutic measures that can be directly applied to fish so aquaculture can develop in a sustainable and environmental friendly way.

Here, structural and functional data on the most important apoptosis related molecules and mechanisms in fish are reviewed. Published data point to the existence in fish of apoptotic pathways equivalent to those of mammals, making fish useful animal models for studying apoptosis, and with applicability for the advance of the knowledge on the role of apoptotic cell death in human apoptosis-related disorders (reviewed elsewhere in this issue). However, and as previously stressed by Bhardwaj and Aggarwal [26], while searching gene and protein databases and literature for fish homologs of mammalian apoptosis molecules and reports on apoptosis, we have faced enormous inconsistencies in the nomenclature, as the same molecule may be given several different names or abbreviations (Table **1**), making the search in the databases extremely difficult. As many molecules from different species will likely appear in the following years it is also expectable that this confusion and complexity will greatly increase. Therefore, we suggest that codes should be updated and rules for nomenclature proposed so everyone could follow. Here, we make a first approach and suggestion and strongly encourage others to adhere/improve or come up with a better system (Table **1**).

2. EXTRINSIC PATHWAY

Death receptors are cell surface receptors that, when bound to specific ligands, transmit signals that induce cell death through the extrinsic apoptotic pathway. All death receptors belong to the tumor necrosis factor receptor (TNFR) superfamily and contain a death domain (DD), required for apoptotic signal transduction, as part of their sequence [26]. The extracellular domains of the receptors of this family contain varying numbers of cysteine-rich domains (CRD), but otherwise display no significant homology, and the cytoplasmic domains are generally small and, except for the DD, also do not share significant homology [26]. None of these receptors have inherent enzymatic activity and they exert their cellular functions by recruiting adaptor molecules, resulting in the formation of the death-inducing signalling complexes (DISCs), composed of the death receptor, FADD, caspase-8 and, when present, caspase-10 [27].

Examples of death receptors are TNFR1, Fas, DR3, DR4, DR5 and DR6 [27]. To exert their cellular functions, death receptors

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must be activated by their respective ligands that include TNF, Fas ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL).

Table 1. Nomenclature and Rules Used in the Present Article for Fish Apoptosis-Related Molecules and Corresponding Names used in Other Reports

Used Abbreviation	Other Abbreviation(s)	Reference(s)
DareBcl-X_L	zBlp1, zfBlp1, zBcl-X_L, zfBcl-X_L	[98, 102, 121]
DareMcl-1a	zMcl-1a, zfMcl-1a	[98, 117, 121]
DareMcl-1b	zMcl-1b	[98, 117]
DareNr-13	zNR13, Nrz	[98, 109]
DareBad	zBad	[98, 115, 117]
DareBik	zBik	[98]
DareBmf2	zBmf2	[98]
DarePuma	zPuma	[98]
DareBax2	zBax2	[98]
DareBok1	zBok1	[98]
DareBok2	zBok2	[98]
DareBcl-2	zBcl-2, zBlp2	[98, 106]
DareBmf1	zBmf1	[98]
DareNoxa	zNoxa	[98]
DareBcl-X_L-L	zfBlp1-L	[102]
DareBcl-X_L-S	zfBlp1-S	[102]
DareBax1	zBax1	[98]
DareBid	zBid	[98]
DareFas	zFas	[30]
DareHDR	zHDR	[30]
DareOTR	zOTR	[30]
IcpuFas	CF Fas	[35]
CtidTRAIL	GC-TRAIL	[29]
DareTRAIL1a	zDL1a	[30]
DareTRAIL1b	zDL1b	[30]
DareTRAIL2	zDL2	[30]
DareTRAIL3	zDL3	[30]
PaolTNFR1	fTNFR1	[41]
DareTNFα1	zTNF1	[30]
DareTNFα1	zTNF2	[30]
DareFADD	zFADD	[30]
DareTRADD	zTRADD	[30]
DareTNFR1	zTNFR1	[30]

The rational behind the nomenclature is as follows: the name and/or abbreviation first described for a certain molecule, or, when the name has been updated and is presently widely used, is maintained for all species. When both species and molecule are abbreviated and merged, a four-letter abbreviation of the species scientific name precedes the molecule abbreviation. The species abbreviation is constructed as proposed for the nomenclature of the major histocompatibility complexes [185], that is, the first two letters are derived from the name of the genus, the last two letters from the name of the species. In both cases, the first two letters of the scientific names are chosen; when the letter combination has already been used to designate the apoptosis molecule of another species, the next letters from the genus or species name are used. Only the first of the four letters form the species abbreviation is capitalized. E.g., Zebrafish (*Danio rerio*) Bid: DareBid.

In mammals, TRAIL is expressed as a type II transmembrane protein (with a short cytoplasmic N-terminal domain and a long C-terminal extracellular receptor-binding domain) that can be cleaved from the cell surface by the activity of a metalloprotease to form a soluble ligand [28]. Both full-length membrane expressed TRAIL and the soluble ligand can rapidly induce apoptosis in a wide variety of human cancer cell lines [28]. It has been shown that TRAIL is expressed constitutively in a wide range of cells and tissues, although studies in mice and humans showed that TRAIL is not expressed at the surface of freshly isolated T-cells, B-cells, monocytes, dendritic cells, natural killer (NK) cells or NKT-cells (reviewed in [28]). After stimulation with interferons most NK-cells, monocytes, peripheral T-cells, and dendritic cells express TRAIL at their surface, suggesting an important role of TRAIL in innate immune responses (reviewed in [28]). In mammals, TRAIL can bind two apoptosis-inducing receptors, DR4 (TRAIL-R1) and DR5 (TRAIL-R2), two additional cell-bound receptors incapable of transmitting an apoptotic signal, DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4), sometimes also called decoy receptors, and, a soluble receptor called osteoprotegerin (OPG) (reviewed in [27, 28]).

TRAIL gene has been cloned from human, mice, chicken and fish (Table **2**). The molecular characterization and expression analysis of TRAIL gene, and the putative transcription factor binding sites involved in regulating TRAIL gene expression from grass carp were reported in 2006 [29]. These authors also found a putative zebrafish TRAIL when grass carp TRAIL (CtidTRAIL) was used to search the zebrafish database [29]. CtidTRAIL and the putative zebrafish TRAIL are 299 amino-acid residues long with 93.3% identity between them [29]. CtidTRAIL has 36.3%, 36.1%, 33.8% and 38.8% identity to human, mouse, rat and chicken TRAILs, respectively [29]. CtidTRAIL and the putative zebrafish TRAIL possess a TNF conserved domain in the C-terminal portion, a feature typically present in members of the TNF ligand family. Similarly to what has been observed in mammals and chicken, TRAIL was found to be expressed in most grass carp tissues examined, which included gill, intestine, head kidney, trunk kidney, spleen and liver [29]. The expression of *CtidTRAIL* in liver contrasts to what has been observed in adult humans, where TRAIL is not expressed in the liver, but is in accordance to what has been reported for chicken [29]. In contrast to the organization of the promoters of related members of the TNF family, such as TNF-α, LT-β, and FasL, human and grass carp TRAIL promoters lack typical "TATA" or "CAAT" boxes [29]. Similar to human TRAIL promoter, *CtidTRAIL* promoter contains many putative transcription factor binding sites, such as the consensus motifs for lymphoid-specific transcription factors NFAT, STAT, GATA, NF-kappaB, Sp1 and Ap1 [29]. *Ctid-TRAIL* has additional specific transcription factor binding sites, such as p53 and IRF1. The presence of all these lymphoid-specific transcription factors in *Ctid-TRAIL* promoter region, together with its constitutive expression in most tissues and with its molecular and genomic structure, may indicate that CtidTRAIL and human TRAIL play similar functions in the apoptosis signalling [29]. It has recently been reported that zebrafish possess four Apo2L/TRAIL relatives (DareTRAIL1a, DareTRAIL1b, DareTRAIL2, DareTRAIL3) and that the genomic region encoding *DareTRAIL2* has conserved synteny with human locus encoding Apo2L/TRAIL [30]. Eimon *et al.* [30] established that three (DareTRAIL1a, DareTRAIL1b, DareTRAIL2) of the four zebrafish Apo2/TRAIL homologs interact with DareHDR and DareOTR. DareHDR and DareOTR were described as DD-containing TNFRs. DareOTR has been shown to be highly expressed in the ovary [31] and several tissues of the embryo, including notochord, intermediate cell mass (ICM) and cells of the lateral line. DareHDR has been implicated in haematopoiesis [32] and found to be expressed in the ventral posterior region of the zebrafish embryo at 10-somite stage, and at the ICM (where primitive haematopoiesis takes place) at later stages of embryonic development [30, 32]. It was not possible to identify mammalian homologues of DareHDR and DareOTR by sequence alone because their extracellular domains (ECDs) resemble CD40, Herpes virus entry mediator (HVEM), and Fas, while their DDs are most homologous to DR4, DR5 and avian TvbS1 [30]. The fact that the ECDs of DareHDR and DareOTR are differ-

ent from those of human Apo2/TRAIL DD-containing receptors DR4 and DR5 and the decoy receptors DcR1, DcR2, and osteoprotegerin, led Eimon and co-workers to conclude that receptors for this ligand do not necessarily share a conserved ECD architecture in nonmammalian vertebrates [30].

FasL is a type II membrane protein belonging to the TNF family (reviewed in [33]). Proteolytic cleavage leads to the generation of membrane-bound N-terminal fragments and a soluble FasL (sFasL) ectodomain [34]. FasL is expressed primarily on activated T cells, some tumor cells, and immune-privileged sites such as the eye and the testis (reviewed in [33]). FasL induces apoptosis in cells expressing the Fas receptor. Fas is a type I membrane protein of the TNFR superfamily that was discovered due to the ability of specific antibodies to induce apoptosis in lymphoid tumor lines expressing the molecule (reviewed by [33]). Fas homologues from catfish and medaka fish were identified (Table **2**) and characterized [35, 36]. Although the amino-acid sequence similarity of catfish Fas (IcpuFas) was low compared with mammalian Fas proteins (approximately 23 %), similarly to mammalian Fas, IcpuFas possesses three CRD, two TNFR/NGFR family signatures and a DD within the predicted cytoplasmic tail [35]. Within the CRD, the percentage identity of IcpuFas to representative mammalian Fas increased to 37% and the cysteine backbone of the molecule was found to be remarkably conserved. Medaka Fas displayed 24% identity and 42% similarity to human Fas, and was found to contain a DD in the cytoplasmic region [36]. It has been reported that the genomic structure of the Fas gene is conserved within vertebrates and the genomic organization around the Fas gene locus exhibits synteny throughout vertebrates suggesting that the Medaka Fas is a true ortholog for mammalian Fas [36]. Experiments where cells were transfected with a vector expressing a chimeric human/oryzias (h/o) Fas showed that Medaka Fas is a functional molecule with killing activity and that the putative DD in Medaka Fas cytoplasmic tail is capable of transmitting apoptotic signals in mammalian cells in response to exogenous death stimuli [36]. Homologs of Fas and FasL of zebrafish have recently been reported, although no expression of these genes was detected during the first 48h of embryonic development [30]. Nevertheless, injection of zebrafish embryos with mRNA encoding *DareFas* induced apoptosis and death of the embryos and overexpression of DareFas in human HeLa cells induced cell death at similar levels to the ones observed when the cells overexpressed human Fas [30]. Expression studies indicated that Fas was expressed in several catfish cell lines and that its transcription was down regulated in response to increasing cell density, this effect being particularly evident in cells in which FasL expression was readily detected in confluent cultures, but showed very low levels of Fas [35]. As discussed by Long *et al.* [35], this parallels what has been reported for several mammalian cell lines that constitutively express FasL and, in response to increasing cell density, down regulate Fas to prevent FasL-induced apoptosis. The expression of Fas and FasL has been studied in natural cytotoxic cells (NCC) of teleosts using commercially available polyclonal or monoclonal antibodies raised against human or mouse Fas or FasL [35, 37, 38]. Bishop and co-workers [37] reported that NCC from tilapia and catfish do not express membrane FasL nor Fas, but constitutively express cytoplasmic soluble FasL (sFasL) and that ligation of NCC with tumour cells caused the release of sFasL that was responsible for lysis of Fas+ tumour target cells. The authors suggested that, unlike mammalian-activated NK or T-cells, the more phylogenetically primitive NCC do not express membrane FasL nor Fas but instead release soluble FasL as a cytokine capable of inducing apoptosis of Fas-bearing target cells [37, 38]. Again using serological approaches, Long *et al.* [35] have shown that catfish cells express a protein which cross-reacts with a monoclonal antibody directed to human FasL and that is regulated in a similar manner. However, efforts to identify the cDNA encoding the catfish homologue of FasL have not been successful [35].

The involvement of Fas and FasL in the apoptosis induced by gonadotropin-releasing hormone (GnHR) in the testes of goldfish has been studied [39]. Using anti-human Fas and FasL polyclonal antibodies, it has been shown that GnHR-iduced apoptosis in the goldfish testes was mediated by increased levels of Fas and FasL proteins as well as elevated levels of activated caspase-3 and -8 [39]. As discussed by Andreu-Vieyra *et al.* [39], although for years GnHR has been proposed to play a role in the regulation of the gonadal function, the exact mechanism underlying its actions has remained elusive. By the time their work was published, it had been shown that up-regulation of Fas was a prerequisite for mammalian germ cell apoptosis and the association of the induction of Fas and FasL expression in mammalian testis with apoptosis in response to a variety of agents/factors (e.g. ionizing radiation, ischemia/perfusion damage, deprivation of serum and growth factors, heat treatment, androgen withdrawal) had been demonstrated. However, no information was available on the regulation of Fas and/or FasL by GnHR in the male gonad. The work developed in goldfish suggests that induction of apoptosis by GnHR in the testis is mediated by increased levels of Fas and FasL, although the underlying mechanism remains to be disclosed [39]. Taken together, the above described reports indicate that the Fas-FasL system of teleosts may have similar functions to the one of mammals.

In mammals, TNFα is a pleiotropic cytokine that mediates apoptosis, cell proliferation, immunomodulation, inflammation, allergy and autoimmune disease, among others, by its interaction with TNFR1 or TNFR2, but only TNFR1 contains a DD (reviewed in [26, 40]). TNFR1 is expressed in most tissues and cell types and is involved in transducing two major types of signals: activation of transcription factor NF-kappaB and apoptosis [26]. TNF genes have been identified and characterised in several species of fish (Table **2**), but their participation in the apoptosis in fish has not been much investigated.

An ortholog of mammalian *TNFR1* was identified and characterised in Japanese flounder (*Paralichthys olivaceus*) [41]. The putative extracellular region of PaolTNFR1 contains three CRD and a site for N-glycosylation and the predicted cytoplasmic domain contains the consensus motif for a DD found in mammalian TNFR1 and Fas [41]. The existence of three CRD in the extracellular domain of PaolTNFR1 contrasts with the existence of four CRD in human TNFR1. Within the DD of PaolTNFR1, the first Trp residue, which is conserved in the death domain of other species, is replaced by a Glu [41]. *DareTNFR1*, an ortholog of mammalian *TNFR1*, was also reported in zebrafish [30]. The identification was made based on the architecture of its ECDs, a dendogram of its DDs and conserved synteny with mammalian counterparts [30]. Although *DareTNFR1* was found to be expressed in several tissues of the embryo, overexpression of DareTNFR1 did not induce apoptosis or mortality in zebrafish embryos and it was suggested that these data are in accordance to what happens in mammals, where TNFR1 is implicated mainly in nonapoptotic responses [30]. However, transient overexpression of DareTNFR1 in human cells induced cell death at levels similar to the ones observed in cells overexpressing human TNFR1 [30]. The zebrafish genome also encodes two orthologs of TNF (*DareTNF1* and *DareTNF2*) and the genomic region encoding *DareTNF2* has conserved synteny with human locus encoding TNFα [30]. Although *DareTNF1* and *DareTNF2* expressions were not detected by *in situ* hybridisation in zebrafish embryos in the first 48 hours post fertilisation (hpf), *DareTNF2* expression was detected by RT-PCR from 8-48 hpf [30]. However, injection of *DareTNF1* and *DareTNF2* mRNAs into zebrafish embryos did not induce apoptosis, discernible defects or death of the embryos [30].

As stated above, it is known from studies in mammalian systems that binding of death ligands to the death receptors results in the formation of signalling complexes where the activation of the initiator caspase-8 and -10 occurs. Binding of FasL to Fas and of

Table 2. Apoptosis Molecules in Fish

Gene	Fish Species	Accession n°		E-Value	Non-Fish Most Similar Gene	Domain
DEATH RECEPTORS						
Trail1a	*Danio rerio*	DQ812112	c	2,0E-59	*Gallus gallus* Trail-like	TNF_2
	Ictalurus punctatus	CV992476	p	1,0E-41	*G. gallus* Trail-like	TNF_2
	Oryzias latipes	DC267723	p	-	-	-
	Pimephales promelas	DT091460	p	1,0E-11	*G. gallus* Trail-like	-
	Salvelinus fontinalis	AAG47644	p	5,0E-11	*G. gallus* Trail-like	-
	Squalus acanthias	EG361075	p	3,0E-27	*Homo sapiens* TNF ligand 10	TNF_2
	Takifugu rubripes	CA589457	p	-	-	-
	Tetraodon nigroviridis	CAF91505	p	7,0E-29	*G. gallus* Trail-like	TNF_2
Trail1b	*Danio rerio*	DQ812113	c	8,0E-54	*G. gallus* Trail-like	TNF_2
	Gasterosteus aculeatus	DT953874	c	1,0E-49	*G. gallus* TNF ligand 10	TNF_2/TNF_1
	Ictalurus punctatus	CV993622	p	5,0E-42	*G. gallus* Trail-like	TNF_2
	Oryzias latipes	AM320871	p	3,0E-06	*G. gallus* Trail-like	-
	Tetraodon nigroviridis	CAF94487	c	6,0E-11	*G. gallus* Trail-like	-
Trail2	*Danio rerio*	BC076005	c	1,0E-59	*G. gallus* TNF ligand 10	TNF_2/TNF_1
	Acipenser transmontanus	DR977007	p	1,0E-46	*Mus musculus* TNF ligand 10	TNF_2
	Ctenopharyngodon idella	AAW22592	c	1,0E-60	*G. gallus* TNF ligand 10	TNF_2/TNF_1
	Gasterosteus aculeatus	DW600037	c	1,0E-49	*G. gallus* TNF ligand 10	TNF_2/TNF_1
	Oncorhynchus mykiss	ABC84583	c	1,0E-52	*G. gallus* TNF ligand 10	TNF_2/TNF_1
	Oryzias latipes	AM301363	p	2,0E-30	*G. gallus* TNF ligand 10	TNF_2/TNF_1
	Osmerus mordax	EL541767	p	1,0E-20	*G. gallus* TNF ligand 10	TNF_2
	Pimephales promelas	DT290095	p	8,0E-50	*G. gallus* TNF ligand 10	TNF_2/TNF_1
	Salmo salar	DW566544	p	4,0E-26	*G. gallus* TNF ligand 10	TNF_2/TNF_1
	Tetraodon nigroviridis	CAF98738	c	2,0E-49	*G. gallus* TNF ligand 10	TNF_2/TNF_1
Trail3	*Danio rerio*	DQ812114	c	5,0E-14	*G. gallus* TNF ligand 10	TNF_2
	Misgurnus anguillicaudatus	BJ832187	p	7,0E-15	*M. musculus* TNF ligand 10	TNF_2
	Gasterosteus aculeatus	DW614538	p	5,0E-41	*H. sapiens* TNF ligand 10	TNF_2
	Pimephales promelas	DT153890	p	-	-	-
	Rutilus rutilus	EG536906	p	1,0E-15	*G. gallus* TNF ligand 10	TNF_2
FasL	*Danio rerio*	DQ812115	c	2,0E-25	*Bos taurus* Fas ligand	TNF_2
	Oncorhynchus mykiss	BX857476	p	1,0E-22	*G. gallus* Fas ligand	TNF_2
	Pimephales promelas	DT145470	c	3,0E-17	*G. gallus* TNF ligand 11	TNF_2
	Salmo salar	EG849778	p	9,0E-22	*G. gallus* Fas ligand	TNF_2
TNF	*Danio rerio*	NM_212859	c	4,0E-19	*Oryctolagus cuniculus* TNF	TNF_2
	Acanthopagrus schlegelii	AAP94278	c	2,0E-22	*O. cuniculus* TNF	TNF_2
	Cyprinus carpio	CAC84642	c	1,0E-19	*Macropus eugenii* TNF	TNF_2
	Dicentrarchus labrax	ABB05053	c	5,0E-21	*M. eugenii* TNF	TNF_2
	Gasterosteus aculeatus	DV010394	p	4,0E-12	*M. eugenii* TNF	TNF_2
	Ictalurus punctatus	CAD10389	c	2,0E-15	*Felis catus* TNF	TNF_2
	Lateolabrax japonicus	AAR02413	c	4,0E-22	*O. cuniculus* TNF	TNF_2
	Oncorhynchus mykiss	CAB89521	c	4,0E-18	*Mustela putorius furo* TNF	TNF_2
	Oreochromis niloticus	AAR88097	c	1,0E-19	*Equus caballus* TNF	TNF_2
	Pagrus major	AAP76392	c	4,0E-22	*E. caballus* TNF	TNF_2
	Paralichthys olivaceus	BAA94969	c	2,0E-21	*M. eugenii* TNF	TNF_2
	Pleuronectes platessa	CAC95226	p	1,0E-06	*E. caballus* TNF	TNF_2
	Pseudosciaena crocea	ABK62876	c	9,0E-21	*O. cuniculus* TNF	TNF_2
	Salmo salar	ABG91800	c	1,0E-17	*M. eugenii* TNF	TNF_2
	Salvelinus fontinalis	AAF86331	c	4,0E-17	*M. eugenii* TNF	TNF_2
	Sparus aurata	Q8JFG3	c	2,0E-21	*O. cuniculus* TNF	TNF_2
	Squalus acanthias	EE627603	p	2,0E-34	*G. gallus* TNF	TNF_2
	Takifugu rubripes	NP_001033074	c	2,0E-19	*Macaca fascicularis* TNF	TNF_2

(Table 2) contd….

Gene	Fish Species	Accession n°		E-Value	Non-Fish Most Similar Gene	Domain
DEATH RECEPTORS						
DR6	*Danio rerio*	DQ812118	c	8,0E-173	*Canis familiaris* TNFR DR6	TNFR_NGFR_2/TNFR_NGFR_1; Death Domain
	Gasterosteus aculeatus	DW651614	p	9,0E-81	*Monodelphis domestica* TNFR DR6	Death Domain
	Pimephales promelas	DT180281	p	7,0E-31	*C. familiaris* TNFR DR6	TNFR_NGFR_2/TNFR_NGFR_1
	Poecilia reticulata	ES371241	p	5,0E-51	*M. domestica* TNFR DR6	-
	Rutilus rutilus	EG545418	p	3,0E-71	*C. familiaris* TNFR DR6	Death Domain
	Salvelinus fontinalis	AAL09310	p	8,0E-41	*M. domestica* TNFR DR6	TNFR_NGFR_2/TNFR_NGFR_1
	Tetraodon nigroviridis	CAG13004	c	2,0E-101	*H. sapiens* TNFR 21	TNFR_NGFR_2/TNFR_NGFR_1; Death Domain
Fas	*Danio rerio*	DQ812117	c	5,0E-27	*G. gallus* Fas antigen	TNFR_NGFR_2/TNFR_NGFR_1; Death Domain; EGF_2
	Gasterosteus aculeatus	DW628534	p	2,0E-13	*Xenopus laevis* death receptor-M1	TNFR_NGFR_2/TNFR_NGFR_1
	Ictalurus punctatus	AAT36327	c	6,0E-28	*Sus scrofa* TNFR6	TNFR_NGFR_2/TNFR_NGFR_1; Death Domain; EGF_2
	Oncorhynchus mykiss	BX867045	p	9,0E-21	*G. gallus* TNFR21	TNFR_NGFR_2/TNFR_NGFR_1/ EGF_2
	Oryzias latipes	AAS91707	c	3,0E-24	*G. gallus* Fas antigen	TNFR_NGFR_2/TNFR_NGFR_1; Death Domain; EGF_2
	Paralichthys olivaceus	C82250	p	2,0E-20	*G. gallus* TNFR21	TNFR_NGFR_2/TNFR_NGFR_1
	Petromyzon marinus	CO545206	p	1,0E-22	*H. sapiens* TNFR14	TNFR_NGFR_2/TNFR_NGFR_1
	Pimephales promelas	DT222533	p	5,0E-10	*G. gallus* Fas antigen	TNFR_NGFR_2/TNFR_NGFR_1/ EGF_2
	Rutilus rutilus	EG542302	p	2,0E-12	*O. cuniculus* B-type Fas antigen	TNFR_NGFR_2/TNFR_NGFR_1/ EGF_2
	Squalus acanthias	EG027601	p	6,0E-21	*C. familiaris* TNFR14	TNFR_NGFR_2/TNFR_NGFR_1
	Thunnus thynnus	EC918662	p	8,0E-11	*Rattus norvegicus* TNFR6	TNFR_NGFR_2/TNFR_NGFR_1
HDR	*Danio rerio*	NM_194391	c	3,0E-26	*G. gallus* TvbS1	TNFR_NGFR_2/TNFR_NGFR_1; Death Domain
	Gasterosteus aculeatus	DW039605	p	1,0E-19	*G. gallus* TNFRSF23.v1	TNFR_NGFR_2/TNFR_NGFR_1
	Oncorhynchus mykiss	BX320658	p	2,0E-20	*G. gallus* TNFRSF23.v1	TNFR_NGFR_2/TNFR_NGFR_1/ EGF_2
	Osmerus mordax	EL534477	p	3,0E-08	*G. gallus* TNFRSF23.v1	-
	Paralichthys olivaceus	BAC65225	c	3,0E-23	*G. gallus* TNFRSF23.v1	TNFR_NGFR_2/TNFR_NGFR_1; Death Domain
	Ptyochromis sp.	BJ696335	p	5,0E-08	*G. gallus* TvbS1	Death Domain
	Rutilus rutilus	EG537778	p	4,0E-06	*M. musculus* TNFR10b	Death Domain
	Salmo salar	DY695219	p	6,0E-22	*G. gallus* TNFRSF23.v1	TNFR_NGFR_2/TNFR_NGFR_1/ EGF_2
	Takifugu rubripes	CA845756	p	1,0E-07	*G. gallus* TvbS1	Death Domain
OTR	*Danio rerio*	DQ812116	c	3,0E-22	*X. laevis* death receptor-M1	TNFR_NGFR_2/TNFR_NGFR_1; Death Domain
	Gasterosteus aculeatus	DW592611	p	5,0E-24	*G. gallus* TNFRSF23.v1	TNFR_NGFR_2/TNFR_NGFR_1
	Oryzias latipes	AV668946	p	7,0E-15	*X. laevis* death receptor-M1	TNFR_NGFR_2/TNFR_NGFR_1
TNFR1	*Danio rerio*	BC068424	c	6,0E-29	*G. gallus* TNFR1A	TNFR_NGFR_2/TNFR_NGFR_1; Death Domain
	Gasterosteus aculeatus	DT959467	c	3,0E-19	*R. norvegicus* TNFR1A	TNFR_NGFR_2/TNFR_NGFR_1
	Ictalurus punctatus	CV992080	p	6,0E-16	*C. familiaris* TNFR1A	TNFR_NGFR_2/TNFR_NGFR_1
	Oncorhynchus mykiss	AAK91758	c	6,0E-40	*H. sapiens* TNFR6b	TNFR_NGFR_2/TNFR_NGFR_1/EGF_2
	Osmerus mordax	EL540430	p	3,0E-13	*G. gallus* TNFR1A	TNFR_NGFR_2/TNFR_NGFR_1; Death Domain
	Pimephales promelas	DT152157	p	3,0E-17	*Mus spretus* TNFR1A	TNFR_NGFR_2/TNFR_NGFR_1
	Salmo salar	EG889868	p	2,0E-09	*X. laevis* death receptor-M1	TNFR_NGFR_2/TNFR_NGFR_1
	Salvelinus fontinalis	AAD56428	p	6,0E-36	*B. taurus* OCIF	TNFR_NGFR_2/TNFR_NGFR_1/EGF_2
	Takifugu rubripes	BU807158	p	2,0E-11	*R. norvegicus* TNFR1A	TNFR_NGFR_2/TNFR_NGFR_1
	Tetraodon nigroviridis	CAF97843	c	2,0E-25	*F. catus* TNFR1A	TNFR_NGFR_2/TNFR_NGFR_1; Death Domain

(Table 2) contd….

Gene	Fish Species	Accession n°		E-Value	Non-Fish Most Similar Gene	Domain
DEATH RECEPTORS						
FADD	*Danio rerio*	BC114285	e	2,0E-24	*B. taurus* FADD	DED/Death Domain
	Fundulus Heteroclitus	CN987778	e	4,0E-21	*G. gallus* FADD	DED/Death Domain
	Ictalurus punctatus	AAS84609	e	2,0E-23	*R. norvegicus* FADD	DED/Death Domain
	Oncorhynchus mykiss	BX862258	e	2,0E-27	*M. domestica* FADD	DED/Death Domain
	Oryzias latipes	AAS91703	e	6,0E-22	*B. taurus* FADD	DED/Death Domain
	Pimephales promelas	DT184519	e	2,0E-28	*B. taurus* FADD	DED/Death Domain
	Salmo salar	EG935843	e	7,0E-27	*M. domestica* FADD	DED/Death Domain
TRADD	*Danio rerio*	BC083425	e	1,0E-56	*G. gallus* TRADD	Prokar_Lipoprotein; Death Domain
	Gasterosteus aculeatus	DW618510	e	7,0E-43	*G. gallus* TRADD	Death Domain
	Hippoglossus hippoglossus	EB041396	p	6,0E-33	*H. sapiens* TRADD	-
	Ictalurus punctatus	CK412440	p	2,0E-27	*G. gallus* TRADD	Death Domain
	Oncorhynchus mykiss	AAU81663	e	1,0E-54	*G. gallus* TRADD	Death Domain
	Oryzias latipes	BJ736410	p	6,0E-44	*G. gallus* TRADD	Death Domain
	Osmerus mordax	EL539023	p	3,0E-35	*G. gallus* TRADD	-
	Paralabidochromis chilotes	BJ678914	p	2,0E-21	*M. domestica* TRADD	-
	Pimephales promelas	DT182601	p	2,0E-51	*G. gallus* TRADD	Death Domain
	Salmo salar	DW554414	p	6,0E-44	*G. gallus* TRADD	Death Domain
	Tetraodon nigroviridis	CAF99560	e	6,0E-47	*G. gallus* TRADD	Death Domain
BCL-2 FAMILY						
Bcl-2	*Danio rerio*	NM_001030253	e	1,0E-49	*R. norvegicus* Bcl-2	BH4_2; Bcl-2 Family; BH1; BH2
	Cyprinus carpio	CA964607	p	3,0E-33	*G. gallus* Bcl-2	Bcl-2 Family; BH2
	Epinephelus coioides	AAW29023	p	3,0E-33	*S. scrofa* Bcl-2 like 1	Bcl-2 Family; BH1
	Oncorhynchus mykiss	CX258326	p	5,0E-47	*M. musculus* Bcl-2	BH4_2; Bcl-2 Family; BH1; BH2
	Oryzias latipes	AM333038	p	1,0E-43	*Macaca mulatta* Bcl-2 like 1	Bcl-2 Family; BH1
	Osmerus mordax	EL530673	p	8,0E-53	*S. scrofa* Bcl-2 like 1	BH4_2; Bcl-2 Family; BH1
	Pimephales promelas	DT247004	p	4,0E-08	*H. sapiens* Bcl-2	BH4_2
	Rutilus rutilus	EG540963	p	3,0E-39	*M. domestica* Bcl-2	BH2; Bcl-2 Family
	Salmo salar	DY734223	p	1,0E-60	*S. scrofa* Bcl-2 like 1	BH4_2; Bcl-2 Family; BH1
	Squalus acanthias	EG360969	p	2,0E-50	*O. cuniculus* Bcl-2 like 1	BH4_2; Bcl-2 Family; BH1; BH2
	Tetraodon nigroviridis	CAF96873	e	6,0E-52	*S. scrofa* Bcl-2 like 1	Bcl-2 Family; BH1
Bcl-X	*Danio rerio*	AF317837	e	3,0E-62	*M. domestica* Bcl-xS	BH4_2; Bcl-2 Family; BH1
	Gasterosteus aculeatus	DN702345	p	9,0E-57	*M. domestica* Bcl-xS	BH4_2; Bcl-2 Family; BH1
	Oncorhynchus mykiss	BX913023	p	1,0E-60	*M. domestica* Bcl-xS	BH4_2; Bcl-2 Family; BH1
	Osmerus mordax	EL530673	p	5,0E-54	*M. domestica* Bcl-xS	BH4_2; Bcl-2 Family; BH1
	Pimephales promelas	DT234095	p	3,0E-60	*M. domestica* Bcl-xS	BH4_2; Bcl-2 Family; BH1
	Poecilia reticulata	ES384082	p	2,0E-50	*B. taurus* Bcl-xL	Bcl-2 Family; BH1
	Rutilus rutilus	EG548666	p	5,0E-62	*M. domestica* Bcl-xS	BH4_2; Bcl-2 Family; BH1
	Tetraodon nigroviridis	CAF93123	p	8,0E-60	*M. domestica* Bcl-xS	BH4_2; Bcl-2 Family; BH1
Mcl-1	*Danio rerio* Mcl-1a	NM_131599	e	9,0E-41	*H. sapiens* Mcl-1	Bcl-2 Family
	Danio rerio Mcl-1b	NM_194394	e	3,0E-34	*M. domestica* Mcl-1	Bcl-2 Family
	Ictalurus punctatus Mcl-1b	ABD85567	p	2,0E-31	*H. sapiens* Bcl-2 homolog Mcl-1	Bcl-2 Family
	Pimephales promelas Mcl-1b	DT356670	e	3,0E-35	*H. sapiens* Mcl-1	Bcl-2 Family
	Salmo salar Mcl-1b	CAJ90909	e	2,0E-37	*H. sapiens* Mcl-1	Bcl-2 Family
	Tetraodon nigroviridis Mcl-1b	CAF95150	e	4,0E-34	*M. domestica* Mcl-1	Bcl-2 Family
Nr13	*Danio rerio*	AF441285	e	1,0E-19	*G. gallus* Nr13	Bcl-2 Family; BH2
	Pimephales promelas	DT365053	e	9,0E-20	*G. gallus* Nr13	Bcl-2 Family; BH2
	Rutilus rutilus	EG545765	e	2,0E-20	*G. gallus* Nr13	Bcl-2 Family; BH2
Bad	*Danio rerio*	AF231017	e	3,0E-11	*F. catus* Bad	-
	Oncorhynchus mykiss	CX137454	p	2,0E-08	*H. sapiens* Bad isoform CRA_a	-
	Oryzias latipes	AM323478	p	1,0E-08	*H. sapiens* Bad isoform CRA_a	-
	Ptyochromis sp.	BJ694186	e	8,0E-07	*R. norvegicus* Bad beta	-
	Takifugu rubripes	AL837664	p	2,0E-09	*H. sapiens* Bad isoform CRA_a	-
	Tetraodon nigroviridis	CAG05742	p	9,0E-07	*H. sapiens* Bad isoform CRA_a	-

(Table 2) contd….

Gene	Fish Species	Accession n°		E-Value	Non-Fish Most Similar Gene	Domain
BCL-2 FAMILY						
Bid	*Danio rerio*	DQ860155	c	-	-	-
	Gadus morhua	CO541487	c	4,0E-08	*G. gallus* Bid	-
	Ictalurus punctatus	CB937949	p	7,0E-06	*G. gallus* Bid	-
	Oncorhynchus mykiss	BX076999	c	4,0E-13	*B. taurus* Bid	-
	Pimephales promelas	DT344981	c	2,0E-05	*G. gallus* Bid	-
	Salmo salar	CX356652	c	3,0E-10	*B. taurus* Bid	-
Bik	*Danio rerio*	DQ860154	c	-	-	-
Bmf	*Danio rerio* Bmf1	DQ860153	c	-	-	-
	Danio rerio Bmf2	DQ860156	c	-	-	-
	Gasterosteus aculeatus Bmf1	CD503276	c	3,0E-17	*M. domestica* BMF	-
	Oryzias latipes Bmf2	DC269898	p	4,0E-06	*M. domestica* BMF	-
	Pimephales promelas Bmf2	DT200938	c	4,0E-04	*M. domestica* BMF	-
	Squalus acanthias Bmf2	CX197062	p	7,0E-12	*M. domestica* BMF	-
	Tetraodon nigroviridis Bmf2	CAG09126	c	8,0E-05	*M. domestica* BMF	-
Noxa	*Danio rerio*	DQ860152	c	-	-	-
	Ictalurus punctatus	CV990645	c	-	-	-
	Rutilus rutilus	EG548025	c	-	-	-
Puma	*Danio rerio*	DQ860151	c	-	-	-
	Gasterosteus aculeatus	DW038040	c	-	-	-
	Pimephales promelas	DT340182	c	-	-	-
Bim	*Danio rerio*	[98]	-	-	-	-
Bax	*Danio rerio* Bax1	NM_131562	c	2,0E-55	*X. laevis* Bax	Bcl-2 Family
	Danio rerio Bax2	DQ860150	c	2,0E-15	*X. laevis* Bax	Bcl-2 Family
	Epinephelus coioides Bax1	AAW29022	p	7,0E-52	*X. laevis* Bax	Bcl-2 Family
	Hippoglossus hippoglossus Bax1	EB033823	c	7,0E-54	*X. laevis* Bax	Bcl-2 Family
	Ictalurus punctatus Bax1	CB940721	p	3,0E-58	*X. laevis* Bax	Bcl-2 Family
	Lipochromis sp. Bax1	DB860790	c	1,0E-58	*X. laevis* Bax	Bcl-2 Family
	Lipochromis sp. Bax2	DB868320	p	1,0E-44	*X. laevis* Bax	Bcl-2 Family
	Misgurnus anguillicaudatus Bax1	BJ824970	c	1,0E-56	*X. laevis* Bax	Bcl-2 Family
	Misgurnus anguillicaudatus Bax2	BJ821498	p	2,0E-16	*H. sapiens* Bax beta	Bcl-2 Family
	Oncorhynchus mykiss Bax1	CX031493	p	1,0E-41	*X. laevis* Bax	Bcl-2 Family
	Oncorhynchus mykiss Bax2	CA349175	p	3,0E-12	*B. taurus* Bax alpha	Bcl-2 Family
	Oryzias latipes Bax1	AM138350	c	3,0E-53	*X. laevis* Bax	Bcl-2 Family
	Pimephales promelas Bax1	DT340537	p	7,0E-51	*X. laevis* Bax	Bcl-2 Family
	Pimephales promelas Bax2	DT090068	p	6,0E-14	*C. familiaris* Bax	Bcl-2 Family
	Ptyochromis sp. Bax1	BJ692232	p	1,0E-53	*X. laevis* Bax	Bcl-2 Family
	Salmo salar Bax1	DY717084	c	1,0E-55	*X. laevis* Bax	Bcl-2 Family; BH3
	Tetraodon nigroviridis Bax1	CAG02784	c	4,0E-56	*X. laevis* Bax	Bcl-2 Family; BH3
Bok	*Danio rerio* Bok1	NM_001003612	c	4,0E-90	*G. gallus* Bok	Bcl-2 Family
	Danio rerio Bok2	DQ860157	c	3,0E-68	*G. gallus* Bok	BH4_2; Bcl-2 Family
	Gadus morhua Bok1	CO542330	c	3,0E-65	*G. gallus* Bok	Bcl-2 Family
	Gasterosteus aculeatus Bok1	DW665608	c	3,0E-89	*G. gallus* Bok	Bcl-2 Family
	Gasterosteus aculeatus Bok2	DT954703	c	4,0E-89	*G. gallus* Bok	Bcl-2 Family
	Lipochromis sp. Bok1	DB874166	p	4,0E-45	*G. gallus* Bok	-
	Oryzias latipes Bok1	BJ734582	c	8,0E-88	*G. gallus* Bok	Bcl-2 Family
	Osmerus mordax Bok2	EL539675	p	2,0E-67	*G. gallus* Bok	Bcl-2 Family
	Pimephales promelas Bok1	DT231066	p	3,0E-76	*G. gallus* Bok	Bcl-2 Family
	Pimephales promelas Bok2	DT142880	c	1,0E-68	*G. gallus* Bok	Bcl-2 Family
	Rutilus rutilus Bok2	EG541085	p	6,0E-30	*M. domestica* Bok	Bcl-2 Family
	Takifugu rubripes Bok1	BU808649	p	4,0E-60	*G. gallus* Bok	Bcl-2 Family
	Tetraodon nigroviridis Bok1	CAF90551	c	7,0E-84	*G. gallus* Bok	Bcl-2 Family
	Tetraodon nigroviridis Bok2	CAG07662	c	2,0E-68	*G. gallus* Bok	Bcl-2 Family

(Table 2) contd….

Gene	Fish Species	Accession n°		E-Value	Non-Fish Most Similar Gene	Domain
CASPASES AND REGULATORS						
Caspy (casp-a)	*Danio rerio*	NP_571580	c	6,0E-51	*Pan troglodytes* casp1	DAPIN; p20/p10; Casp family his/cys active site
Caspy2 (casp-b)	*Danio rerio*	NP_690840	c	3,0E-42	*X. laevis* casp1a	DAPIN; p20/p10; Casp family his/cys active site
Caspase-b like	*Danio rerio*	XM_689455	c	1,0E-39	*H. sapiens* casp5/c	DAPIN; p20/p10; Casp family hist/cys active site
Caspase-c	*Danio rerio*	BC090436	c	1,0E-41	*C. familiaris* casp4	p20; Casp family his/cys active site
Caspase-c like	*Danio rerio*	XM_686399	c	4,0E-52	*E. caballus* casp1	p20/p10; Casp family his active site
Caspase-2	*Danio rerio*	NP_001036160	c	2,0E-113	*G. gallus* casp2	CARD; p20/p10; Casp family his/cys active site
	Gasterosteus aculeatus	DN732234	p	3,0E-43	*G. gallus* casp2	Card; p20
	Oncorhynchus mykiss	CA370435	p	8,0E-68	*G. gallus* casp2	p20/p10; Casp family his/cys active site
	Oryzias latipes	BJ730254	p	1,0E-56	*G. gallus* casp2	p20/p10; Casp family cys active site
	Pimephales promelas	DT268910	p	4,0E-75	*M. domestica* casp2	p20/p10; Casp family his/cys active site
	Salmo salar	DY739782	p	2,0E-15	*X. laevis* casp2	p20
	Tetraodon nigroviridis	CAF97856	p	2,0E-94	*G. gallus* casp2	Card; p20/p10; Casp family his/cys active site
	Thalassophryne nattereri	CO816596	p	4,0E-38	*G. gallus* casp2	p10
Caspase-3	*Danio rerio casp3a*	NP_571952	c	5,0E-85	*G. gallus* casp3	p20/p10; Casp family his/cys active site
	Danio rerio casp3b	DQ812120	c	3,0E-87	*G. gallus* casp3	p20/p10; Casp family cys active site
	Chrysiptera parasema	AAY88536	p	7,0E-57	*G. gallus* casp3	p20/p10; Casp family his/cys active site
	Dicentrarchus labrax	ABC70996	c	3,0E-90	*G. gallus* casp3	p20/p10
	Gasterosteus aculeatus	DN688504	p	4,0E-79	*G. gallus* casp3	p20/p10; Casp family his/cys active site
	Gobius niger	AAU94306	p	4,0E-66	*G. gallus* casp3	p20/p10; Casp family his/cys active site
	Hippoglossus hippoglossus	EB035311	p	9,0E-67	*G. gallus* casp3	p20/p10; Casp family his/cys active site
	Ictalurus punctatus	CV991805	p	3,0E-79	*G. gallus* casp3	p20/p10; Casp family cys active site
	Misgurnus anguillicaudatus	BJ83066	p	8,0E-60	*G. gallus* casp3	p20/p10; Casp family cys active site
	Oncorhynchus mykiss	CA366923	p	4,0E-65	*G. gallus* casp3	p20/p10; Casp family his/cys active site
	Oryzias latipes casp3a	BAC00949	c	4,0E-82	*G. gallus* casp3	p20/p10; Casp family his/cys active site
	Oryzias latipes casp3b	BAC00948	c	4,0E-93	*G. gallus* casp3	p20/p10; Casp family his/cys active site
	Pimephales promelas	DT295835	p	3,0E-63	*G. gallus* casp3	p20/p10; Casp family his/cys active site
	Salmo salar	DY708186	p	2,0E-76	*Cricetulus griseus* casp3	p20/p10; Casp family his/cys active site
	Squalus acanthias	EB688145	p	2,0E-74	*G. gallus* casp3	p20/p10; Casp family cys active site
	Takifugu rubripes	AAM43816	c	4,0E-90	*G. gallus* casp3	p20/p10; Casp family his/cys active site
	Tetraodon nigroviridis	CAF96609	c	3,0E-91	*G. gallus* casp3	p20/p10; Casp family his/cys active site
Caspase-6	*Salmo salar casp6b*	AAY28974	c	2,0E-112	*P. troglodytes* casp6	p20/p10; Casp family his/cys active site
	Salmo salar casp6a	AAY28971	c	4,0E-112	*M. domestica* casp6	p20/p10;Casp family his/cys active site
	Danio rerio casp6a	NP_001018333	c	1,0E-107	*P. troglodytes* casp6	p20/p10; Casp family his active site
	Danio rerio casp6b	BC083437	c	5,0E-83	*C. familiaris* casp6	p20/p10; Casp family his active site
	Danio rerio casp6c	BC114318	c	2,0E-78	*H. sapiens* casp6	p20/p10; Casp family his active site
	Gasterosteus aculeatus	DW644136	c	8,00E-103	*P. troglodytes* casp6	p20/p10; Casp family his/cys active site
	Oncorhynchus mykiss	AAF73848	c	3,0E-11	*P. troglodytes* casp6	p20/p10; Casp family his/cys active site
	Oryzias latipes	AM318681	p	3,0E-73	*M. domestica* casp6	p20/p10; Casp family his/cys active site
	Pimephales promelas	DT174613	p	5,0E-83	*C. familiaris* casp6	p20/p10; Casp family his/cys active site
	Tetraodon nigroviridis	CAG11470	c	3,0E-104	*M. domestica* casp6	p20/p10; Casp family his active site
Caspase-7	*Salmo salar*	AAY28975	c	2,00E-116	*Xenopus tropicalis casp7*	p20/p10;Casp family his/cys active site
	Danio rerio casp7a	NP_001018443	c	7,00E-127	*X. tropicalis casp7*	p20/p10; Casp family his/cys active site
	Danio rerio casp7b	DN834451	p	1,0E-50	*X. tropicalis casp7*	p20/p10; Casp family his/cys active site
	Gasterosteus aculeatus	DW641650	c	2,00E-121	*X. tropicalis casp7*	p20/p10; Casp family his/cys active site
	Oncorhynchus mykiss	CU073080	p	4,00E-91	*X. tropicalis casp7*	p20/p10;Casp family his/cys active site
	Oryzias latipes	BJ907004	p	1,00E-73	*X. tropicalis casp7*	p20; Casp family his/cys active site
	Tetraodon nigroviridis	CAG12603	c	5,00E-103	*X. tropicalis casp7*	p20/p10; Casp family his active site

(Table 2) contd….

Gene	Fish Species	Accession n°		E-Value	Non-Fish Most Similar Gene	Domain
CASPASES AND REGULATORS						
Caspase-8	*Danio rerio Casp8a*	BC081583	c	3,0E-80	*R. norvegicus* casp8	2 DED;p20/p10; Casp family cys active site
	Danio rerio Casp8a like	DQ812121	c	5,0E-50	*H. sapiens* isoform CRA_f	p20/p10; Casp family cys active site
	Danio rerio Casp8b	DQ812123	p	2,0E-20	*M. domestica* mch4	2 DED
	Danio rerio Caspase (card)	CT693310	p	2,0E-29	*H. sapiens* FLICE 2	CARD; p20; Casp family cys active site
	Danio rerio CaspXa	ABG91572	c	2,0E-49	*G. gallus* casp8	CARD; p20/p10; Casp family cys active site
	Danio rerio CaspXb	ENSDARG00000058343	p	6,00E-50	*H. sapiens* proMch4	p20/p10
	Gasterosteus aculeatus	DN668731	p	4,0E-56	*G. gallus* casp8	p20/p10; Casp family his/cys active site
	Gasterosteus aculeatus (card)	ENSGACT00000017012	c	8,0E-49	*B. taurus* casp8	CARD; p20; Casp family cys active site
	Ictalurus punctatus (card)	AAT37512	c	5,0E-55	*G. Gallus* casp8	CARD; p20/p10; Casp family his/cys active site
	Oncorhynchus mykiss	CU072654	p	7,0E-45	*C. familiaris* casp8	p20/p10; Casp family cys active site
	Oryzias latipes	AAS91704	c	2,0E-65	*R. norvegicus* casp8	2 DED;p20/p10; Casp family cys active site
	Oryzias latipes (card)	AM144512	p	-	-	CARD; p20
	Osmerus mordax	EL542275	p	7,0E-36	*C. familiaris* casp8	p20/p10; Casp family cys active site
	Pimephales promelas	DT346669	p	2,0E-40	*G. gallus* casp8	p20/p10/Casp family cys active site
	Salmo salar	DW567373	p	1,0E-42	*C. familiaris* casp8	p20/p10; Casp family cys active site
	Tetraodon nigroviridis	CAG09993	p	5,0E-65	*G. gallus* casp8	p20/p10; Casp family cys active site
Caspase-9	*Dicentrarchus labrax*	ABC70998	c	4,0E-111	*M. domestica* ICE-LAP6	CARD; p20/p10; Casp family his/cys active site
	Astatotilapia burtoni	DY630579	p	3,0E-41	*M. domestica* ICE-LAP6	CARD; p20
	Danio rerio	AAH97103	c	1,0E-105	*M. domestica* ICE-LAP6	CARD; p20/p10; Casp family his/cys active site
	Gadus morhua	EG642284	p	8,0E-27	*M. domestica* ICE-LAP6	p10
	Hippoglossus hippoglossus	EB040889	p	3,0E-85	*M. domestica* ICE-LAP6	p20/p10; Casp family his/cys active site
	Misgurnus anguillicaudatus	BJ818778	p	9,0E-69	*M. domestica* ICE-LAP6	p20/p10; Casp family his/cys active site
	Oncorhynchus mykiss	AAY88917	c	9,0E-108	*M. domestica* ICE-LAP6	CARD; p20/p10; Casp family his/cys active site
	Oryzias latipes	BJ711475	p	8,0E-53	*H. sapiens* casp9	p20/p10; Casp family cys active site
	Pimephales promelas	DT261605	p	1,0E-52	*M. domestica* ICE-LAP6	p20/p10; Casp family cys active site
	Poecilia reticulata	ES376943	p	2,0E-51	*C. familiaris* casp9	p20; Casp family his active site
	Pseudopleuronectes americanus	AW013063	p	5,0E-36	*C. familiaris* casp9	p20; Casp family his active site
	Salmo salar	EG883628	p	4,0E-39	*M. musculus* casp9	CARD; p20
	Squalus acanthias	EC093373	p	4,0E-41	*M. domestica* ICE-LAP6	CARD; p20
	Tetraodon nigroviridis	CAG01765	c	1,0E-106	*M. domestica* ICE-LAP6	CARD; p20/p10; Casp family his/cys active site
Caspase-10	*Danio rerio*	XP_685430	c	1,0E-58	*G. gallus* proMch4	p20/p10; Casp family his/cys active site
	Gasterosteus aculeatus	DN668731	c	4,0E-56	*G. gallus* casp8	p20/p10; Casp family his/cys active site
	Hippoglossus hippoglossus	EB039021	p	1,0E-48	*H. sapiens* casp10d	p20/p10; Casp family his active site
	Oncorhynchus mykiss	CX262072	p	6,0E-40	*G. gallus* proMch4	p20/p10;Casp family cys active site
	Paralichthys olivaceus	BAE98149	c	1,0E-79	*G. gallus* proMch4	2 DED; p20/p10; Casp family his/cys active site
	Takifugu rubripes	CA329181	p	1,0E-42	*G. gallus* proMch4	p20/p10; Casp family cys active site
	Tetraodon nigroviridis	CAG09993	p	5,0E-65	*G. gallus* casp8	p20/p10;Casp family cys active site
FLIP	*Danio rerio*	BC116570	c	2,0E-62	*C. familiaris* c-FLIP	2 DED; p20
Apaf-1	*Danio rerio*	AAF67189	c	0,0E+00	*M. domestica* Apaf-1	CARD; WD_REPEATS_REGION; WD_REAPTS 2/1
	Lipochromis sp.	DB872448	p	3,0E-55	*C. familiaris* Apaf-1	WD_REPEATS_REGION; WD_REAPTS 2/1
	Oncorhynchus mykiss	CU073112	p	3,0E-68	*M. domestica* Apaf-1	WD_REPEATS_REGION; WD_REAPTS 2/1
	Osmerus mordax	EL547605	p	5,0E-79	*C. familiaris* Apaf-1	WD_REPEATS_REGION; WD_REAPTS 2/1

(Table 2) contd….

Gene	Fish Species	Accession n°		E-Value	Non-Fish Most Similar Gene	Domain
CASPASES AND REGULATORS						
Apaf-1	*Pimephales promelas*	DT309473	p	2,0E-80	*M. domestica* Apaf-1	WD_REPEATS_REGION; WD_REAPTS 2/1
	Rutilus rutilus	EG531088	p	7,0E-63	*M. domestica* Apaf-1	CARD
	Salmo salar	DY723319	p	3,0E-90	*M. musculus* Apaf-1	-
	Tetraodon nigroviridis	CAG01878	c	0,0E+00	*M. domestica* Apaf-1	CARD; WD_REPEATS_REGION; WD_REAPTS 2/1
XIAP	*Danio rerio*	AAL32047	c	1,0E-93	*R. norvegicus* baculoviral IAP	BIR_REPEAT_2; ZF_RING_2
	Cyprinus carpio	EC392263	p	3,0E-37	*M. domestica* XIAP	BIR_REPEAT_2
	Fundulus heteroclitus	DN957101	p	1,0E-30	*X. tropicalis* birc4	BIR_REPEAT_2
	Gasterosteus aculeatus	DT972782	p	2,0E-68	*X. tropicalis* birc4	BIR_REPEAT_2/1
	Hippoglossus hippoglossus	EB038688	p	1,0E-28	*P. troglodytes* baculoviral IAP	BIR_REPEAT_2
	Oncorhynchus mykiss	CA364711	p	3,0E-48	*M. domestica* XIAP	BIR_REPEAT_2
	Oryzias latipes	BJ728878	p	1,0E-70	*X. laevis* xXIAP	BIR_REPEAT_2/1; ZF_RING_2
	Osmerus mordax	EL539900	p	2,0E-48	*X. tropicalis* birc4	BIR_REPEAT_2
	Pimephales promelas	DT234214	p	3,0E-53	*G. gallus* baculoviral IAP	BIR_REPEAT_2/1
	Rutilus rutilus	EG535112	p	1,0E-52	*X. tropicalis* birc4	BIR_REPEAT_2/1
	Salmo salar	DW575197	p	2,0E-50	*X. tropicalis* birc4	BIR_REPEAT_2/1
IAP	*Danio rerio*	AAP04483	c	0,0E+00	*G. gallus* baculoviral IAP	CARD; BIR_REPEAT_2/1; ZF_RING_2
	Gasterosteus aculeatus	DW676977	p	5,0E-105	*G. gallus* baculoviral IAP	BIR_REPEAT_2/1
	Ictalurus furcatus	CK405937	p	5,0E-65	*M. domestica* MIHB	CARD; ZF_RING_2
	Ictalurus punctatus	AAO24632	c	0,0E+00	*G. gallus* baculoviral IAP	CARD; BIR_REPEAT_2/1; ZF_RING_2
	Misgurnus anguillicaudatus	BJ819591	p	4,0E-97	*G. gallus* baculoviral IAP	BIR_REPEAT_2
	Oncorhynchus mykiss	BX874909	p	2,0E-88	*G. gallus* baculoviral IAP	BIR_REPEAT_2
	Osmerus mordax	EL544094	p	2,0E-106	*G. gallus* baculoviral IAP	BIR_REPEAT_2/1
	Pimephales promelas	DT359490	p	2,0E-87	*G. gallus* baculoviral IAP	BIR_REPEAT_2/1
	Salmo salar	DY716703	p	2,0E-83	*G. gallus* baculoviral IAP	BIR_REPEAT_2/1
	Tetraodon nigroviridis	CAG00512	c	0,0E+00	*G. gallus* baculoviral IAP	CARD; BIR_REPEAT_2/1; ZF_RING_2
Smac/diablo *	*Danio rerio*	NP_956640	c	9,0E-40	*P. troglodytes* Smac	-
	Cyprinus carpio	CF661050	p	2,0E-32	*P. troglodytes*Smac	-
	Pimephales promelas	DT361691	p	5,0E-33	*B. taurus* diablo homolog	-
	Rutilus rutilus	EG531199	p	1,0E-39	*P. troglodytes* Smac	-
	Tetraodon nigroviridis	CAG08981	c	2,0E-18	*P. troglodytes* Smac	-
HtrA2 *	*Danio rerio*	XP_690107	c	6,0E-124	*M. musculus* serine protease	PDZ; PROTEIN_KINASE_ATP
	Gasterosteus aculeatus	DT953033	p	5,00E-80	*M. musculus* serine protease	-
	Pimephales promelas	DT239426	p	8,00E-33	*M. musculus* serine protease	-
	Platichthys flesus	DV567298	p	7,0E-96	*M. musculus* serine protease	-
	Squalus acanthias	EG026836	p	5,0E-87	*G. gallus* serine protease	-

Shaded molecules were used as query for searching respective fish homologs by BLAST search on non-redundant, expressed sequenced tags (EST) and genomic (Ensembl) databases. These were then Blasted for searching non-fish most similar genes. Domains were predicted using PROSITE (www.expasy.org/prosite). Expected values (E-value) were calculated by BLASTP; c- complete sequence; p- partial sequence. * BLAST search was performed with human Smac/Diablo (Accession n° EAW98311) and HtrA2 (Accession n° O43464) for obtaining the *Danio rerio* homologs and then these were used to BLAST search other fish homologs.

TRAIL to DR4 or DR5, results in the recruitment of the adaptor protein FADD where its DD directly interacts with the DD of DR4 and DR5 (reviewed in [27, 42]). FADD also contains a DED that interacts with the DED of pro-caspase-8 and -10 forming the death-induced signalling complex (DISC), where these caspases are auto-catalytically activated (reviewed in [27, 42]). Upon stimulation, TNFR1, *via* its DD, recruits the adapter protein TNFR1-associated DD protein (TRADD), which in turn recruits receptor-interating protein (RIP) and TNF-receptor associated factors (TRAFs), forming the membrane-bound complex I for IKK and NF-kB activation (reviewed in [42]). In a second step, TRADD dissociates from TNFR1 and associates with FADD and caspase-8 to form a cytoplasmic complex II for caspase activation (reviewed in [42]). As discussed by these authors, the regulated assembly of complex I and complex II may underlie TNF's ability to induce either cell death or cell survival under different cellular contexts [42].

A catfish homolog to mammalian FADD (Table **2**) was identified by sequencing a clone from a spleen cDNA library [43]. It was shown that the gene is expressed in the spleen, brain and head kidney [43] and at all stages of growth cycle of several lymphoid cells and cell lines of catfish [35]. A FADD homolog was also identified

and characterised in medaka fish [36]. It has been found that medaka FADD molecule possesses both the DED and DD and displays 34 % identity and 52% similarity to human FADD [36]. The genomic structure and the genomic organization of medaka *fadd* are similar to the ones of other vertebrates, including mammals, suggesting that this gene shares its origin within vertebrates [36]. Exogenous expression of medaka FADD induced cell death in HeLa and mouse embryonic fibroblast tranfectants, indicating that the medaka FADD is able to transmit apoptotic signals into transfected mammalian cells in a caspase-8 dependent way [36]. Homologs of FADD and TRADD were identified in zebrafish (Table **2**) [30]. Injection of *DareFADD* and *DareTRADD* mRNAs in zebrafish embryos induced widespread apoptosis and death of the embryos, and overexpression of DareFADD in mammalian cells induced high levels of cell death [30]. Using morpholinos, it was shown that DareFADD but not DareTRADD is required to the induction of apoptosis by DareTRAIL1a and DareTRAIL1b in zebrafish embryos [30].

In conclusion, since fish have the basic components of the extrinsic apoptosis pathway known to exist in mammals, they represent highly valuable models for the research in death receptor-dependent apoptotic cell death.

3. INTRINSIC PATHWAY

Since the discovery of the antiapoptotic function of the bcl-2 oncogene [44], Bcl-2 family members have been extensively studied in mammalian systems, manly due to their importance as key regulators of apoptosis, playing a central role in some pathological conditions (reviewed in [45]) including cancer (reviewed in [46]), neurodegenerative disorders (reviewed in [47-50]), autoimmune diseases (reviewed in [51]) and viral infections (reviewed in [52, 53]). This resulted in an increasing number of articles being published in the last decade and a quite extensive reviewing on the subject (reviews published during the past five years include e.g., [8, 15, 16, 54-63]). Briefly, Bcl-2 family members include proteins with sequence and structural similarity in the so-called Bcl-2 homology (BH1 to BH4) domains (Fig. **1**) and regulate the response to diverse cytotoxic conditions such as UV- and γ-irradiation, antineoplasic drugs, virus, bacteria, deprivation of cytokines, neurotrophins and growth factors, and anoikis, normally having little impact on the signals induced by the death receptors of the TNF family [64]. Bcl-2 members can be antiapoptotic or proapoptotic.

Antiapoptotic members are mainly characterised by containing three to four BH domains (Fig. **1**), three of which (BH1/BH2/BH3) forming a hydrophobic groove where scaffold or adaptor proteins and pro-apoptotic Bcl-2 members are likely to compete for binding. The antiapoptotic members induce cell survival by sequestering/inhibiting pro-apoptotic scaffold or adaptor proteins and pro-apoptotic members needed for activation of caspases, and/or by maintaining organelle integrity (reviewed in [8, 15, 16, 65]).

Proapoptotic members can be subdivided into BH3-only proteins ("activators" and "de-repressors/facilitators"), which, as the name stands for, only contain the third BH domain (reviewed in [16, 57, 62, 63, 65, 66]), or multidomain proapoptotic proteins. These share a high degree of structural similarity with prosurvival Bcl-2 family members, containing two to three BH domains, although all having the BH3 domain responsible for the proapoptotic activity (Fig. **1**) (reviewed in [15, 16, 67, 68]). Contrary to the prosurvival members, which have the BH3 domain as integral part of their hydrophobic pocket and thus not available for binding other Bcl-2 hydrophobic grooves, the multidomain proapoptotic Bcl-2 proteins appear to expose, after a post-translational modification and/or conformational change [69], and nestle their BH3 domains into the hydrophobic groove of prosurvival Bcl-2 members where they are kept inactive. Alternatively, multidomain proapoptotic proteins would be loosely attached to the membrane, their hydrophobic pockets still being intact binding to either the phospholipid bilayer or to an unknown inhibitory molecule [15]. Depending on the apoptotic stimulus and/or cellular context, a specific BH3-only protein can be activated either by transcriptional induction (e.g., PUMA/Bbc3 and Noxa activation by the transcription factor p53 in apoptosis induced by DNA-damage) or post-translational modifications by phosphorylation (e.g., Bad and Bik phosphorylation in apoptosis induced by cytokine/growth factor deprivation, anoikis

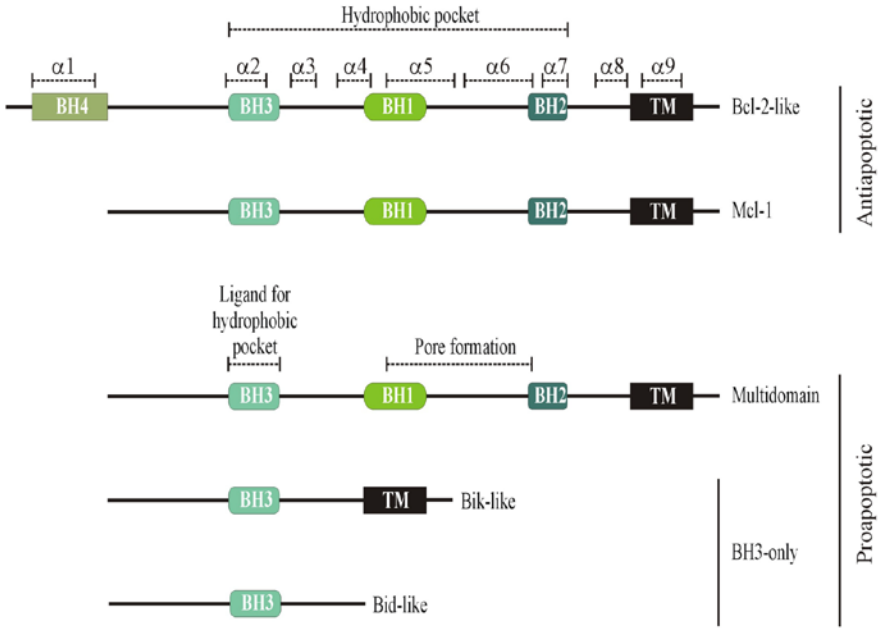

Fig. (1). Bcl-2 family members. Diagrammatic representation of the antiapoptotic and proapoptotic (multidomain and BH3-only) Bcl-2 family members. α-helices, hydrophobic pocket, ligand for hydrophobic pocket and α5 and α6 pore forming units are according with Borner *et al.* [15]. See text for details.

and death receptor ligation) or proteolysis (e.g., cleavage of Bid into truncated Bid by death receptor activated caspase-8) (reviewed in [15, 65]). Moreover, BH3-only proteins can be kept inactive by sequestration to macromolecular structures (e.g., Bim and Bmf sequestration by the dynein light chain of the dynein motor complex of microtubles and of the actin cytoskeleton-based myosin V motor complex, respectively) (reviewed in [8, 15, 65]). Once activated, "de-repressors/facilitators" BH3-only proteins (e.g., Bad) interact with Bcl-2 antiapoptotic family members on the outer mitochondrial or nuclear/endoplasmic reticulum membranes, triggering the release of proapoptotic scaffold or adaptor proteins and BH3 proapoptotic family members. On the other hand, "activators" BH3-only proteins (e.g., truncated Bid) directly activate multidomain proapoptotic Bcl-2 family members (reviewed in [8, 16, 63, 70, 71]). Then, multidomain proapoptotic proteins undergo a conformational change (eventually assisted by some BH3-only proteins) and insert into the outer mitochondrial membrane where they may directly form or induce the formation, *via* the binding to other channel proteins (reviewed in [54, 72-76]) of pores in the mitochondrial membrane and the concomitant mitochondrial membrane permeabilization (MMP). MMP is characterised by: (i) the release of mitochondria-residing factors that promote caspase-dependent cell death (holocytochrome *c*, Smac/DIABLO and Omi/HtrA2); (ii) the release of mitochondria-residing factors that promote caspase-independent cell death (apoptosis-inducing protein (AIF), endonuclease G (EndoG) and Omi/HtrA2); (iii) an alteration of the inner mitochondrial transmembrane potential ($\Delta\Psi_m$); and (iv) a bioenergetic catastrophe including the arrest of oxidative phosphorilation, as well as the accumulation of reactive oxygen species (ROS) (reviewed in [8, 15, 16, 54, 55, 77-79]). At least in some instances, cell death can still be prevented at this stage by antiapoptotic Bcl-2 members depending on their relative abundance (rheostat model, [80]) and therefore inhibiting the releasing of apoptogenic molecules and triggering of apoptosis (reviewed in [15, 16]). In addition, Bcl-2 family members are also involved in the regulation of several types of proapoptotic signals that are generated in organelles other than mitochondria (reviewed in [8]), such as nucleus [81-83], ER [71, 84-86] and lysosomes [87-89], and in the cytosol [90-93] and cytoskeleton [94, 95]. Whereas the various Bcl-2 members have specific functions in distinct cell types, their related members of the family can compensate for the missing/blocked family member, presumably due to redundancy and partially overlapping expression patterns (reviewed in [15, 16, 96]). Finally, a plethora of cellular factors outside the Bcl-2 family may also directly interact with Bcl-2 family members to modulate their action (reviewed in [8, 16]).

Intrinsic Pathway in Fish

That fish have similar functionally conserved Bcl-2 family members and an intrinsic apoptotic pathway as mammals has been shown by several lines of evidence. Due to the advantage and increasing relevance of zebrafish as a model in the study of apoptosis-related human disorders and development (reviewed elsewhere in this issue) in addition to the almost completion of its genomic sequencing, most of the data related with the Bcl-2 family and the intrinsic apoptotic pathway refer to zebrafish. Thus, blasting non-redundant and expressed-sequence tags (EST) public databases for searching zebrafish Bcl-2 protein homologs indicated that zebrafish genome contains homologs of most mammalian Bcl-2 family members [23, 97, 98]. Bcl-2 family members in other fish species can also be retrieved using zebrafish and/or mammalian orthologs as query in blast searching non-redundant and EST public databases as well as genomic sequence databases (Table **2**). Several fish Bcl-2 family genes have two copies (Table **2** and [98]) resulting from the early whole-genome duplication in the teleost lineage after the divergence from tetrapods about 450 million years ago [99-101]. A syntenic relationship has apparently been kept between most of the fish and human Bcl-2 family gene orthologs, as suggested from the fact that *DareBcl-x$_L$*, *DareMcl-1a*, *DareMcl-1b*, *DareNr-13*, *DareBad*, *DareBik*, *DareBmf2*, *DarePuma*, *DareBax2*, *DareBok1* and *DareBok2* genes are syntenic with the human homologs [98].

Detailed primary and secondary structure and sequence analysis has been only reported for some of the zebrafish Bcl-2 family members. Thus, regarding antiapoptotic Bcl-2 family members, a Bcl-x$_L$ homolog has been cloned (DareBcl-x$_L$) [102]. DareBcl-x$_L$ contains four BH domains and corresponding α helices as well as a putative C-terminal transmembrane domain [102], which accords well with the structure of other known Bcl-x$_L$ proteins (reviewed in [15]). Its sequence has about 47% identity to other mammalian counterparts with higher identity found in the BH domains [102]. Surprisingly, signalling peptide scanning identified a putative ER retention signal at the C-terminal end of the DareBcl-x$_L$ protein, while no such signal has been identified in any other Bcl-x$_L$ proteins, in accordance with the apparent subcellular co-localization of DareBcl-x$_L$ and mitochondria and ER in zebrafish cells [102]. The different localization comparing to mammalian Bcl-x$_L$ proteins [103, 104] led the authors to hypothesise that this zebrafish homolog may perform a novel function(s) not found in its Bcl-x$_L$ relatives [102]. Additional differences were found in the *DareBcl-x$_L$* sequence, namely it contains only Thr^{115} but not Thr^{47}, both residues in human Bcl-x$_L$ shown to be phosphorilated by the stress-induced protein kinase JNK. Moreover, *DareBcl-x$_L$* lacks the mammalian-specific Asp^{61} residue as a caspase-1 and -3 cleavage site, which after cleavage renders a Bcl-x$_L$ C-terminal product of 16 kDa with potent apoptotic activity [105]. However, an Asp residue has been conserved in zebrafish and chicken sequences slightly downwards the mammalian one, so whether fish Bcl-x$_L$ homologs do not use the caspase-mediated proteolysis as a way of regulation to modulate their Bcl-x$_L$ protein activities [102] or whether other Asp residue/caspase-cleavage site is used for such a purpose needs further investigation.

A zebrafish Bcl-2 homolog (DareBcl-2) has been cloned and showed to share a high level of similarity with other Bcl-2 family members, particularly within the BH functional domains, where 95%, 80%, 56% and 68% similarity have been reported for BH1, BH2, BH3 and BH4, respectively [106]. No further detailed primary and secondary structural and sequence analysis has been reported.

Two copies of the *Mcl-1* gene have been found in zebrafish ([23]; here referred as *DareMcl-1a* and *DareMcl-1b*). However, detailed characterization has only been reported for *DareMcl-1a* [107]. A single Mcl-1 product was obtained by suppressive subtractive hybridisation in salmon (SasaMcl-1) and has also been characterised [108]. Accordingly, fish Mcl-1 proteins contain two PEST sequences, a putative nuclear localization signal and a transmembrane domain. Seven α-helices and the BH-3, -1 and -2 domains located to corresponding helix counterparts as in other Bcl-2 family proteins have also been predicted [107, 108]. As for other Mcl-1 proteins, fish Mcl-1 lacks a clear sequence homology to the BH4 domain of Bcl-2, although an α-helix not corresponding to any BH domains has been predicted in the N-terminal half of DareMcl-1a protein [107]. DareMcl-1a and SasaMcl-1 amino acid sequence share 46% identity, showing both about 40% identity with the human *Mcl-1*, but with a higher sequence identity within the BH and transmembrane domains [107, 108]. Moreover, it was shown that DareMcl-1a has a nuclear location which accords with the identified nuclear localization signal [107].

Another prosurvival member that has been identified [23] and its primary and secondary structures characterised [109], is the zebrafish ortholog of chicken *Nr-13* [110], which was named Nrz/zNR13 (here referred as *DareNr-13*) [57, 109]. The *DareNr-13* gene exhibits one single intron at the same position as chicken *Nr-13* and herpes virus (HVT) *Vnr-13*, and the deduced protein from the cDNA sequence shows the four typical BH domains and the C-

terminal hydrophobic tail characteristic of most antiapoptotic Bcl-2 family members [109]. At protein level DareNr-13 has higher identity with chicken (39.7%) and HVT Nr-13 (38.7%) than with the mammalian Nr-13 orthologs [109], such as mouse Boo/DIVA [111, 112] and human nrh/Bcl-B [113, 114]. Arnaud *et al.* [109] have also shown that, as Nr-13 itself and related apoptosis inhibitors, DareNr-13 is a *bona fide* apoptosis inhibitor (presumably an antagonist of Bax) localised and acting at the mitochondrial level.

The only other description of a fish Bcl-2 family member with detailed primary and secondary structures and sequence analysis regards the zebrafish proapoptotic BH3-only protein Bad (DareBad) [115]. Despite its low identity (29-34%) and similarity (37-45%) to the mammalian counterparts, the BH3 domain is highly conserved [98, 115]. Moreover, DareBad conserves the two serine residues [97, 98] whose phosphorylation by Akt/Pkb and perhaps other kinases, has been shown in mammals to facilitate Bad sequestration into the cytosol by the 14-3-3 chaperone with the consequent abrogation of its pro-apoptotic activity. Further support for a similar posttranslational mechanism in fish is the association of Akt activity and cell survival shown after optic nerve injury in goldfish [116]. Furthermore, comparison of the aminoacid sequences of human and zebrafish Bad revealed the presence of one putative consensus caspase-3 recognition sequence in DareBad [115], suggesting that the proapoptotic activity of DareBad may be enhanced by caspase cleavage as shown for mammals [117]. In agreement with the lack of any transmembrane domain in the DareBad protein sequence, DareBad was shown to localize in the cytosol of DareBad transfect COS-1 cells, migrating to the mitochondria upon apoptotic stimulus by serum deprivation [115]. Regarding the other zebrafish BH3-only homologs, their overall aminoacid sequence has been shown to diverge significantly from mammalian counterparts; however, their BH3 domain retains a high degree of similarity, emphasizing the functional importance of this domain [98]. Zebrafish *Bik* and *Puma* (*DareBik* and *DarePuma*, respectively) are the most divergent zebrafish BH3-only genes [98]. Moreover, whereas in the case of DarePuma the BH3 domain aligns most closely with mammalian homologs, the BH3 domain of DareBik bears a striking resemblance to the mammalian BH3 domain of Bid. However, as DareBik lacks a predicted caspase cleavage site and is syntenic with human Bik, it has been considered the zebrafish ortholog of human Bik [98]. Additional sequence features supporting the homology of some of the zebrafish BH3-only family members, include conservation not only of the BH3-region but also of the caspase-8 cleavage site (LQTD) in fish *Bid* sequences [97, 98], allowing the generation of a truncated Bid-like fragment, and the presence of putative dynein light-chain-binding domains in DareBmf1 and DareBmf2 which, as in mammalian Bmf, would allow their sequestration to the actin cytoskeleton until activation [98].

To the best of our knowledge, there are no reports regarding detailed primary and secondary structures and sequence analysis of Bcl-2 proapototic multidomain family members. Of notice is the fact that even though zebrafish Bax2 (DareBax2) displays a significantly higher sequence similarity to mammalian Bax, it is functionally similar to human Bak (see below) [98]. This may justify why a fish *Bak* sequence homolog has not yet been found in the EST and genomic databases and that, as suggested by Kratz *et al.* [98], the DareBax2 fish homologs have evolved to display similar selectivity to the human Bak.

Recently, a detailed expression study of the Bcl-2 gene family throughout zebrafish early embryonic development and adult tissue has been reported [98]. With the exception of *DareBik* and *DareBok1*, those authors detected maternal expression of all the other Bcl-2 family members. After the initiation of zygotic transcription, the levels of *DareBid*, *DareBik*, *DareBad*, *DareBmf1*, *DareNoxa*, *DareBax1*, *DareMcl-1a*, *DareMcl-1b*, *DareBcl-x_L*, and *DareNR-13* were fairly constant, whereas the transcription of *DareBax2*, *DareBmf2*, and *DareBcl-2* decreased dramatically after the 1000-cell stage before increasing again from 24 to 72 hours post-fertilization. *DareBok2* was exclusively from maternal origin while *DareBok1* was from zygotic origin. Regarding their expression in adult tissues the same authors [98] found that, with the exception of *DareBik* and *DareBmf2*, ovary was especially rich in all *Bcl-2* family members, whereas liver was the organ with less *Bcl-2* family members expression, and, those expressed (Bid, Mcl-1a and Mcl-1b), with only weak expression levels [98]. A remarkably specific expression pattern at the RNA level has also been observed for the zebrafish BH3-only genes with *DareBik* being expressed in kidney and eye, *DareBad* and Dare*Bmf1* exclusively in ovary, *DareNoxa* in ovary and less intensively in kidney and eye, and *DarePuma* strongly in kidney and brain and weakly in ovary, eye and very slightly in heart [98]. Chen and collaborators [102] reported that *DareBcl-x_L* gene expressed four different mRNA transcripts with different embryonic expression profiles. Whereas two of the transcripts were only transiently expressed in the very early stages of embryonic development, marked differences were observed in the expression of the other two transcripts. Indeed, the major short form of *DareBcl-x_L* (*DareBcl-x_L-S*) was found to be extensively expressed during zebrafish embryonic development and was detected in unfertilized eggs, suggesting a maternal origin, and the major long form of *DareBcl-x_L* (*DareBcl-x_L-L*) was expressed only from the gastrula stage onwards and, therefore, clearly produced from the zygotic copy of the *DareBcl-x_L* gene [102]. Expression patterns consistent with those detected by Kratz and colleagues during zebrafish early embryonic stage have been also described by others for *DareMcl-1a* and *DareMcl-1b* [107], and *DareNr-13* [109]. However, results from Arnaud *et al.* [109] suggest that knockdown of *DareNr-13* during somitogenesis triggers apoptosis whereas in gastrulation its effect is apoptosis-independent, controlling gastrulation by negatively regulating *snail-1*, a transcription factor that regulates the expression of major adhesion proteins [118]. Maternally derived *DareBad* mRNA has also been reported by Hsieh *et al.* [115]. However, while Kratz and colleagues [98] reported expression of *DareBad* up to 72h after fertilization, Hsieh *et al.* [115] have found constant expression levels of *DareBad* for up to 5h after fertilization but gradually declining afterwards. In adult fish, some differences have also been found. While high expression levels in ovary were reported in those studies [98, 115], high expression in intestine and moderate expression in kidney and muscle were reported by Hsieh *et al.* [115]. To clarify whether prosurvival family members play similar roles in zebrafish embryogenesis as in mice, Kratz *et al.* [98] performed studies which showed that *DareMcl-1a* and *DareMcl-1b*, but not *DareBcl-2*, are critical to normal early zebrafish development, suggesting that during early embryogenesis in vertebrates Mcl-1 plays a crucial antiapoptotic role, whereas Bcl-2 is dispensable.

Functional and structural conservation between mammalian and fish Bcl-2 family members have been further supported by results from ectopic expression of zebrafish proapoptotic and antiapoptotic Bcl-2 family members. Thus, injection of mRNA encoding *DareBid*, *DareBik*, *DareBmf1*, *DareBmf2*, *DareNoxa*, *DarePuma*, *DareBax1* and *DareBax2* triggered dose-dependent apoptosis characterized both by disintegration of the blastomeres and yolk cell and dramatic increase in activated caspase-3 [98]. Surprisingly, injection of mRNA encoding *DareBad*, *DareBok1* and *DareBok2*, even at high mRNA doses, did not induce significant apoptosis [98], although overexpression of *DareBad* in COS-1 cells resulted in apoptosis [115]. On the other hand, functional assays using RNA-injected zebrafish embryos indicated that the EGFP-DareBcl-2 fusion protein can suppress normal developmentally regulated apoptosis [106]. Moreover, in a transgenic zebrafish model, the same authors [106] showed that expression of EGFP-DareBcl-2 in the lymphoid compartment increased T- and B-cell population. Overexpressing *DareBcl-x_L* or *DareMcl-1a* under the control of the liver-specific promoter L-FABP [119, 120] also led to liver hyperplasia in transgenic zebrafish larvae. The hyperplasia of the liver

resulting from overexpression of either *DareMcl-1a* or *DareBcl-x$_L$* has been attributed by those authors to interrupted hepatocyte apoptosis or an accelerated cell proliferation. Recently, it has also been shown that DareMcl-1a and DareBcl-x$_L$ prevent cytochrome *c* release from cells undergoing betanodavirus-induced apoptosis [121]. Apoptosis induced by overexpression of a minimum lethal dose of the proapoptotic family members could be rescued by coexpression of *DareMcl-1a*, *DareMcl-1b*, *DareBcl-x$_L$* or *DareBcl-2*. However, *DareBcl-2* failed to inhibit apoptosis triggered by ectopic expression of *DareBax2*, suggesting that DareBax2 does not interact with the zebrafish homolog of mammalian Bcl-2, similar to what happens with human Bak [63, 98]. Furthermore, a weak interaction between DareBik and DareBcl-2 has also been reported which, together with the above data, support the existence of selective interactions between certain prosurvival and proapoptotic family members [98] in a similar way as do mammalian Bcl-2 family members [122, 123]. Moreover, in the same study Kratz and colleagues [98] showed that, as happens in certain mammalian cell types, *DareMcl-1* modulates sensitivity to Apo2L/TRAIL-induced apoptosis and, therefore, Mcl-1 may be a key modulator of death ligand-induced apoptosis in cells that require engagement of both the extrinsic and intrinsic pathways for commitment to apoptosis.

One of the well-established activators of the intrinsic apoptotic pathway is DNA damage, induced by genotoxic and nongenotoxic stresses such as radiation, viruses and repair errors, which may in turn lead to cancer. To prevent this, multicellular organisms have evolved an efficient control system with the tumor-suppressor protein p53 playing a central role in repair, apoptosis and senescence [124]. In addition to DNA damage other cellular stresses such as oncogene activation, hypoxia, growth factor stimulation and oxidative stress can activate mammalian p53. Due to the potential toxicity for nontumorigenic cells, the activity of p53 is tightly regulated through the interaction with various partner proteins and several posttranslational mechanisms leading to protein stabilization [124]. p53 has been cloned and characterised in several fish species and its importance during embryogenesis and DNA damage-induced apoptosis elicited by UV or by the anti-cancer compound camptothecin has been reported [125-132]. Moreover, data mining the p53 pathway in the Fugu genome has indicated conservation of the apoptotic pathway [133]. In this context, exposure of zebrafish embryos to γ-irradiation was shown to induce substantial increase in p53-dependent activation of caspase-3 [98]. Moreover, ectopic expression of prosurvival Bcl-2 family members, such as *DareMcl-1a*, *DareMcl-1b*, *DareBcl-x$_L$* or *DareBcl-2* was shown to protect embryos from γ-irradiation-induced apoptosis [98]. The same study has shown that, as found in mammalian cells [134, 135], the BH3-only protein DarePuma is the primary initiator of the apoptotic process triggered by γ-irradiation; knockdown of *DarePuma* attenuated caspase-3 activation as effectively as did knockdown of *p53*, whereas knockdown of the other BH3-only genes *DareBik*, *DareBmf1*, *DareBid* or *DareNoxa* did not. These data were further corroborated by quantitative RT-PCR analysis which revealed that, while γ-irradiation induced a 3- to 4-fold upregulation of *Dare Noxa*, it augmented *DarePuma* expression almost 100-fold, both in a p53-dependent way. No other BH3-only gene has shown increased transcription. Finally, those authors have shown that knockdown of *DareBax1* and *DareBax2* also abrogates γ-irradiation-induced apoptosis. This clearly supports the existence of a conserved DNA damage-induced apoptosis pathway in vertebrates with the transcriptional factor p53 playing a key role activating Puma, which, through activation of Bax, leads to MMP, release of cytochrome *c* and apoptosis. On the other hand, comparing to higher vertebrates, several differences in p53 expression and regulation have been reported in fish such as absence of tumorigenesis in zebrafish embryos injected with the p53-negative regulator Mdm2 [136] as well as lack of p53 induction in fish cells by model chemotherapeutics, albeit those same chemotherapeutics induced apoptosis [137]. Moreover, the low prevalence of p53 mutations found in wild fish [138, 139] as well as in laboratory-exposed fish [129, 130, 140], compared to the high prevalence (>50%) of p53 mutations in human cancers, further suggest that p53 activation and function in fish species may differ from that of other vertebrates [137]. However, it should be further investigated whether the differences mentioned above may be due to the fact that the experiments have been performed *in vitro*, as well as whether the low prevalence of p53 mutations reported in fish are associated to the fact that the studied fish were not old enough or were not allowed to live long enough in captivity for mutations to occur.

Altogether, these data indicate that fish have in their genomes functional homologs of virtually all mammalian Bcl-2 family members regulated by similar transcriptional and posttranslational mechanisms, which, together with the presence of a similar structural and functional caspase-9 in fish (see below), points to the existence of a conserved functional intrinsic pathway in vertebrates.

4. CASPASES

The death effector machinery, leading to the morphological [141, 142] and biochemical [143, 144] changes associated with apoptosis, is manly orchestrated by an evolutionarily conserved family of intracellular **c**ysteinyl **asp**artate-specific prote**ases** known as caspases. These peptidases employ a cysteine residue as the catalytic nucleophile and are among the most specific proteases, with a remarkable specificity for cleavage on the carboxyl side of aspartic acid residues (for recent reviewing of mammalian caspases see e.g., Cell Death and Differentiation special issue on caspases, volume 14(1):1-78 and references therein). The cleavage aspartate residues are recognized within the context of tetrapeptide motifs that differ significantly among caspases and explains the diversity of their biological functions (reviewed in [145, 146]). Functionally, caspases can be classified as (Fig. **2**): i) upstream initiator caspases (caspase-2, -8, -9 and -10) whose proforms possess large NH2-terminal prodomains, containing either death effector domains (DED) (caspases-8 and -10) or a caspase-associated recruitment domain (CARD) (caspases-2 and -9); (ii) downstream executioner or effector caspases (caspase-3, -6 and -7), whose proforms contain only short NH2-terminal prodomains thought to be involved in subcellular targeting [147, 148]; and (iii) pro-inflammatory caspases (caspase-1, -4, -5, -11 and -12), whose main role lies in cytokine maturation rather than apoptotic activity (reviewed in [2, 146, 149-153]). Given the potentially devastating effect of inadvertent caspase activation, each caspase is synthesised as a minimally active precursor (caspase zymogen or procaspase). Procaspases consist of a prodomain of variable length, followed by a large (p20) and a small (p10) subunit, both containing the residues forming the recognition and catalytic sites. Although cleavage of the zymogen is not always an obligatory requirement for caspase activation [154], generation of active enzymes usually involves a minimum of two cleavages, one separating the large and small subunits and another separating the prodomain from the large subunit (reviewd in [151, 152, 155]). Generally, the prodomain of the initiator caspases interacts with a specific scaffold or adaptor protein whose role is to cluster the respective initiator caspase (reviewed in [156]). This proximity enhances the autoproteolysis of the caspase in order to form an active dimeric complex, consisting of two small and two large subunits (reviewed in [150, 151]), which then, activate effector caspases through cleavage at specific internal aspartic acid residues in order to amplify the apoptotic signal [157]. The proximity-induced activation of certain procaspases can be blocked by anti-apoptotic members of the Bcl-2 family and other cellular polypeptides (reviewed in [151, 152]). For example, activation of procaspase-9 by the Apaf-1/cytochrome *c* scaffold is primarily regulated by anti-apoptotic Bcl-2 family members. The activation of caspase zymogens by death receptor-associated scaffolds can be regulated by a polypeptide termed FLICE-inhibitory protein (FLIP), which contains DEDs, and can bind to the prodomains of procaspases-8 and -10, thereby inhibiting their recruitment to CD95/

Fas and TNFR1 induced activation complexes. Caspase activation and activity can also be regulated by interactions with inhibitor of apoptosis proteins (IAPs) (reviewed in [158]), which bind to the active site of caspases, acting as competitive inhibitors. Nevertheless, this anti-apoptotic effect of IAPs can be neutralized upon release of certain mitochondrial proteins, such as Smac/DIABLO, that sequester the IAPs (reviewed in [151, 159]), allowing the progression of the apoptotic process. Further regulation of the caspase activity can be done at transcriptional and post-translational levels, including *S*-nitrosylation and phosphorylation (reviewed in [151, 158]).

In the following review we mostly deal with fish homologs of mammalian apoptotic caspases.

Fish Homologs of Mammalian Initiator Caspases

Caspase-8 (also known as FLICE/MACH1/Mch5) is an essential component of the extrinsic/death-receptor pathway [154]. The identification and characterisation of caspase-8 has recently been done for some fish species [36, 160, 161]. At the genomic level, whereas zebrafish caspase-8 genes show a similar structure as that found in their mammalian equivalents, being organised in 10 exons and 9 introns with coincident splice junctions sites [36, 161], the medaka and stickleback caspase-8 genes present 12 exons and 11 introns, with the additional exons located at the first death effector and protease domains [36]. Moreover, the *caspase-8* gene is apparently duplicated in the fish genome (Table **2**). Chromosome mapping showed that the chromosomal segment around the zebrafish, medaka, fugu and stickleback *caspase-8* genes has an organisation syntenic with that of other vertebrates [36, 161]. Fish caspase-8 molecules revealed about 35% identity and 55% similarity to the human counterpart, which together with the above data supports fish *caspase-8* genes as orthologs of mammalian *caspase-8*. As their amphibian and mammalian homologs, fish caspase-8 possesses two DEDs, at their N-terminal prodomains, and a carboxyl-terminal catalytic domain (CASc) including conserved amino acids essential for catalytic activity and the characteristic pentapeptide active site QACQG (QACRG in zebrafish caspase-8b) (Fig. **2**) [36, 161]. That zebrafish caspase-8 associates with the adaptor molecule FADD through homophilic DED interactions was shown by detecting zebrafish caspase-8 and human FADD in immunoprecipitates of HEK293T cells coexpressing HA-tagged human FADD with Flag-tagged zebrafish caspase-8 [161]. Additionally, exogenous expression of zebrafish caspase-8 in *caspase-8*-deficient mouse embryonic fibroblast (MEF) cells triggered cell death with or without ligation of Fas, the former showing an increased sensitivity, with 80% of the transfectants dying following Fas ligation. This indicates that zebrafish caspase-8 directly transmits apoptotic signals *via* Fas to downstream molecules, including, as shown in zebrafish caspase-8-expressing HeLa cells, caspase-3; the same holds true for TNFα induced-cell death [161]. Similarly, exogenous expression of medaka caspase-8 induced apoptotic death in transfected HeLa cells, but apoptotic death was inhibited by either zVAD-fmk, the antiapoptotic cowpox virus serpin cytokine response modifier A (CrmA) [162], or the pan-caspase inhibitor p35 [36]. Furthermore, coinjection of zebrafish FLIP, or CrmA, reduced zebrafish caspase-8a-mediated embryonic mortality, confirming that caspase-8 functions similarly in zebrafish and mammals [30]. The long splice form of the extrinsic pathway inhibitor cellular FLIP (cFLIP) is a key regulator of the extrinsic pathway of caspase-8 activation, inhibiting the recruitment and activation of caspase-8 by death receptors (reviewed in [154, 163, 164] and references therein). Like mammalian FLIP, which contains two DEDs that interact with the DED in FADD, zebrafish FLIP is structurally similar to caspase-8 and -10 and lacks an active-site cysteine [30]. Inhibition of caspase-8-mediated apoptosis in early zebrafish embryos has been also shown to occur through interactions of Yaf2 with the DED of caspase-8, with implications on cell survival during zebrafish embryogenesis [160]. Yaf2 is a member of the Rybp/Yaf2 protein family, with Rybp (also known as DEDAF) being described in mammals as interacting with DED-containing proteins, including FADD, caspase-8, caspase-10, and DEDD, for promoting Rybp pro-apoptotic activity [165]. Finally, induction of extensive apoptosis in zebrafish embryos injected with zebrafish caspase-8a mRNAs [30] as well as detection of caspase-8 transcription by RT-PCR in both maternal and zygotic transcripts during zebrafish embryonic development, with ubiquitous expression in whole embryos [161], suggested that caspase-8 plays an important role during embryonic development of fish embryos similarly to mammalian/amphibian embryos. The above data confirm that, like its mammalian homolog [166, 167], fish caspase-8 is an initiator caspase recruited to the DISC *via* binding to the adaptor protein FADD, transmitting apoptotic signals from death receptors to downstream effector caspases; it can also mediate cross-talk between the extrinsic and intrinsic apoptotic pathways, since fish Bid sequences reveal conservation of the caspase-8 cleavage site (see above).

Caspase-10 is similar to caspase-8 (Fig. **2**), containing also two DEDs and a CASc, but its function in apoptosis remains debatable, although some data suggest that human caspase-10 may have a function overlapping with caspase-8 in the Fas ligand-mediated cell death pathway (reviewed in [154]). To our knowledge, detailed characterization of a fish caspase-10 is still missing, despite *caspase-10*-like genes with high degree of similarity to human *caspase-10* have been reported [161] and can be found in the databases (Table **2**).

Since, in addition to caspase-8 and caspase-10, other components essential for forming the DISC, as well as death ligands and the BH3-only member Bid, have been identified in fish (see above), these data strongly point to the existence of a structural and functional conserved extrinsic/death receptor apoptotic pathway in vertebrates.

Although **caspase-2** was one of the first apoptotic caspases discovered, its physiological function and exact mechanism of activation remains a matter of considerable debate (reviewed in [154]). Caspase-2 has already been identified in fish (Table **2**) [23, 30], but, to our knowledge, detailed structural and functional characterization has not been reported so far. The fact that, as its mammalian counterparts, fish caspase-2 contains a CARD in its prodomain (Fig. **2**) in addition to presenting a conserved synteny with the human ortholog, indicates that this protease will likely play a similar role in the apoptotic process as its mammalian homologs.

Caspase-9 (also known as ICE-LAP6/MCH-6/Apaf-3) is a member of the CED-3 subfamily, bearing high similarity to caspase-3, and is a key component of the intrinsic/mitochondrial death-pathway [154, 168]. *Caspase-9* genes have been identified in some fish species (Table **2**), and the primary and secondary structures of sea bass caspase-9 were recently characterized by our group [169]. BLAST analysis revealed homology between sea bass caspase-9 and its vertebrate equivalents, and, as expected, presented higher similarity (>75.4%) and identity (>67%) with other fish counterparts than with that of non-fish vertebrates (47-48% identity and 62-64% similarity). Sea bass *caspase-9* gene is organized in 10 exons and 9 introns, which, when compared with the human homolog, represents an extra exon located in the predicted prodomain. Fish *caspase-9* gene is apparently a single copy gene, as supported by Southern blot analysis of sea bass genomic DNA and by the fact that only a single *caspase-9* gene has been identified in other fish species (Table **2**) [30]. Additionally, fish caspase-9 genes may preserve a conserved synteny with the human ortholog, as indicated by the zebrafish *caspase-9* [30]. Moreover, sea bass caspase-9 conserves characteristic caspase-9 features (Fig. **2**) (reviewed in [2]) such as a CARD in the N-terminal prodomain, a large (p20) and small (p10) subunits, a caspase family signature, and all important catalytic residues, including the active site pentapeptide QACGG. A CARD was also reported in zebrafish caspase-9 [23], which is relevant taking into account that fish apoptotic protease-activating

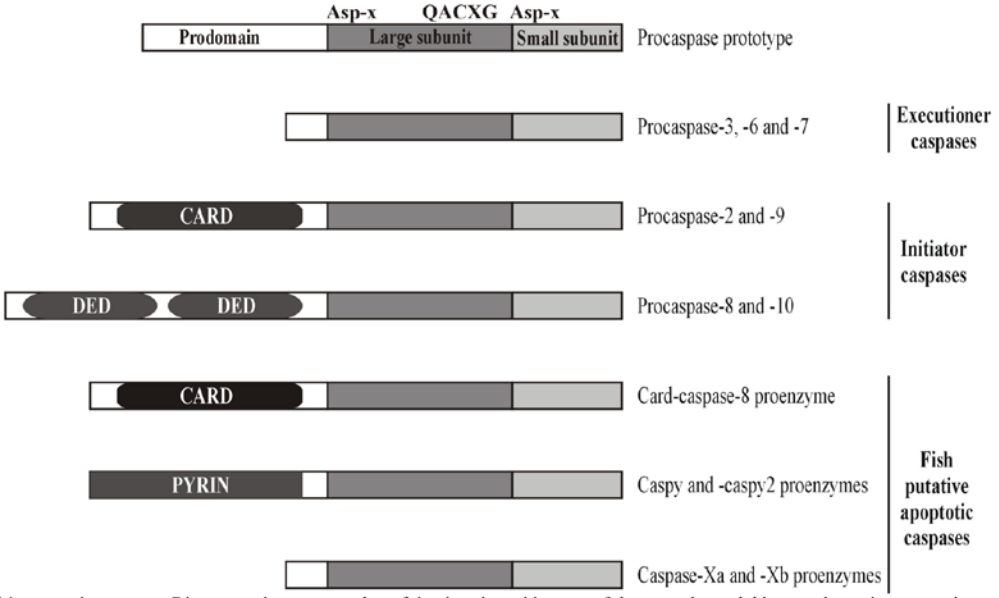

Fig. (2). Fish apoptotic caspases. Diagrammatic representation of the domain architecture of the executioner, initiator and putative apoptotic caspase proenzymes of fish. See text for details.

factor 1 (Apaf-1) was identified and contains CARD, a nucleotide-binding oligomerization domain (NO) and WD-40 repeats (Table **2**) [23]. This suggests that, like in mammalians [170], fish might regulate caspase-9 activity in a Apaf-1/cytochrome *c*-dependent manner within the apoptosome context (reviewed by [155]). In addition, a homolog of Nod1, an Apaf-1-like molecule that regulates apoptosis and NF-kB activation [171, 172], was found in zebrafish [23]. As with the mammalian equivalents (see [154, 163, 164] and references therein), further regulation of fish caspase-9 may be also achieved through inhibition by IAPs, as both IAP and XIAP homologs have been identified in fish (Table **2**) [23, 30]. Structural studies suggest that the N-terminal BIR2 linker region of XIAP occupies the catalytic site in active caspase-3 and -7, whereas the XIAP BIR3 domain is required for inhibiting caspase-9 (see [154, 163, 164] and references therein). The identification of Smac/DIABLO homologs and several serine proteases highly similar to HtrA2/Omi in fish (Table **2**) also indicates that the inhibitory effect of IAPs may be neutralized in a similar way as in mammals, i.e., during apoptotic signalling, Smac/DIABLO and HtrA2 are released from the mitochondria into the cytoplasm where they bind to XIAP and prevent its inhibitory activity (see [154, 163, 164] and references therein). Indications of caspase-9 function and involvement in apoptosis in fish were obtained by detection of caspase-9 transcription by RT-PCR in head kidney of sea bass infected with the apoptotic-inducing bacteria *Photobacterium damselae* ssp *piscicida* (*Phdp*; reviewed elsewhere in this issue). The increased expression of caspase-9 correlated with the presence of apoptotic cells and putative caspase-9 activity in the head kidney, the latest detected by a luminescence assay using a proluminescent substrate containing the caspase-9 specific cleavage tetrapeptide LEHD [169]. Taken together, the above data represent strong evidence that apoptotic processes involving caspase-9 and its regulation are evolutionarily conserved among vertebrates, which, as stated in the Bcl-2 section, points to the existence of a conserved functional intrinsic pathway in vertebrates.

Fish Homologs of Mammalian Executioner Caspases

Caspase-3 is the main downstream effector caspase that cleaves the majority of the cellular substrates in apoptosing cells after activation following cleavage by initiator caspases, except caspase-2 (reviewed in [152, 154, 173]). Caspase-3 is the most well characterized caspase in fish, including zebrafish [174, 175], salmon [176] and sea bass [177]. BLAST search revealed a close relation between fish caspase-3 and other vertebrate caspase-3 molecules, which is supported by the relatively high percentage of aminoacid identities (50-70%) reported between fish and non-fish vertebrate caspase-3 molecules [174, 177]. Fish *caspase-3* presents smaller introns than the human equivalent, and presents a 6 exon/5 intron (7 exon/6 intron in zebrafish) structure, while the human gene has an 8 exon/7 intron structure. However, they all conserve an identical gene organization within the coding region. Although Southern blotting has suggested sea bass *caspase-3* as a single copy gene [177], it is likely that it is present in all fish genomes as a duplicate gene, as two *caspase-3* genes have been identified in other fish species (Table **2**). Whether the duplicates have acquired distinct spatiotemporal functions requires further investigation. As their mammalian homologs, fish caspase-3 presents a short N-terminal prodomain and a catalytic domain containing both caspase p20 and p10 subunits, a caspase family signature and all important catalytic residues, including the characteristic pentapeptide active-site QACRG (Fig. **2**) [174, 176, 177]. The apoptotic function of fish caspase-3 has been widely supported. Thus, high proteolytic activity towards the mammalian caspase-3 and -7 substrate Ac-DEVD-MCA was reported for the recombinant zebrafish caspase-3, whereas it had low activity against the caspase-1, caspase-6 and -8, and caspase-9 substrates (Ac-YVAD-MCA, Ac-IETD-MCA and Ac-LEHD-MCA, respectively), indicating a similar substrate specificity to that of mammalian caspase-3 [174]. Moreover, overexpression of the zebrafish caspase-3 induced apoptosis in cultured fish cells and zebrafish embryos, which could be effectively repressed by the inhibitor of mammalian caspase-3 Z-DEVD-FMK [174]. Zebrafish embryos microinjected with a zebrafish caspase-3 expression vector also had high Ac-DEVD-MCA-hydrolysing activity. In the same study, Yabu *et al.* also showed that the expression of the zebrafish *caspase-3* gene was regulated in both a tissue- and stage-specific manner, with caspase-3 mRNA from maternal origin present in early embryogenesis and after gastrulation by zygotic genome activation, especially in the pectoral fin bud, optic vesicle and hindbrain. Finally, an increase of caspase-3 expression was observed in the head kidney of sea bass infected with the apoptotic-

inducing bacteria *Phdp*, which was accompanied by the occurrence in that organ of cells with apoptotic morphology and activated caspase-3 [177].

Caspase-7 is highly similar to caspase-3 (Fig. **2**) and has similar substrate specificity (reviewed in [152, 154]). However, at least for apoptosis induced *via* the intrinsic pathway, a combined function of caspase-3 and –7 is apparently required for cell death execution (reviewed in [154, 178]). Caspase-7 has been identified in several fish species as a duplicated gene (Table **2**) and its primary and secondary structures recently characterized in salmon [176]. The reported *caspase-7* gene from salmon is organized in a 5 exon/4 intron structure, differing therefore from the mouse and human counterparts [176]. *Caspase-7* mRNA was exclusively detected by whole-mount *in situ* hybridization in the lens of salmon embryos, while *caspase-3b* and *caspase-6a* and *-6b* were expressed in multiple organs of heat-stressed and control salmon embryos. Moreover, cell death induced in heat-stressed salmon embryos could be correlated with the expression of caspase-7, as well as caspase-6 and -3, in a similar way as in mouse embryos [176].

Although structurally similar to caspase-3 and –7, **caspase-6** (Fig. **2**) has somewhat different substrate specificity, its optimal substrate being VEHD rather than DEVD, the preferred caspase-3 and –7 substrate [157]. The function of caspase-6 remains a mystery, as it does not seem to be essential for apoptosis induced by various agents [154]. In fish it has been characterized in trout [179] and salmon [176], being identified in fish as a duplicate [176] or even triplicate [30] gene (Table **2**). As caspase-3 and -7, fish caspase-6a and -6b have a relatively short N-terminal prodomain followed by a catalytic C-terminal domain comprising the large and small subunits, the caspase family signature as well as the amino acids involved in catalysis, including the QACRG catalytic site, and those forming the P1 carboxylate binding pocket. Caspase-6a and -6b share high identity (98% amino acid identity between salmon caspase-6a and -6b) as well as with other vertebrate counterparts (>60% amino acid identity). At genomic level salmon *caspase-6a* presents a 7 exon/6 intron organization, as its human homolog, whereas the insertion of an intron within the first exon generated an additional exon in the *caspase-6b* gene, similar to the chicken *caspase-6* gene [176]. In human *caspase-6*, intron 3 is involved in activation by binding of the pro-apoptotic transcription factor p53 [180]. However, as hypothesized by Takle *et al.* [176] for salmon, the different genomic structure of *caspase-6a* and *-6b* may cause different regulation of the two genes that may be worth to investigate. RT-PCR analysis revealed that the trout *caspase-6* gene is expressed in brain, blood, gill, liver, head kidney and spleen [179]. In salmon, caspase-6a and -6b were also expressed in multiple tissues of heat-exposed and control embryos, although cardiac expression of caspase-6a and -6b was only observed in heart of the heat-stressed embryos, with an apparent correlation with cell death [176]. Trout caspase-6 expression could be specifically increased by addition of LPS to head kidney leukocytes, although only in the presence of cortisol (and following a preincubation with cortisol) [179]. This effect was mediated by glucocorticoid receptors, as incubation with the glucocorticoid receptor blocker RU486 abrogated the expression of caspase-6 in LPS-stimulated trout leucocytes. Moreover, a marked up-regulation of caspase-6 expression in the trout head kidney was observed upon *in vivo* induction of confinement stress. Although both glucocorticoids and stress are regarded as inducers of apoptosis in fish [181, 182], no correlation of the observed induction of caspase-6 with cell death was reported by Laing *et al.* [179].

The above data clearly indicate that fish possess a caspase cascade, similar to that characterized in mammals.

Fish Putative Apoptotic Caspases

A *caspase-8*-like gene, showing high similarity to human and other vertebrate *caspase-8* and *caspase-10* sequences, but possessing a CARD at their N-terminal prodomains instead of DEDs (therefore tentatively named *card-caspase-8*), has been reported in fish (Fig. **2**) [35, 36, 161]. The *card-caspase-8* in not a third *caspase-8/-10* gene as the one identified in the frog and chicken genomes; instead, it apparently results from replacement of its DED by a CARD upon a tandem duplication of the ancestral *caspase-8* gene over the course of evolution [36, 161]. In addition to the CARD in its prodomain, card-caspase-8 contains the caspase family signature, the active catalytic site, and the large (p20) and small (p10) subunits at the CASc. Moreover, card-caspase-8 exhibits proapoptotic activity [35, 36]. However, it would be interesting to find out whether fish card-caspase-8 is activated by interacting with death receptors *via* a CARD-containing adapter molecule such as RAIDD or by recruitment to large multiprotein complexes (as caspase-2; see [154]), or by heterotypic interaction (DED-CARD) with FADD.

Other caspases mostly homologous to mammalian caspase-8 (zebrafish caspase-Xa and -Xb), but containing a QACRG active site and lacking obvious N-terminal DEDs, have also been recently reported (Table **2** and Fig. **2**) [30].

Two pyrin domain-containing caspases (Fig. **2**), caspy and caspy2, as well as the zebrafish ortholog of apoptosis-associated speck-like protein containing CARD (ASC), were also identified in zebrafish [183]. Whether these caspase molecules play initiator or executioner roles in apoptosis is not clear, although the presence of long prodomains and, at least for caspy, the need of oligomerization for its activity (see below), mostly resemble initiator caspases. Both caspy and caspy2 contain long N-terminal pyrin domains [184] and C-terminal caspase catalytic domain, the latest including conserved amino acid residues essential for proteolytic catalysis, and residues essential for interaction with critical core residues of substrates, as well as the pentapeptide active site QACRG. Whereas the N-terminal pyrin domain of caspy and caspy2 were most homologous to that present in zebrafish ASC (87% similarity) and to that of human pyrin-domain containing Nod-family protein Cryopyrin/PYPAF1 (46% similarity), respectively, the catalytic domains shared closer similarity with that of human caspase-1 and -5, respectively [183]. Additionally, the pyrin domain of caspy2 exhibits homology not only to pyrin domains but also to the CARDS of *Xenopus* caspase-1 (49% similarity) and bovine caspase-13 (45% similarity) [183]. As its mammalian counterparts, zebrafish ASC is composed of an N-terminal pyrin domain linked to a C-terminal CARD [183]. Functionally, both caspy and caspy2 induced apoptosis when transfected into mammalian cells, being its effect attenuated by p35 and inhibited by zVAD-fmk, whereas only caspy was inhibited by the human caspase-1 inhibitor CrmA. Moreover, caspy and caspy2 exhibit different substrate specificity, with caspy preferentially cleaving the caspase-1 substrate AcYVAD-AMC, whereas caspy2 preferentially cleaves the caspase-5 subtrate AcWEHD-AFC [183], which, together with the above data, supports the interpretation that both are active enzymes with substrate specificities. Finally, it was shown by co-immunoprecipitation and expression assays that only caspy interacts with zebrafish ASC through a homophilic pyrin-pyrin domain interaction, with the interaction occurring in the same speck [183]. However, proteolytic processing of caspy and induction of caspy-dependent apoptosis can only be achieved by enforced oligomerization of zebrafish ASC, which is presumably induced through an interaction between the C-terminal CARD of zebrafish ASC and a putative upstream regulator yet to be identified, therefore indicating ASC as an adaptor molecule [183]. Last but not least, morpholino antisense oligonucleotide-based knockdown of caspy in zebrafish embryos was shown to affect development of the jaw and posterior pharyngeal arches with no other abnormalities identified in any other part of the body, suggesting that caspy is involved in the execution of a program of neural crest cell death required for proper developmental and assembly of cartilage [183]. However, it cannot be ruled out that caspy is in-

volved in processing the zebrafish IL-1/IL-18 and/or another factor that is required for normal development of pharyngeal arch cartilages [183]. The fact that truly mammalian caspase-1 homologs, with CARD at their prodomain, have been identified in fish (Table **2**), suggests that caspy, and probably caspy2, can play a different role yet to be found in mammalian homologs.

As a concluding remark, and despite the fact that many other molecules important in the apoptotic process have been identified and characterised in fish, but were not included in the present review due to space constrains, there is compelling evidence that fish do contain functional homologs of virtually all members involved in the apoptotic machinery (Table **2**), making fish excellent models for studying apoptosis-related genetic or infectious disorders as well as for drug development.

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