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Genetic characterization of the chemokine receptor CXCR4 gene in lagomorphs: comparison between the families Ochotonidae and Leporidae

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Summary

Chemokines receptors are transmembrane proteins that bind chemokines. Chemokines and their receptors are known to play a crucial role in the immune system and in pathogen entry. There is evidence that myxoma virus, the causative agent of myxomatosis, can use the chemokine receptor CXCR4 to infect cells. This virus causes a benign disease in its natural host, *Sylvilagus*, but in the European rabbit (*Oryctolagus cuniculus*) it causes a highly fatal and infectious disease known as myxomatosis. We have characterized the chemokine receptor CXCR4 gene in five genera of the order Lagomorpha, *Ochotona* (Ochotonidae), and *Oryctolagus*, *Lepus*, *Bunolagus* and *Sylvilagus* (Leporidae). In lagomorphs, the CXCR4 is highly conserved, with most of the protein diversity found at surface regions. Five amino acid replacements were observed, two in the intracellular loops, one in the transmembrane domain and two in the extracellular loops. *Oryctolagus* features unique amino acid changes at the intracellular domains, putting this genus apart of all other lagomorphs. Furthermore, in the 37 European rabbits analysed, which included healthy rabbits and rabbits with clinical symptoms of myxomatosis, 14 nucleotide substitutions were obtained but no amino acid differences were observed.

Introduction

Chemokines receptors are seven-transmembrane domain G-protein coupled receptors that allow binding of chemokines (chemotactic cytokines), a superfamily of small heparin-binding proteins that share a sequence homology and are characterized by four conserved cysteines residues linked to disulfide bonds. Chemokines and their receptors play a central role in directing the migration of circulating leucocytes during immune surveillance, inflammation and the establishment of immunity, and are involved in organogenesis, hematopoiesis, angiogenesis, tumor growth, metastasis and dendritic cell maturation (reviewed in Rossi & Zlotnik, 2000). Chemokines receptors have also been associated with pathogen entry (e.g. Horuk *et al*., 1993; Feng *et al*., 1996). CXCR4, CCR1 and CCR5 are among the candidate receptors used by the myxoma virus to infect cells (Lalani *et al*., 1999).

The myxoma virus is the causative agent of myxomatosis, which can be a highly fatal disease in the European rabbit (*Oryctolagus cuniculus*) although benign in its natural host, species of *Sylvilagus* (Fenner, 1983). This virus belongs to the family Poxviridae and like all the members of this family has a large, linear, double-stranded DNA genome that encodes more than 20 host-related proteins (Cameron *et al*., 1999; Zúñiga, 2002; Seet *et al*., 2003). A previous study on CCR5 in *Oryctolagus* revealed the replacement of a specific peptide motif of the second extracellular loop (ECL2) of the CCR5 protein by a motif which in other species characterizes the CCR2 molecules. This replacement was found in all the *Oryctolagus* specimen analysed while absent in *Sylvilagus* and *Lepus* species (Carmo *et al*., 2006).

Here, we have characterized the genetic diversity of the CXCR4 gene among lagomorphs. The CXCR4 has an open-reading frame that consists of two exons separated by an intron of 2.1kb. The first exon includes the sequence corresponding to the first five amino acids of the CXCR4 protein and a 5′ UTR region and the second exon codes for the last 347 amino acids and a-3′ UTR region (Wegner *et al*., 1998). We have sequenced the exon 2 of the receptor for *Ochotona, Bunolagus*, *Lepus*, *Oryctolagus* and *Sylvilagus*. Gene regions will be defined and named in accordance with the structural domains of the CXCR4 protein they encode, which consist, respectively, of the N-terminal domain (N-tail), the C-terminal domain (C-tail), the extracellular loops (ECL1, ECL2, ECL3) and the intracellular loops (ICL1, ICL2, ICL3) and the seven transmembrane domains (TM1-TM7).

The order Lagomorpha includes two families, the Leporidae and the Ochotonidae. The Ochotonidae family differentiated from the Leporidae family in the Oligocene (~35 Mya) (Yu *et al*., 2000) and is represented by one

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extant genus, *Ochotona* (pikas), with approximately 30 species restricted to Asia and North America. The Leporidae family comprises 11 genera, *Brachylagus*, *Bunolagus*, *Caprolagus*, *Lepus*, *Nesolagus*, *Oryctolagus*, *Pentalagus*, *Poelagus*, *Pronolagus*, *Romerolagus* and *Sylvilagus*, with near 60 species with a worldwide distribution. The evolutionary relationships between leporid genera are not clearly established (Matthee *et al*., 2004; Robinson & Matthee, 2005). *Oryctolagus* is the only leporid genus with a European origin and comprises two subspecies, *O. cuniculus cuniculus* and *O. cuniculus algirus*. The subspecies *O. c. algirus* is restricted to the south-west of Iberian Peninsula and a few Atlantic Islands (reviewed in Ferrand & Branco, 2007) while *O. c. cuniculus* has a worldwide distribution, man-made mainly, and includes all domestic breeds.

Materials and methods

Blood or tissue samples of 37 European rabbits belonging to both the subspecies *O. c. cuniculus* and *O. c. algirus* were obtained from wild specimen collected in Portugal, Spain and France. Eight of the samples presented clinical symptoms of myxomatosis. A sample of a domestic rabbit breed (*Fauve de Borgogne*) was also included. Ten leporid species and two ochotonid species were analysed: *Bunolagus monticularis, Sylvilagus floridanus*, *Sylvilagus brasiliensis, Lepus americanus*, *Lepus townsendii, Lepus californicus*, *Lepus castroviejoii*, *Lepus europaeus*, *Lepus granatensis* and *Lepus saxatilis; Ochotona daurica* and *Ochotona princeps*.

In lagomorphs, the genomic organization of the CXCR4 gene is unknown. Total RNA was isolated from spleen samples from *Oryctolagus* with TRIzol, following the manufacturer's instructions (Life Technologies, Grand Island, NY, USA). cDNA was prepared as described (Krug & Berger, 1987) and was polymerase chain reaction (PCR) amplified using the primers CXCR4_F1 and CXCR4_R1 (for description of the primers used in this study see Table 1) designed according to conserved regions of the gene in mammals. PCR products of approximately 1050 bp in length were gel purified and cloned into pGEM-T Easy vector (Promega, Madison, WI, USA).

Table 1. Sequences of the primers used for amplification by polymerase chain reaction and schematic representation of their localization. The nucleotide positions are according to the human cDNA sequence of CXCR4 GenBank accession number NM_001008540

Primer	Composition (5′–3′)	Positions (according to the human cDNA sequence)
CXCR4_F1	ATGGAGGGGATCAGTATATAC	1–21
CXCR4_R1	TTAGCTGGAGTGAAAACTTGA	1039–1059
CXCR4_F2	ACTTCAGACAACTACACGGA	22–41
CXCR4_F3	CATAAAGGAGCCCTGCTTCC	69–88

Total genomic DNA was extracted using a Qiagen extraction kit (Qiagen, Vienna, Austria). The PCRs were carried out in a 25 μL reaction volume containing 50 ng of genomic DNA, 1 × reaction buffer, 2.5 μL of DMSO, 3 mM $MgCl_2$, 0.5 mM of each dNTP, 0.4 μM of each primer and 1 U of Taq polymerase (EcoTaq, Ecogen, Spain). For *Oryctolagus*, PCR amplification was performed with the primers CXCR4_F2 and CXCR4_R1. For the other lagomorph genera, a different forward primer had to be used, CXCR4_F3, which was designed based on the rabbit cDNA sequence of CXCR4. The amplification conditions were 5 min at 94 °C followed by 35 cycles at 94 °C (45 s), 54 °C (30 s) and 72 °C (1 min), with a final extension at 72 °C (7 min). Sequencing was performed on an ABI PRISM 310 Genetic Analyser (PE Applied Biosystems, Foster City, CA, USA) following the ABI PRISM BigDye Terminator Cycle sequencing protocols. PCR products were sequenced in both directions.

The sequences obtained were aligned with published CXCR4 gene sequences available in NCBI GenBank using ClustalW (Thompson *et al*., 1994) and adjusted by visual inspection. The nucleotide and inferred protein sequences appear in the tables and figures with the following denominations and GenBank accession numbers: Homo (*Homo sapiens*: NM_001008540), Macaca (*Macaca mulata*: NM_001042645), Sus (*Sus scrofa*: NM_213773), Bos (*Bos taurus*: NM_174301), Canis (*Canis familiaris*: NM_001048026), Felis (*Felis catus*: NM_001009826), Rattus (*Rattus norvegicus*: NM_022205), Mus (*Mus musculus*: NM_009911), Gallus (*Gallus gallus*: NM_204617) and Xenopus (*Xenopus laevis*: Y17895). Nucleotide sequence data obtained in this study have been submitted to GenBank and have been assigned the following accession numbers: *Oryctolagus cuniculus* EU258276 to EU258306 and EU265728 to EU265733; *Bunolagus monticularis* EU258264; *Lepus europaeus* EU258265; *Lepus townsendi* EU258266; *Lepus castroviejoi* EU258267; *Lepus saxatilis* EU258268; *Lepus americanus* EU258269; *Lepus granatensis* EU258270; *Lepus californicus* EU258271; *Sylvilagus floridanus* EU258272; *Sylvilagus brasiliensis* EU258273; *Ochotona daurica* EU258274 and *Ochotona princeps* EU258275. Measures of nucleotide diversity were estimated using the software package DnaSP (Rozas *et al*., 2003). The number of synonymous substitutions per synonymous site (dS) and the number of nonsynonymous substitutions per nonsynonymous site (dN) were calculated using the MEGA 3.1 software (Kumar *et al*., 2004). Seven sequences from European rabbit were excluded from the analyses due to the short length. Phylogenetic trees were obtained by neighbour-joining. The reliability of the trees was tested by bootstrap (Felsenstein, 1985).

Results

The exon 2 of CXCR4 was sequenced for the lagomorph species aforementioned. All the sequences obtained cover the three intracellular, the three extracellular loops and the seven transmembrane domains of the CXCR4 protein.

Table 2. Nucleotide variation at the CXCR4 CDS region among 30 individuals of *Oryctolagus cuniculus*. The nucleotide positions are numbered according to the putative initiation site of human CXCR4 gene. R = A/G, M = A/C, Y = C/T, represent polymorphic positions; '.' represents identity with the reference sequence (GenBank accession number EU258276); the code of each sample is in brackets; the rabbits with clinical symptoms of myxomatosis are shaded in grey

		Position													
		2	2	2	4	5	6	6	7	7	7	8	8	9	9
		3	4	7	7	1	3	9	1	4	8	1	9	0	6
		1	3	6	4	3	3	3	4	4	9	3	4	1	6
	Population/Breed	G	G	C	C	T	G	C	C	C	C	G	T	T	A
O.c.algirus	Sernancelhe (Sern2)	.	.	.	.	C	.	Y	.	.	.	.	Y	.	.
	Sernancelhe (Sern4)	.	.	.	.	.	.	.	.	.	M	.	.	.	.
	Torres Vedras (TV7)	.	.	.	Y	C	.	.	.	.	.	.	.	.	.
	Torres Vedras (TV8)	.	.	.	.	Y	.	.	.	.	.	.	.	.	.
	Almodôvar (Alm8)	.	.	.	.	C	.	.	.	.	.	.	.	.	.
	Flores (FL16)	.	.	.	.	C	.	.	.	.	.	R	.	.	.
	S.J.Pesqueira (CB31)	A	.	.	.	.	.	.	.	.	.	.	C	C	G
	S.J.Pesqueira (CB32)	R	R	.	.	.	.	.	.	.	M	.	Y	Y	R
	Portimão (CB82)	R	.	.	.	Y	.	.	.	.	.	.	Y	.	.
	Portimão (CB93)	.	.	.	.	C	.	.	Y	.	.	.	C	.	.
O.c.cuniculus	Algete (Alg1)	.	.	.	.	Y	K	.	.	Y	.	.	.	.	R
	Algete (Alg2)	.	.	.	.	.	T	.	.	T	.	.	.	.	G
	Algete (Alg3,4)	.	.	.	.	.	K	.	.	T	.	.	.	.	R
	Algete (Alg5,16)	.	.	.	.	.	K	.	.	.	.	.	.	.	.
	Algete (Alg6,13,17)	.	.	.	.	.	K	.	.	Y	.	.	.	.	R
	Algete (Alg10)	.	.	M	.	.	K	.	.	T	.	.	.	.	R
	Algete (Alg12)	.	.	.	.	.	.	.	.	.	A	.	.	.	.
	Algete (Alg18)	.	.	.	.	Y	.	.	.	.	.	.	.	.	.
	Alicante (Alic18)	.	.	.	.	.	.	.	.	.	A	.	.	.	.
	Causse (Cau1)	.	.	.	.	.	.	.	.	.	A	.	.	.	.
	F.Borg. (fba18)	.	.	.	.	.	.	.	.	.	M	.	.	.	.
	Domestic (CD1,3,4)	.	.	.	.	.	.	.	.	.	A	.	.	.	.
	Domestic (CD2)	.	.	.	.	.	.	.	.	.	M	.	.	.	.

Table 3. Estimates of polymorphism in the CXCR4 nucleotide sequences of lagomorphs. *N*, number of individuals; *S*, number of polymorphic sites; Syn and Nonsyn, number of polymorphic sites that are synonymous and nonsynonymous, respectively; π, average number of nucleotide differences per site between two sequences; θ, proportion of nucleotide sites that are expected to be polymorphic. Number of synonymous substitutions per synonymous site (dS) and the number of nonsynonymous substitutions per non-synonymous site (dN) were calculated using the formulae of Li, Wu & Luo (1985)

	N	*S*	Syn	NonSyn	dS	dN	dN/dS	π	θ
Overall	45	95	83	12	0.04189 ± 0.00572	0.00212 ± 0.00083	0.05061	0.00984	0.02371
Bunolagus	1	0	0	0	0.00000	0.00000	—	—	—
Oryctolagus	30	14	14	0	0.01251 ± 0.00433	0.00000	—	0.00269	0.00348
Lepus	7	10	9	1	0.01759 ± 0.00512	0.00056 ± 0.00054	0.03184	0.00303	0.00410
Sylvilagus	2	6	5	1	0.01724 ± 0.00776	0.00095 ± 0.00091	0.05510	0.00445	0.00445
Ochotona	2	14	14	0	0.07464 ± 0.02119	0.00000	—	0.01559	0.01559

The data for the carboxyl- and the amino-terminal domains are incomplete since these regions were used as template for the primers.

Nucleotide polymorphism

Table 2 presents the nucleotide variation at the CXCR4 CDS region among 30 individuals of *Oryctolagus cuniculus*. These included healthy rabbits as well as rabbits with clinical symptoms of myxomatosis. Each of the 14 nucleotide polymorphisms observed were synonymous. Table 3 shows the results of the parameters of gene diversity. All the parameters calculated suggest a low level of nucleotide diversity. The overall average number of nucleotide differences per site between two sequences was lower than 0.001. Nucleotide differences

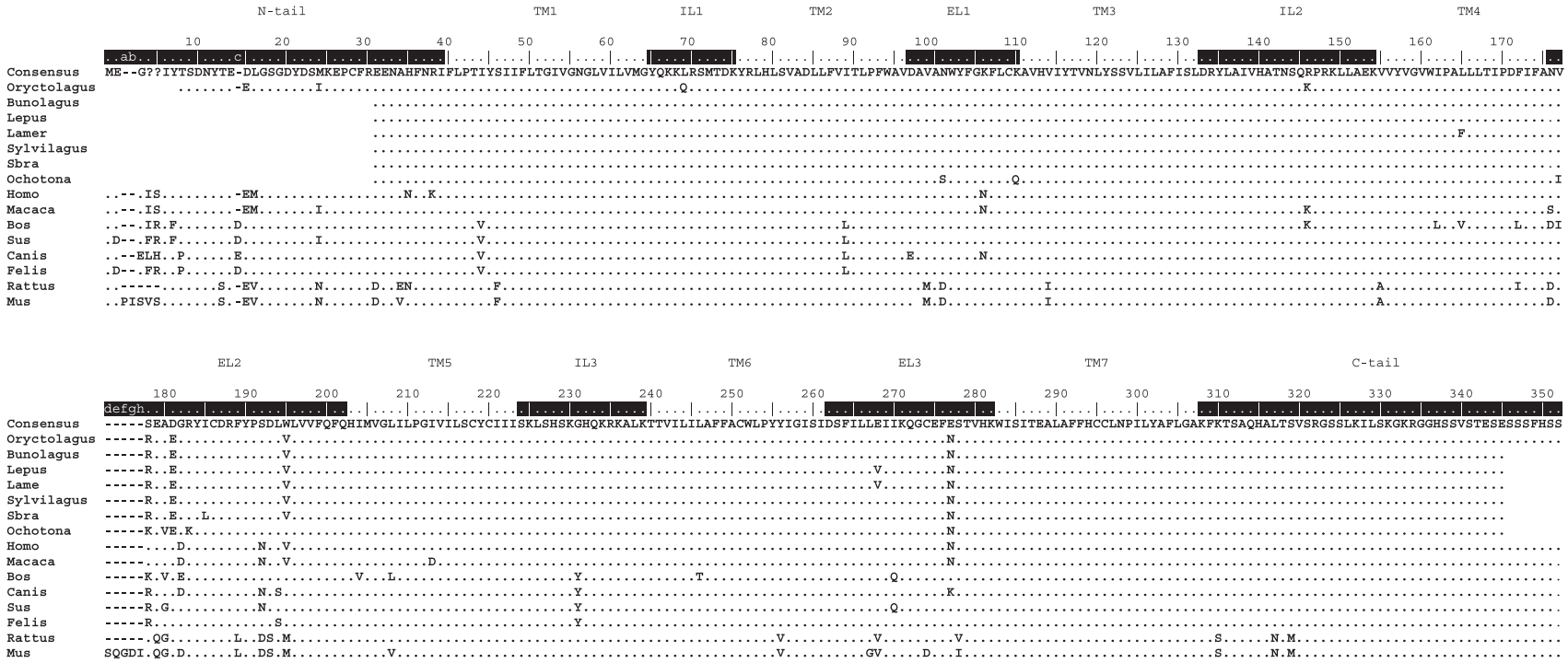

Figure 1. Differences on the deduced amino acid sequences of CXCR4. The amino acid positions are numbered according to the amino acid sequence of human CXCR4. The different domains of the CXCR4 protein are according to the topology accepted for human. '.' represents identity with the consensus sequence, and '-' represents alignment gaps. '?' in the consensus sequence represents nonconsensual amino acid. Nonconsensual amino acids were checked visually and when it was not possible to decide on an amino acid a '?' was used. The *Oryctolagus* sequence is a consensus based on the 37 sequences of CXCR4 of *Oryctolagus cuniculus*; Lame, *Lepus americanus*; Sbra, *Sylvilagus brasiliensis*.

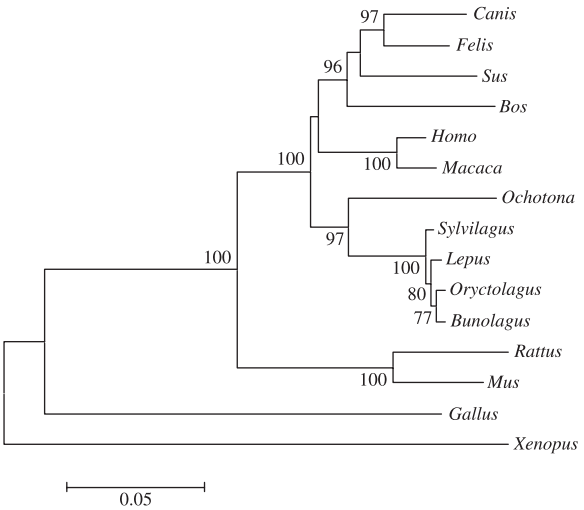

Figure 2. Neighbour-joining tree for CXCR4 nucleotide sequences. The tree was constructed using MEGA 3.1, option p-distance and complete deletion. Bootstrap probabilities values (1000 replicate runs) are indicated at the nodes and values lower than 75% are not shown.

found among lagomorph species were predominantly synonymous.

Amino acid polymorphism

The amino acid sequences of CXCR4 were inferred from the nucleotide data and were aligned with published CXCR4 sequences of mammals. The amino acid differences found among genera are highlighted in Fig. 1. The consensus sequence is based on the amino acid CXCR4 sequences of mammals other than lagomorphs and was constructed using BIOEDIT 7.0.5.3 (Hall, 1999) with a threshold frequency for inclusion of 80%.

The overall degree of diversity among lagomorphs was low with the largest distance found between ochotonid and leporid species and did not exceed 2% of amino acid differences. The genus *Oryctolagus* harboured two amino acid changes, Q69L and K146R, that are clearly apomorphic. *Bunolagus* presented no exclusive amino acids. The genus *Lepus* showed two amino acid substitutions, the alteration $I_{269}V$ and the apomorphic change $L_{165}F$ in *Lepus americanus*. *Sylvilagus brasiliensis* had one apomorphic alteration, $I_{185}L$. The genus *Ochotona* had seven apomorphic amino acids changes, $N_{101}S$, $K_{110}Q$, $V_{177}I$, $S_{178}K$, $A_{180}V$, $R_{183}K$ and $V_{196}L$. With the exception of the amino acid changes in *Oryctolagus* and in *L. americanus*, all the other changes affect exposed regions of the protein. In contrast, the distribution of the nucleotide polymorphisms is more uniform.

Phylogenetic relationships

A neighbour-joining tree for CXCR4 nucleotide sequences was constructed using MEGA3.1 software, option p-distance and complete deletion (Fig. 2). The tree was constructed using the nucleotide sequences because of the low degree of polymorphisms in the amino acid sequences. The family Ochotonidae forms a clearly separated group apart from the family Leporidae. The genera *Oryctolagus* and *Bunolagus* form a well-supported group, which is more closely related to the genus *Lepus* than to the genus *Sylvilagus*.

Discussion

CXCR4 was reported as the most evolutionary conserved of all CXCRs (Murphy, 1993; Huising *et al*., 2003; Lio & Vannucci, 2003) and despite the redundancy within the chemokine system, it has only one ligand. Its wide range of functions and wide variety of tissues where it is expressed seem to be implicated in this specificity and conservative status. For example, mice lacking CXCR4 or SDF-1 die perinatally and show profound defects in the haematopoietic and nervous systems (Ma *et al*., 1998).

As expected, the characterization of CXCR4 in lagomorphs showed that genetic diversity at this locus is very limited. More in particular, for the seven transmembrane domains (157 amino acids) no variation was found among lineages that have evolved independently for more than 35 myr. Only one polymorphism was recorded for these regions in an individual sample of *Lepus americanus* ($L_{165}F$). Pikas appear as the most divergent group compared to the mammalian consensus, with two substitutions in ECL1 and eight substitutions in ECL2. Also for the other species, most changes, when occurring, affect ECL regions. These observations suggest that the internal regions (ICL and TM) of the CXCR4 protein are under strong purifying selection. *Oryctolagus* appears as a notable exception, where both ICL1 and ICL2 show amino acids replacements, respectively, $L_{69}Q$ and $R_{146}K$. Among lagomorphs, these replacements are unique to rabbit. They are apparently apomorphic mutations, although the former is also present in chicken and the latter in rhesus monkey and in cattle (Fig. 1).

In a recent study on CCR5 in rabbit, a remarkable gene conversion was described between this gene and CCR2. A specific peptide motif of the second extracellular loop of the CCR5 protein is replaced by a motif that in other species characterizes the CCR2 protein (Carmo *et al*., 2006). Here we report two amino acid changes in intracellular domains of the CXCR4 protein. The intracellular regions are known to be involved in signal transduction that influences patterns of cellular immune response and causes cell death (Vlahakis *et al*., 2002; Roland *et al*., 2003).

The clinical symptoms that follow the myxoma infection observed among susceptible lagomorph hosts are not the same. In the members of *Sylvilagus* species the infection is restricted to the inoculation site and results in the formation of skin tumours but no mortality has been observed. In European rabbits the infection is much more severe and reaches high mortality rates. Following the infection, myxoma virus replicates in class II MHC^+ cells with a

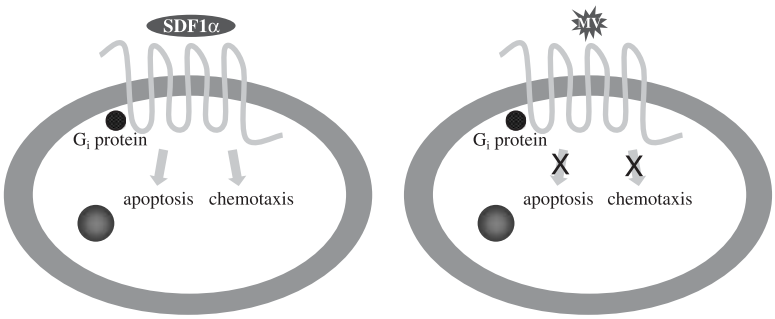

Figure 3. Possible signalling pathways affected by the use of the CXCR4 protein by myxoma virus (MV) to infect cells.

dendritic shape and then in T lymphocytes of the draining lymph node that probably disseminate the virus through the body (Zúñiga, 2002).

The different outcome between *Sylvilagus* and *Oryctolagus* strongly suggests the existence of species-specific differences at genes involved in the acquired and innate immune response. The use of chemokine receptors by myxoma virus may also interfere in the type of immune response since chemokines and chemokine receptors can regulate leucocyte trafficking, angiogenesis and inflammation. There is clear evidence of a different cellular immune responses in rabbits that died from myxomatosis compared to those that overcome the infection (Best & Kerr, 2000a) and apoptosis only occurs in cells with no detectable viral antigen but adjacent to infected cells (Best *et al*., 2000b). The intracellular pathways and the cellular messengers activated after the infection are still unknown but the possibility exists that the unique features found in the intracellular domains of the *Oryctolagus* CXCR4 protein (Fig. 3) may somehow be involved in the different course of the disease. Functional studies should evaluate the possible effect of these alterations.

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