



**UBIQUITINATION AND DEUBIQUITINATION
IN THE MAMMALIAN PEX5-MEDIATED
PEROXISOMAL PROTEIN IMPORT PATHWAY**

CLÁUDIA PATRÍCIA OLIVEIRA GROU

Tese de doutoramento em Ciências Biomédicas

2009

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PEROXISOMAL PROTEIN IMPORT PATHWAY**

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas, submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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PRECEITOS LEGAIS

Nesta tese foram utilizados os resultados dos trabalhos publicados abaixo indicados. A autora desta dissertação declara que interveio na concepção e execução do trabalho experimental, na interpretação de resultados e na redacção dos resultados publicados, sob o nome de “**Grou, C. P.**”.

The published experimental results stated below were used in this thesis. The author of this thesis declares that she participated in the planning and execution of the experimental work, in the data interpretation and in the preparation of the published results, under the name “**Grou, C. P.**”.

Grou, C. P., Carvalho, A. F., Pinto, M. P., Huybrechts, S. J., Sá-Miranda, C., Fransen, M. and Azevedo, J. E. (2009). Properties of the ubiquitin-pex5p thiol ester conjugate. J. Biol. Chem. 284, 10504-13.

Grou, C. P., Carvalho, A. F., Pinto, M. P., Wiese, S., Piechura, H., Meyer, H. E., Warscheid, B., Sá-Miranda, C., and Azevedo, J. E. (2008). Members of the E2D (UbcH5) family mediate the ubiquitination of the conserved cysteine of Pex5p, the peroxisomal import receptor. J. Biol. Chem. 283, 14190-7.

Este trabalho foi financiado pela Fundação para a Ciência e Tecnologia (FCT) através de uma bolsa de Doutoramento (SFRH/BD/29847/2006), pelo programa PTDC/BIA-BCM/64771/2006, pelo Fundo Europeu de Desenvolvimento Regional, Portugal, e por uma bolsa de Doutoramento (FRAMEWORK-PR221402-1-BD) atribuída no contexto do projecto financiado pelo European Union VI Framework program (Grant LSHGCT-2004-512018, Peroxisomes in Health and Disease).

This work was supported by Fundação para a Ciência e Tecnologia through a PhD fellowship (SFRH/BD/29847/2006), by the PTDC/BIABCM/64771/2006 program, by the Fundo Europeu de Desenvolvimento Regional, Portugal, and by a PhD fellowship (FRAMEWORK-PR221402-1-BD) financed by the European Union VI Framework program (Grant LSHG-CT-2004-512018, Peroxisomes in Health and Disease).

AGRADECIMENTOS/ACKNOWLEDGMENTS

Gostaria de aproveitar esta oportunidade para agradecer a todos aqueles que me apoiaram durante os últimos quatro anos.

Em primeiro lugar agradeço ao Prof. Doutor Jorge Azevedo por me ter aceite no seu laboratório, por ter sido o principal promotor e ter participado activamente no desenvolvimento da minha “veia Bioquímica”. A finalização deste projecto não teria sido possível sem a sua dedicação e excelente orientação.

À Doutora Clara Sá-Miranda agradeço pela sua amizade, apoio e preocupação em proporcionar as melhores condições de trabalho possíveis.

Queria agradecer a todos os elementos que fazem ou fizeram parte do grupo OBF com quem aprendi e tive o prazer de trabalhar: Carla, João, Andreia, Manel, Inês, Tony, Marta e Tânia. Apesar de ter partilhado por pouco tempo a bancada comigo, a Carla deixou saudade. Ao João gostaria de agradecer por me ter torrado a paciência sempre que me tratava por “Dra. C. P. Grou”. À Andreia agradeço os bons momentos passados nessas maratonas laboratoriais nocturnas, a sua contribuição para esta tese e por ter suportado ouvir (por vezes intensivamente...) David Fonseca. À Inês pela sua preocupação. Ao Tony agradeço-lhe pelo seu humor refrescante e muito peculiar. Agradeço à Marta por, aquando do processo de escrita, me perguntar diariamente quantas páginas tinha escrito. Para a Tânia, a última aquisição do grupo, terei de fazer um agradecimento especial (porque ela assim o exigiu!). Agradeço-lhe pela energia permanente com que passa os dias, pela constante disponibilidade e preocupação. É como uma mãe mas “em ponto pequeno”. Com ela sei que “estamos cá uns para os outros”.

Finalmente, agradeço ao Manel por me fazer abrandar o ritmo, reflectir e analisar o que faço. Obrigado pela preciosa ajuda sempre que era necessário preparar modelos ou figuras em PowerPoint! Estou-lhe sobretudo grata por ter estado sempre ao meu lado e me dar ânimo se eventualmente as coisas não corriam muito bem.

Não posso deixar agradecer aos ex- e actuais membros da UniLipe e LBG pela sua amizade e boa disposição: à Lorena pelas gargalhadas contagiantes; ao Daniel que nos primórdios do Doutoramento me ajudou com a medição de actividades enzimáticas; à Andrea pela dica de sobrevivência “Perguntar a quem sabe!”; ao Paulo pelas intervenções musicais sempre oportunas; à Inês M.; ao Ricardo; à Margaretta (unidas pelo

nervosismo que antecedeu as nossas apresentações no *IBMC Retreat*), à minha homónima Cláudia B.; à Vânia C., “a loira” (mas no bom sentido).

À Maria João, “uma menina cujo coração bate mais rápido que o das outras pessoas” e vaporizadora oficial de placas de TLC, que teve de me aturar no laboratório e durante os 2 últimos anos também em casa. Obrigado também pela rápida, mas muito clara, explicação sobre formatação de cabeçalhos e rodapés.

Ao Frederico queria agradecer a sua ajuda e paciência sempre que os sistemas cromatográficos da UP3 resolviam não colaborar comigo (certamente teriam defeito!). Obrigado por poder contar com a tua confiança e amizade.

Agradeço ao Miguel Pais Vieira pela sua “boa onda, 100% cool”!

I am very grateful to Sofie Huybrechts and Dr. Marc Fransen for conducting the *in vivo* studies presented in this thesis.

Agradeço ainda a European Union VI Framework Program (Grant LSHG-CT-2004-512018, Peroxisomes in Health and Disease) e à Fundação para a Ciência e Tecnologia pelas bolsas de Doutoramento atribuídas (FRAMEWORK-PR221402-1-BD e SFRH/BD/29847/2006, respectivamente), e à Fundação Calouste Gulbenkian, *FEBS* e *EMBO* pelo apoio financeiro para participação em congressos científicos.

Às minhas *mui* grandes amigas R(rrr)aquele e Dri agradeço-lhes a amizade constante (mesmo à distância!).

Por último resta-me agradecer aos membros do “clã Grou-Claro-Martinho” residentes nessas belas localidades de Pelicanos e Porto Carvalho. Um agradecimento especial à minha irmã pelas tiradas humorísticas oportunas e sempre inesperadas que me fazem rir. Este trabalho é particularmente dedicado aos meus pais que nunca se queixaram das horas a que me iam buscar ou levar ao comboio, que sempre me apoiaram onde quer que eu me encontrasse. Espero que sintam orgulho neste trabalho, pois vocês contribuíram de uma forma determinante para a sua conclusão e sucesso (espero!).

A TODOS MUITO OBRIGADO!!

ABSTRACT/RESUMO/RÉSUMÉ

Abstract

PEX5, the peroxisomal cycling receptor, plays a key role in the import of matrix proteins. According to current models, newly synthesized proteins are recognized by PEX5 in the cytosol and are then targeted to the organelle membrane. There, strong protein-protein interactions between PEX5 and components of the docking/translocation machinery are established resulting in the insertion of PEX5 into the docking/translocation machinery with the concomitant translocation of cargo proteins across the organelle membrane. PEX5 is then exported back into the cytosol by an ATP-dependent machinery to promote further cycles of protein transportation. Monoubiquitination of mammalian PEX5 at a conserved N-terminal cysteine residue was recently uncovered and found to be a mandatory event for the export step. This phenomenon has also been described for yeast PEX5 and shown to be mediated by PEX4, a specialized ubiquitin-conjugating enzyme (E2), anchored to the peroxisomal membrane through PEX22. As the mammalian PEX4 functional counterpart was not known, we set up a protocol to isolate E2s from rat liver, coupled with a peroxisome-dependent PEX5-ubiquitination assay. We have partly purified the PEX4-like activity. Five rat liver E2s candidates were identified by mass spectrometry analysis: E2L3 (UbchH7), E2N (UbchH13), and the three closely related E2s E2D1 (UbchH5a), E2D2 (UbchH5b), and E2D3 (UbchH5c). Subsequent work in our laboratory using human recombinant versions of the five E2s identified in this study revealed that E2D1/2/3 (UbchH5a/b/c) are indeed the mammalian functional counterparts of yeast PEX4.

PEX5 monoubiquitination at its N-terminal conserved cysteine residue is rather intriguing as this type of ubiquitination seems to be quite rare. Data on protein thiolester conjugates are scarce and its relevance in this peculiar protein traffic pathway unknown. The properties of docking/translocation machinery-embedded and soluble monoubiquitinated PEX5 species (Ub-PEX5) are described in this study. We show that Ub-PEX5 is deubiquitinated by a combination of context-dependent enzymatic and non-enzymatic mechanisms. Soluble Ub-PEX5 retains the capacity of nonmodified PEX5 to interact with the docking/translocation machinery in a cargo-dependent manner. Furthermore, the replacement of the conserved cysteine residue by a lysine yielded a functional protein both *in vitro* and *in vivo*. Altogether these findings indicate that neither the thiolester bond nor the cysteine are crucially important for those steps occurring at the peroxisomal membrane. The use of unconventional cysteine-based ubiquitination instead of the classical lysine-based one might be related to the chemical lability of soluble Ub-PEX5 thiolester conjugate, as it can be easily disrupted by physiological nucleophilic compounds. Consequently, its half-life is shortened, thereby decreasing the probability of

being degraded by the proteasome. Alternatively, some regulatory pathway that relies on cysteine susceptibility to be modified in numerous ways in response to cellular metabolic states may also justify why PEX5 N-terminal cysteine residue is evolutionary conserved.

We believe that the findings reported in this study are an important contribution not only for the understanding of peroxisomal biogenesis, but also for the ubiquitination research field.

Resumo

A PEX5, o receptor peroxissomal cíclico, desempenha um papel fulcral na importação de proteínas matriciais. De acordo com os modelos vigentes, proteínas peroxissomais recém sintetizadas são reconhecidas pela PEX5 no citosol e direccionadas por esta para a membrana do organelo. Na membrana peroxissomal, fortes interações proteína-proteína estabelecidas entre a PEX5 e os componentes da maquinaria de *docking*/translocação resultam na sua inserção na maquinaria de *docking*/translocação e translocação concomitante da proteína transportada através da membrana peroxissomal. A PEX5 é exportada para o citosol por uma maquinaria dependente de ATP para promover mais ciclos de transporte proteico. A monoubiquitinação do resíduo de cisteína conservado presente no N terminus da PEX5 foi recentemente descoberta em mamíferos e demonstrada ser obrigatória para a exportação da PEX5. Em levedura este fenómeno foi também descrito e foi demonstrado ser mediado pela PEX4, uma enzima conjugadora de ubiquitina (E2) especializada, ancorada à membrana peroxissomal pela PEX22. Visto que a homóloga funcional da PEX4 em mamíferos não era conhecido, foi desenvolvido um protocolo para isolar E2s a partir de citosol de fígado de rato. A actividade da homóloga funcional da PEX4 foi parcialmente purificada. A análise por espectrometria de massa identificou cinco E2s candidatas de fígado de rato: E2L3 (UbchH7), E2N (UbchH13), e as três E2s intimamente relacionadas E2D1 (UbchH5a), E2D2 (UbchH5b), e E2D3 (UbchH5c). Trabalho desenvolvido posteriormente no nosso laboratório utilizando proteínas humanas recombinantes correspondentes às cinco E2s identificadas neste estudo revelou que as proteínas E2D1/2/3 (UbchH5a/b/c) são as homólogas funcionais da PEX4 em levedura.

A monoubiquitinação do resíduo de cisteína conservado N-terminal da PEX5 é um fenómeno interessante, visto que este tipo de ubiquitinação é aparentemente raro. Dados sobre proteínas conjugadas via uma ligação tiol-éster são escassas. A sua relevância nesta via de transporte proteico é desconhecida. Neste estudo são descritas as propriedades das espécies monoubiquitinadas da PEX5 (Ub-PEX5) inseridas na maquinaria de *docking*/translocação e solúveis. É mostrado que Ub-PEX5 é desubiquitinada por uma combinação de mecanismos enzimáticos e não enzimáticos que actuam diferencialmente consoante a localização do conjugado ubiquitinado. A Ub-PEX5 solúvel retém a capacidade da PEX5 não modificada de interagir, de uma forma dependente de proteínas transportadas, com a maquinaria de *docking*/translocação. Mais ainda, a substituição do resíduo de cisteína conservado por uma lisina origina uma proteína funcional *in vitro* e *in vivo*. Todos estes dados indicam que nem a ligação tiol-éster, nem a cisteína são particularmente relevantes para os passos que ocorrem na

membrana peroxissomal. A utilização de uma ubiquitinação não convencional de cisteínas em vez da ubiquitinação clássica de lisinas poderá estar relacionada com a labilidade química do conjugado tiol-éster Ub-PEX5 solúvel, dado que este pode ser facilmente destruído por compostos nucleofílicos fisiológicos. Consequentemente, a semi-vida destes conjugados é encurtada, diminuindo a possibilidade de serem degradados pelo proteossoma. Por outro lado, a conservação evolutiva da cisteína N-terminal da PEX5 pode também estar relacionada com uma via de regulação baseada na susceptibilidade destes resíduos a várias modificações em resposta a estados metabólicos celulares.

Crê-se que os dados apresentados neste estudo constituem uma contribuição importante não só para a compreensão da biogénese peroxissomal, mas também para o campo de investigação da ubiquitinação.

Résumé

PEX5, un récepteur peroxysomal, joue un rôle clé dans l'importation des protéines de la matrice. D'après des modèles récents, les protéines peroxysomales néo-synthétisées sont reconnues par PEX5 dans le cytosol et sont ensuite adressées à la membrane de l'organelle. À ce niveau de fortes interactions protéine-protéine sont établies entre PEX5 et les composants du complexe d'arrimage/translocation résultant en son ancrage dans le complexe de translocation et la translocation simultanée des protéines cargo, liées à PEX5, à travers la membrane peroxisomale. PEX5 est par la suite exporté de nouveau dans le cytosol par un complexe ATP-dépendant afin d'entamer de nouveaux cycles de importation. La monoubiquitination de PEX5 de mammifères sur un résidu cystéine N-terminal a été récemment mise en évidence comme étant un événement indispensable à l'exportation de PEX5. Ce phénomène a aussi été décrit chez la levure où la monoubiquitination de PEX5 est médié par PEX4, enzyme spécialisée conjuguant l'ubiquitine (E2), ancrée dans la membrane peroxysomal par PEX22. Étant donné que l'homologue fonctionnel de PEX4 chez les mammifères est inconnu, nous avons développé un protocole pour isoler E2 de foie de rat couplé avec un essai d'ubiquitination de PEX5 dépendant du peroxysome. Nous avons partiellement purifié l'activité *PEX4-like*. Cinq candidats E2s de foie de rat ont été identifiés par analyse de spectrométrie de masse: E2L3 (UbchH7), E2N (UbchH13) et trois proches de E2s, E2D1 (UbchH5a), E2D2 (UbchH5b), et E2D3 (UbchH5c). Des travaux subséquents menés dans le laboratoire en utilisant des versions humaines recombinantes de cinq E2s identifiées dans cette étude ont révélé que E2D1/2/3 (UbchH5a/b/c) sont effectivement les homologues de la PEX4 de levure chez les mammifères.

La monoubiquitination de PEX5 sur son résidu cystéine N-terminal est intrigante car ce type d'ubiquitination semble rare. Les données sur des conjugués protéiques thio-ester sont rares et leur rôle dans ce type de voie de transport est inconnu. Les propriétés du complexe d'arrimage/translocation et des espèces solubles PEX5 monoubiquitinylées (Ub-PEX5) sont décrites dans cette étude. On montre que Ub-PEX5 est dé-ubiquitinylé par une combinaison de mécanismes enzymatiques et non enzymatiques dépendants du contexte. L'Ub-PEX5 soluble retient la capacité de PEX5 non modifié d'interagir avec le complexe d'arrimage/translocation dépendant du cargo. De plus, la substitution du résidu cystéine par un lysine a originé une protéine fonctionnelle à la fois *in vitro* et *in vivo*. Dans l'ensemble ces résultats indiquent que ni le lien thio-ester ni le résidu cystéine sont particulièrement importants pour les étapes qui se produisent dans la membrane peroxisomale. L'utilisation d'une ubiquitination non conventionnelle sur le résidu cystéine, au lieu de l'ubiquitination classique sur le résidu lysine peut être due à la labilité chimique

du conjugué formé par l'Ub-PEX5 et le thiol-ester, puisqu'il peut être facilement rompu par des composants physiologiques nucléophiles. Par conséquent la demi-vie de ce conjugué est courte, réduisant ainsi la probabilité d'être dégradée par le protéasome. Alternativement, quelques voies de régulation basées sur la susceptibilité de la cystéine à être modifiée en réponse à des états métaboliques de la cellule peuvent aussi justifier le fait que le résidu cystéine N-terminal de PEX5 est été conservé au long de l'évolution.

Nous pensons que les résultats reportés dans cette étude apportent une contribution importante dans la compréhension de la biogenèse peroxisomal et ainsi que dans le domaine de recherche de l'ubiquitination.

ABBREVIATIONS

AAA	ATPases associated with diverse cellular activities
ATP γ S	Adenosine 5'-O-(thiotriphosphate)
BSA	Bovine serum albumin
cDNA	Complementar deoxyribonucleic acid
CHO	Chinese hamster ovary
Cys11	Cysteine residue at position 11 of mammalian PEX5
DTM	Docking/translocation machinery
DTT	Dithiothreitol
DEAE	Diethylaminoethyl
DUB	Deubiquitinating enzyme
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
E4	Ubiquitin-chain elongation factor
E-64	N-(<i>trans</i> -epoxysuccinyl)-L-leucine 4-guanidinobutylamide
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
GSH	Glutathione
GST	Glutathione-S-transferase
HECT	Homologous to the E6-AP carboxyl terminus
IAA	Iodoacetamide
IgG	Immunoglobulin
IRD	Infantile Refsum disease
MOPS	4-morpholinepropanesulfonic acid
mPTS	Membrane peroxisomal targeting sequence
MWCO	Molecular weight cut-off
NALD	Neonatal adrenoleukodystrophy
NEM	N-ethylmaleimide
PAGE	Polyacrylamide gel electrophoresis
PBD	Peroxisomal biogenesis disorders
PEG	Polyethylene glycol
PES	Polyethersulfone
PEX	Peroxin

PIM	Peroxisomal import machinery
PMP	Peroxisomal membrane protein
PMSF	Phenylmethylsulfonyl fluoride
PNS	Postnuclear supernatant
PTS1	Peroxisomal targeting sequence type 1
PTS2	Peroxisomal targeting sequence type 2
RCPD	Rhizomelic chondrodysplasia punctata
REM	Receptor export module
RING	Really interesting new gene
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SDS	Sodium dodecylsulfate
SEM	Buffer containing sucrose, ethylenediaminetetraacetic acid and 4-morpholinepropanesulfonic acid
SH3	Src homology 3 domain
SIE	Buffer containing sucrose, imidazole and ethylenediaminetetraacetic acid
STI	Soybean trypsin inhibitor
TCA	Trichloroacetic acid
TPR	Tetratricopeptide repeat
Tris	Tris(hydroxymethyl)aminomethane
Ub	Ubiquitin
Ubal	Ubiquitin aldehyde
Ub-PEX5	Monoubiquitinated PEX5 species
UCC	Ubiquitin-conjugating cascade
WD	Tryptophan-aspartate repeat
X-ALD	X-linked adrenoleukodystrophy
ZS	Zellweger syndrome

I. INTRODUCTION

1 - Peroxisome structure and function

Peroxisomes are nearly ubiquitous organelles within eukaryotic cells. Rodhain first identified these structures as microbodies [1], but due to their conserved hydrogen peroxide-based metabolism, de Duve and Baudhuin later introduced the name “peroxisomes” [2]. In sharp contrast to mitochondria and chloroplasts, peroxisomes possess a finely granular proteinaceous matrix devoid of DNA, surrounded by a single membrane. Their abundance, size and morphology may vary according to the organism, tissue, cell type and environmental conditions. Typically peroxisomes present themselves as nearly spherical structures ranging from 0.1-1 μm in diameter [3-5]. However, they can also form elongated, tubular structures and tubular-reticular networks [6].

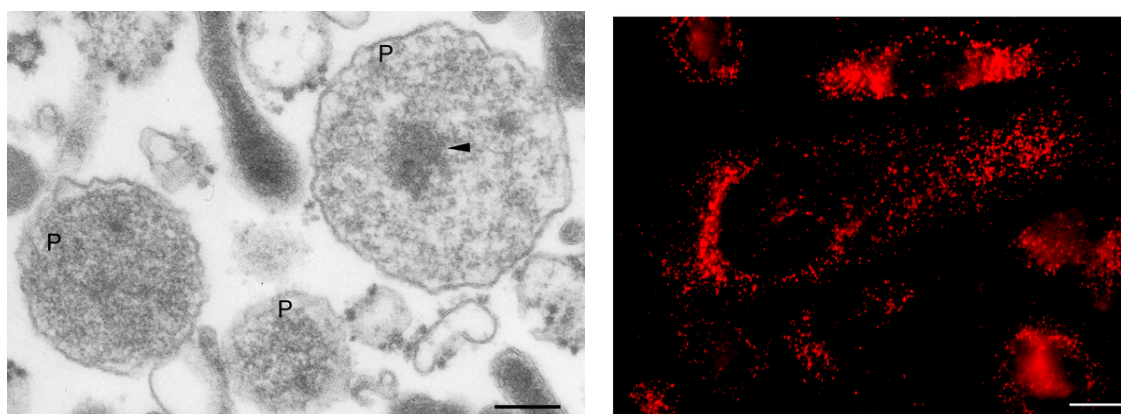


Figure 1 - Peroxisomes. An electron micrograph of peroxisomes (*P*) in an organelle pellet from a rat liver postnuclear supernatant. Crystalline cores of urate oxidase (*arrowhead*) are frequently observed in rodent hepatic peroxisomes [7]. *Scale bar*, 0.2 μm (*left panel*; kindly taken by Prof. Dr. Manuel Teixeira da Silva, IBMC, Porto, Portugal). Chinese Hamster Ovary (CHO) cells stained with an antibody against PEX14, a peroxisomal membrane protein, show the presence of peroxisomes throughout the cytoplasm with a typically punctate distribution pattern. *Scale bar*, 10 μm (*right panel*; from [8]).

Peroxisomes are involved in a wide range of metabolic pathways, many of which tissue- and organism-specific. Generation and disposal of hydrogen peroxide [2,9] and β -oxidation of fatty acids [10,11] are their core activities. In lower eukaryotic organisms (including yeast) and plants, β -oxidation of fatty acids is carried out exclusively by peroxisomes [12,13], whereas in mammals this function is shared with mitochondria [10,14]. In contrast, α -oxidation of fatty acids is only fulfilled by peroxisomes [15]. Mammalian peroxisomes are also involved in the catabolism of purines, polyamines (spermine and spermidine) and D-amino acids [16,17]. The biosynthesis of plasmalogens is accomplished by the cooperation between peroxisomes and the endoplasmic reticulum (ER) [18,19]. Mammalian and plant peroxisomes have been implicated in cellular

signalling due to their involvement in the metabolism of oxygen free radicals and nitric oxide [20-24]

The metabolic diversity displayed is often reflected in peroxisomal specializations. Plants have glyoxysomes (responsible for the glyoxylate cycle) [25]; in trypanosomes part of the glycolytic pathway occurs in glycosomes [26,27]; and filamentous ascomycetes (e.g. *Neurospora crassa*) contain Woronin bodies (structures that seal septal pores in response to cellular damage) [28].

2 - Peroxisomal disorders

For many years peroxisomes were considered the cell's dust bin and their importance was underestimated. However, if one considers their widespread distribution and participation into a multitude of essential biochemical and metabolic processes, it is not surprising that their malfunction or absence has severe consequences. Indeed, the relevance of peroxisomes in human development and health is highlighted by the existence of a group of rare inherited diseases with profound phenotypic consequences. Peroxisomal disorders are subdivided into two major categories: single enzyme deficiencies and peroxisomal biogenesis disorders (PBDs) [29-31].

Disorders within the first group can be further organized according to the metabolic pathway affected [30] (Table 1). X-linked adrenoleukodystrophy (X-ALD) is the prototype and most common single enzyme peroxisomal disorder [32,33].

Table 1 - Peroxisomal single enzyme deficiencies

Metabolic pathway affected	Peroxisomal disease	Enzyme defect
Ether phospholipid synthesis	Rhizomelic chondrodysplasia punctata Type 2	DHAP acyltransferase
	Rhizomelic chondrodysplasia punctata Type 3	Alkyl-DHAP synthase
Peroxisomal β -oxidation	X-linked adrenoleukodystrophy	Adrenoleukodystrophy protein
	Acyl-CoA oxidase deficiency	Acyl-CoA oxidase-1
	D-bifunctional protein deficiency	D-bifunctional protein
	2-Methylacyl-CoA racemase deficiency	2-Methylacyl-CoA racemase
	Sterol carrier protein X deficiency	Sterol carrier protein X
Peroxisomal α -oxidation	Refsum disease	Phytanoyl-CoA hydroxylase
Glyoxylate detoxification	Hyperoxaluria Type 1	Alanine:glyoxylate aminotransferase
H ₂ O ₂ -metabolism	Acatalsaeemia	Catalase

DHAP, dihydroxyacetone phosphate (adapted from [30]).

PBDs, contrarily to the single enzyme deficiencies group, present multiple functions affected, a consequence of defects in the assembly of the organelle. PBDs comprise a genetically heterogeneous group of autosomal recessive disorders that can be classified into two distinct clinical categories: the Zellweger spectrum and rhizomelic chondrodysplasia punctata (RCDP) type I [31,34]. Patients within the Zellweger spectrum fall into three groups distinguishable by their clinical severity: Zellweger syndrome (ZS; most severe), neonatal adrenoleukodystrophy (NALD; intermediate severity) and infantile Refsum disease (IRD; mildest). Patients with ZS rarely survive to their first year, whereas IRD patients reach childhood or even young adulthood. Survival in RCDP type I patients is also variable [35]. Although some RCDP type I clinical phenotypes overlap those of Zellweger spectrum, these patients present unique biochemical and clinical features. In fact, this disorder involves mutations in one single gene (*PEX7*) [36], whereas the Zellweger spectrum can be caused by mutations in any of 12 different genes [29] (Table 2).

Different experimental approaches including complementation analysis, functional cloning and homology probing were successfully used to assign the 13 presently known genes causative of human PBDs (reviewed in [29,37]; see also Table 2).

Table 2 - PEX gene defects and complementation groups in PBD

Gene	CG-Dutch	CG-Japan	CG-KKI	Clinical phenotypes
<i>PEX1</i>	2	E	1	ZS, NALD, IRD
<i>PEX2</i>	5	F	10	ZS, IRD
<i>PEX3</i>		G	12	ZS
<i>PEX5</i>	4		2	ZS, NALD
<i>PEX6</i>	3	C	4,6	ZS, NALD, IRD
<i>PEX7</i>	1	R	11	RCDP1
<i>PEX10</i>		B	7	ZS, NALD
<i>PEX12</i>			3	ZS, NALD, IRD
<i>PEX13</i>		H	13	ZS, NALD
<i>PEX14</i>		K		ZS
<i>PEX16</i>		D	9	ZS
<i>PEX19</i>		J	14	ZS
<i>PEX26</i>		A	8	ZS, NALD, IRD

CG, complementation group; Dutch, group at University of Amsterdam; Japan, group at Gifu University School of Medicine; KKI, Kennedy Krieger Institute; ZS, Zellweger syndrome; NALD, neonatal adrenoleukodystrophy; IRD, infantile Refsum disease; RCDP1, rhizomelic chondrodysplasia punctata type 1 (adapted from [29]).

3 - Peroxisomal biogenesis

Peroxisome assembly and maintenance requires the concerted action of a set of essential proteins, the peroxins (PEXn) [38]. Mutations in genes encoding peroxins (*PEX* genes) may abrogate partial- or completely their function, leading to the aforementioned PBDs.

Peroxins can be grouped based on their particular function in peroxisomal biogenesis, *i.e.* peroxisomal membrane assembly, import of matrix proteins, and growth and division. Thirty two peroxins have been identified among the studied organisms (reviewed in [39,40]). However, only 16 mammalian homologs have been found. It should be underscored the evolutionary conservation of most peroxins (see Ref. 41 and Table 3). Please note that in some cases a peroxin may not have been conserved, but its function was. This is the case of PEX18/PEX21 (*Saccharomyces cerevisiae*) and PEX20 (other yeasts and fungi) (see also section 3.2, *Introduction*), whose roles have been embraced by PEX5 in plants and mammals. Still, it is remarkable how the mechanistic features of peroxisomal biogenesis have been conserved throughout evolution from fungi to mammals [40,41].

Since peroxisomes contain no DNA, peroxisomal proteins are encoded by nuclear DNA, synthesized on free polyribosomes and targeted posttranslationally into the organelle by cytosolic receptors which recognize their targeting sequences [5,42-44].

Distinct machineries are devoted to the sorting of membrane and matrix proteins to peroxisomes [39,41,45]. Details on the two independent peroxisomal protein import pathways will be presented in sections 3.1 and 3.2.

The peroxisomal division machinery is beyond the scope of this thesis and will not be addressed, but for recent reviews on this topic see Refs. 46 and 47.

3.1 - Peroxisomal membrane biogenesis

A longstanding controversial issue far from being solved concerns the origin of peroxisomes. Do peroxisomes multiply by growth and division as autonomous organelles or do they arise from the ER? The majority of the published data favours the growth and division model [42,48-50] with the ER providing the lipids. Mitochondria [51] and lipid droplets [52] have also been suggested to be the source of peroxisomal lipids. Thereby it is generally accepted that peroxisomal membrane proteins (PMPs) route directly to peroxisomes via *cis*-acting targeting signals, the membrane peroxisomal targeting signals (mPTSs). In contrast to targeting signals of peroxisomal matrix proteins (see section 3.2, *Introduction*), mPTSs are not defined by a specific amino acid sequence but rather by

amino acid physicochemical properties and structural elements (see Ref. 53 and reviewed in [54]). Presently PEX3 and PEX16 are the only PMPs reported to be trafficked through the ER but only in cells lacking peroxisomes where their respective encoding-genes have been introduced [55,56].

The assembly of peroxisomal membrane appears to require a rather simple machinery comprising only three peroxins in mammals: PEX3, PEX16, intrinsic proteins of the peroxisomal membrane, and PEX19, an acidic mainly cytosolic protein. PEX3 and PEX19 are proposed to be the key players in the sorting of PMPs [57], whereas the role of PEX16 in peroxisomal biogenesis remains obscure [58].

Several studies favour that PEX19 acts as a cytosolic receptor/chaperone for PMPs [8,59-62]. PEX19 can bind a broad range of newly synthesized PMPs [8,61-66]. Furthermore, PEX19 was shown to interact with PMPs during or immediately after their translation, preventing them from aggregating by probably shielding its hydrophobic domains, thereby keeping them in an import-competent status and directing them to the peroxisomal membrane [8,66]. Currently it is accepted that PEX3 is the docking factor for PEX19-PMP complexes at the peroxisomal membrane [67,68]. In fact, cargo-loaded PEX19 displays higher binding affinity towards PEX3 [8].

Despite the simplicity of the PMP sorting machinery, mechanistic details on the docking/insertion of PMPs at/into the peroxisomal membrane are still scarce.

3.2 - Peroxisomal matrix protein sorting

Peroxisomes confine within their membrane boundaries more than 50 enzymes that are imported posttranslationally [5,17].

Sorting of proteins into the peroxisomal matrix depends on peroxisomal targeting signals (PTS) which are specifically recognized by receptors. The vast majority of matrix proteins contains a PTS1, the tripeptide (S/A/C)-(K/R/H)-(L/M), located at their extreme C terminus [69-71]. The PTS1 has been further extended to the eight residues upstream the tripeptide as they might influence the receptor affinity to the cargo protein in a species-specific manner ([72] and reviewed in Ref. 73). A few matrix proteins are targeted by a distinct signal, the PTS2, an N-terminal degenerated nonapeptide complying the consensus sequence (R/K)-(L/V/I)-(X)₅-(H/Q)-(L/A) [74,75], often cleaved at the peroxisomal matrix [74,76,77].

Table 3 - Proteins implicated in peroxisomal biogenesis (Peroxisins)

Peroxin	Organisms	Localization	Domains	Proposed function
PEX1	M, P, F, Y	cytosol/membrane	AAA ATPase	Matrix protein import, export of PEX5
PEX2	M, P, F, Y	membrane	Zinc RING finger	Matrix protein import
PEX3	M, P, F, Y	membrane		Membrane biogenesis, PMP import
PEX4	P, F, Y	cytosol/membrane	E2 Ubiquitin-conjugating enzyme	Matrix protein import, ubiquitination of PEX5
PEX5^a	M, P, F, Y	cytosol/membrane	Natively unfolded domain, TPRs	Matrix protein import, PTS1 (and PTS2 in M, P) receptor
PEX6	M, P, F, Y	cytosol/membrane	AAA ATPase	Matrix protein import, export of PEX5
PEX7	M, P, F, Y	cytosol/membrane	WD repeats	Matrix protein import, PTS2 receptor
PEX8	F, Y	matrix/membrane		Matrix protein import
PEX9	YI	(ORF misidentified, antisense sequence of PEX26)		
PEX10	M, P, F, Y	membrane	Zinc RING finger	Matrix protein import
PEX11^b	M, P, F, Y	membrane		Division and proliferation
PEX12	M, P, F, Y	membrane	Zinc RING finger	Matrix protein import
PEX13	M, P, F, Y	membrane	SH3	Matrix protein import
PEX14	M, P, F, Y	membrane	Coiled-coil	Matrix protein import
PEX15	Sc	membrane		Matrix protein import, PEX1/PEX6 anchor
PEX16	M, P, F, YI	membrane		Membrane biogenesis
PEX17	Y	membrane	Coiled-coil	Matrix protein import
PEX18	Sc	cytosol/membrane		Matrix protein import, PTS2 import
PEX19	M, P, F, Y	cytosol/membrane	Farnesylation motif	Membrane biogenesis, PMP import
PEX20	F, Y	cytosol/membrane		Matrix protein import, PTS2 import
PEX21	Sc	cytosol/membrane		Matrix protein import, PTS2 import
PEX22	P, F, Y	membrane		Matrix protein import, PEX4 anchor
PEX23	F, Y	membrane	Dysferlin	Proliferation
PEX24	F, Y	membrane		Proliferation
PEX25	Y	membrane		Proliferation
PEX26	M, F, Y ^c	membrane		Matrix protein import, PEX1/PEX6 anchor
PEX27	Sc	membrane		Proliferation
PEX28	Sc	membrane		Proliferation (PEX24 ortholog)
PEX29	Y	membrane		Proliferation
PEX30	Sc	membrane	Dysferlin	Proliferation (PEX23 ortholog)
PEX31	Sc	membrane	Dysferlin	Proliferation
PEX32	Y	membrane	Dysferlin	Proliferation

M, mammals; P, plants; F, filamentous fungi; Y, yeasts; YI, *Yarrowia lipolytica* only; Sc, *Saccharomyces cerevisiae* only; ^aMammals contain two main isoforms, PEX5S and PEX5L, the latter harbouring a PEX7-binding site; ^bMammalian cells contain three *PEX11* genes encoding PEX11 α , PEX11 β and PEX11 γ ; ^cPEX26 is absent in Sc and related yeasts [31,40,41,45,78]. Colour coding is according to the involvement of peroxins in peroxisomal biogenesis *i.e.* membrane assembly (**violet**), import of matrix protein (**blue**) and growth and division (**brown**).

PTS1-containing proteins are recognized by PEX5 through its tetratricopeptide repeats (TPRs) (see section 3.2.1.1, *Introduction*) located within C-terminal half of the receptor [79-83]. On the other hand, PTS2-containing proteins are routed to peroxisomes via PEX7, a member of the WD-repeat protein family [75,84]. PEX7 itself is not able to accomplish the targeting of PTS2-containing proteins. It requires species-specific PTS2 co-receptors to fulfil this task (recently reviewed in [85,86]). These co-receptors are: PEX18/PEX21 in *S. cerevisiae* [87], PEX20 in most yeasts and fungi [88,89] and PEX5 in plants and mammals [90-92]. Lower eukaryote PTS2 co-receptors share functional and structural properties with the large isoform of mammalian PEX5, including a PEX7-binding domain (see Fig. 3), and therefore are frequently referred to as PEX5-like proteins [93-95].

As pointed out, in higher eukaryotes PEX5 mediates the sorting of the two classes of matrix proteins. In addition, a growing number of examples of proteins in which the PTS1 is absent, weak or not essential have been reported to be target to the peroxisome in a PEX5-dependent manner (reviewed in [96]). *S. cerevisiae* acyl-CoA oxidase is one of the most cited examples. This protein does not contain a PTS1, yet it reaches the peroxisomal lumen via PEX5. It is suggested the existence of an internal PTS within the protein that interacts with the N-terminal half of PEX5 [97]. The N-terminal region of PEX5 emerges as an additional recognition domain for cargoes [95,97-99]. Alternatively, it has been proposed that some PTS-lacking matrix proteins might reach the peroxisomal lumen by “piggybacking” on other proteins bearing PTS [100,101].

This model is in agreement with the not yet understood ability of peroxisomes to translocate already folded, co-factor-bound and oligomeric proteins [100-102]. Despite the absence of a permanent pore and the impermeability of the peroxisomal membrane to bulky solutes (e.g. ATP and the co-factors NAD/H, NADP/H) [103,104], peroxisomes still allow the entrance of large particles (SKL-tagged gold particles, diameter of 9 nm, [105]). This is indeed a noteworthy property that differentiates the peroxisomal translocation machinery from other organelles machineries. The peroxisomal import machinery (PIM) does not require substrates to be unfolded as the mitochondrial, chloroplast and the ER machineries do (for further details see Refs. 106-108).

3.2.1 - PEX5

PEX5 is present in all peroxisome-containing organisms, from protozoa to mammals. In mammals the *PEX5* transcript undergoes alternative splicing and two main PEX5 isoforms are synthesized: a larger (PEX5L) and a smaller (PEX5S) one. PEX5L has an internal insertion of 37-amino acids, encoded by exon 8 and absent in PEX5S [80,109,110]. This amino acid stretch provides PEX5L a domain to interact with PEX7 and

thus a role in the PTS2-protein import [90,91]. Lower eukaryotes, however, have only one PEX5 isoform, equivalent to the mammalian PEX5S.

3.2.1.1 – Structural/Functional relationship

According to structural and functional data, PEX5 can be divided into two main domains: the N-terminal and C-terminal halves. Alignment of PEX5 sequences from several organisms reveals a highly conserved C-terminal domain, in sharp contrast with its N-terminal one (see Fig. 2). It is not surprising if one considers that the C-terminal region harbours the PTS1-binding domain comprising seven TPRs (Figs. 3 and 4). At first, deletion and mutagenesis studies directly implied TPRs in the recognition of PTS1-containing proteins [79,111,112]. More recently, the resolution of the crystal structure of this region of human PEX5 alone and in complex with a PTS1-containing peptide [113,114] or with a functional PTS1-cargo protein [114] provided the molecular basis for this cargo/receptor interaction (see Fig. 4). In the cargo-loaded structure of the receptor, the PTS1-motif is located within the ring-like structure formed by the two TPRs triplets 1-3 and 5-7 which are connected by the flexible hinge comprising TPR4. Apparently, TPR4 is not directly involved in PTS1-binding.

It is within the N-terminal segment, that PEX5 bears the domains for interaction with other peroxins (see Fig. 3). Of main importance are the conserved multiple pentapeptide WXXXF/Y repeats, which mediate the interaction with PEX13 and PEX14 [115-120]. Plant PEX5 and the long mammalian isoform also harbour in this region the PEX7-binding site [90,92,93,109,117,121]. It should also be highlighted the presence of an evolutionary conserved cysteine-containing motif (Cys- X_n -Asn-Pro-Leu, where X_n is any 3–9 amino acid residues) near the N terminus of the peroxin [122]. In contrast to the TPRs segment, the N-terminal region of PEX5 does not behave as a globular and folded protein [123,124]. Biophysical and biochemical data described this domain as a natively unfolded pre-molten globule-like domain [123]. This feature explains why PEX5, a monomeric protein, displays an anomalously large Stokes radius [124]. The flexible structure of the N-terminal segment should allow PEX5 to simultaneously interact with a multitude of partners (peroxins, PTS2- and non-PTS-cargo proteins).

Hs	MAMRELVEA-- EC GA-----NPLMKLAGHFTQDKALRQE-----GLRPGWPWPGAPASEAASKPLGVAS	58
Dr	MAMRELVEA-- EC GA-----NPLMKLTGHMTQEGGAWRH-----RSTP-TIPP-----TPIEAT	48
Ce	--MKGVVEG-- QC GQ-----NALVGLANTFGTSNQRVAP-----SNAAASLLP-----SSSM	44
At	MAMRDLVNGGAA-CAVPGSSSSSNPLGALTNALLGSSSKTQERLKEIPNANRSGPRPQFYSEDQIRSLPGESE	73
Sc	MDVGS----- CS VG-----NNPLAQLHKHTQQNKSQFNQ--KNNGRNLNESPLQGTNKPGEAFISNVNAI	60
	: . * * *	
Hs	EDELVAEFLQDQNAPLVSRAPQTFKMDLLAEMQQIEQS-----	97
Dr	EDELVAEFLQGP-----QRPPHSFDMGQLLEEMQQIDQQ-----	82
Ce	GEQMANEFLRQQAR---TMAPTSMKSMQNNLPQAS-----	78
At	DQPLLQPGAQGGSEFFRGFRSDQNLGAAWDEVQGGPMPMPGMPFEPVQPTFEGPPQRVLSNFLHSFVSSSR	146
Sc	SQENMANMQRFINGEPLIDDKRMEIGPSSGRLPPFSNVHS-----	101
	: : : .:	
Hs	-----NFRQAPQRAPGVADLALSENWAQEF--AAGDAVDVT---QDYN-----	136
Dr	-----NYRQAPQRAPDVAALALSGDWASEFL--STADAASSSGQAALDPA-----	125
Ce	-----ASSSLAANWTKEFQ--PRQNQLASQ-----	101
At	GGIPFRPAPVPVLGLSQSDKQICIRDRSSIMARHFFADRGEFFINSQVNALLSSLDIDGIIQARGHVPGRFREL	219
Sc	-----LQTSANPTQIKGVNDISHWSQEFQ--GSNSIQNRNADTGNSE-----	141
	: . ** :	
Hs	-----ETDWSQEFISEVTDPLSVSPARWAEYLEQSEEKWLWGEPEG-----TATDR-WY	185
Dr	-----DADWTREFINEVAD---PGRWAEYLEQSEEKWLWGLDLGE-----REQDKWEA	170
Ce	-----WSQQTSSAPS-----MESAWRVQVQAPSMTS-----TSSHQPIT	135
At	DDYWNESQAVVKPNLHPADNWAEEFNQHGMDHG--GPDSSWVSFEQQHGVNGWATEFEQQGSQMLSSQMRMSMD	290
Sc	-----KAWQRGSTTASSRFQY--PNTMMNNYAYASMNLSLGSRLQSPAFMNPQQSGRSKE	194
	* :	
Hs	DEYHPEEDLQHTASDFVAKVDDPKLANSEFLKFVRQIGEGQVSLESAGSGRAQAEQWAAEFIQQGGTSDAWV	258
Dr	QEYQSGEELRQTANELVAKVDDPKLQN-----TEVSAES-----AESWV	209
Ce	DAGMWSSEYLDTVDTSLTKSSG-----TQNW	162
At	MQNIAAMEQTRKLAHTLSQDGNPKFQNSRFLQFVSKMSRGELIIDENQVKQASAPGEWATEYEQQYLGPSSWA	363
Sc	GVNEQEQPWTQFEKLEKEVSENLDIN-----DEIEKEENV	231
	: : :	
Hs	DQFTRPVNTSA-----LDMEFERAKSAIESDVDFWDKLQAELEEMAKRDAEAHPWLSDDYDLTS--	317
Dr	DEFAT-----YGPDPQQAKAIVESDVDFWEKLQQEWEEMAKRDAEAHPWLSDFDQMLS--	262
Ce	DDFME-----QQDNYGMENTWKDAQAFQERWEEKR-----DMEKDES--	200
At	DQFANEKLSHGPEQWAEFASGRGQQTAEADQWVNEFSLKLVDDWIDEFAEGPVGDSADAWANAYDEFLNEK	436
Sc	SEVEQNKPETVE-----KEEGVYGDQYQSDQFQEVWDSIHKDAEEVLPSELVNDLNLGEDYLYKYL	292
	 *	
Hs	---ATYDKGYQFEENPLRDHPQPFEEGLRRLQEGD-LPNAVLLFEAAVQQDPKHMEAWQYLGTQAENEQEL	386
Dr	---SSYDKGYQFEEDNPYLSHEDPFAEGVKRMEAGD-IPGAVRLFESAVQRQPDNQLAWQYLGTQAENEQEF	331
Ce	---LQSPENYVYQEANPFTTMSDPLMEGDNLNRNGD-IGNAMLAYEAAVQKDPQDARAWCKLGLAHAENEKDD	269
At	NAGKQTSGVYVFSMDNYPVGHPEPMKEGQELFRKGL-LSEAALALEAEVMKNPENAEGRWLLGVTHAENDDDQ	508
Sc	-GRVNGNIEYAFQSNNEYFNPNAYKIGCLLMENGAKLSEAALAFEAAVKEKPDHVDAWLRLGLVQTQNEKEL	364
	* : . . * . . * : . * * : * * : * *	
Hs	LAISALRRCLLEKLPDNTALMALAVSFTNESLQRQACETLRDWLRYTPAYAHLVTPAEEGAGGAGLGPSCR--	457
Dr	AAISALRRCELEKKNLTALMALAVSFTNESLHRQACETLRDWMHNPKYRIILEQHEREKQREGAREREKES	404
Ce	LAMQAFQKCLQIDAGNKEALLGLSVSQANEGMENEALHQLDKWMSSYLGSNSTQVTTTPP-----	329
At	QAIAAMMRAQEADPTNLEVLALGVSHNTNELEQATALKYLYGWLNRNHPKYGAIAAPP-----	564
Sc	NGISALEBCKLDPKNLEAMKTLAISYINEGYDMSAFTMLDKWAETKYPEIWSRIKQDDDKFQ-----	427
	. : * . . . * . : * : * * . * * *	
Hs	-ILGSLSDSLFLEVKEFLAAVRLDPTSIDPDVQCGLGVLFNLSGEYDKAVDCFTAALSVRPNDYLLWNKLG	529
Dr	ERFGSLPEALFGEVQTLFLNAAAAPESQVDPQLCGLGVLFNLSGEYDKAVDCFSAAHSVTPQDYLLWNKLG	477
Ce	-LYSSFLSDTFNRVEARFLDAARQQGATPDPLQNALGVLYNLNRNFAFARVDSLKLAI SKNPTDARLWNRLG	401
At	---ELADSLYHADIARLFNEASQLNPE--DADVHIVLGVLYNLSREFDRAITSFQTALQLKPNDSYSLWNKLG	631
Sc	-KEKGFTHIDMNAHITKQFLQLAN-NLSTIDPEIQLCLGLLFYTKDDFDKTIDCFESALRVNPNDELWNRLG	498
	: : * : * : * : * : * : * : *	
Hs	ATLANGNQSEEAAYRRALQLPGYIRSRYNLGISCNLGAHREAVEHFLEALNMQRKS-----RGPR	593
Dr	ATLANGNRSEEAAYRRALQLPGFVRSRYNLGISCNLGAHREAVEHFLEALSLQRAAGDGEAGRGPG	550
Ce	ATLANGDHTAEAISAYREALKLYPTYVRARYNLGISCMQLSSYDEALKHFLSALELQKGG-----	461
At	ATQANSVQSADAI SAYQALDLKPNYVRWANMGI SYANQGMKYKESIPYVVRALAMNPKA-----	691
Sc	ASLANSNRSEEA IQAYHRAQLKPSFVRARYNLAVSSMNIGCFKEAAGYLLSVLSMHEVN----TNNKKGDV	567
	* : * . : : * : * : * : * : * : *	
Hs	GEGGAMSENIWSTLRRLALSMGL--QSDAYGAADA-RDLSTLLTMFGLPQ----	639
Dr	AAATIMSDNIWSTLRMALSMG--ESSLYSAADR-RDLDTLLTHFSHREGAE	600
Ce	---NDASGIWTTMRSAAIRTSNVPDNLRAVER-RDLAAVKASLV-----	602
At	-----DNAWQLRLSLSCAS--RQDMIACES-RNLDLLQKEFFPL-----	728
Sc	SLLNTYNDTVIETLKRVIAMN--RDDLLQEVKPGMDLKRFGESF-----	612
	. : : . . : *	

Figure 2 - Sequence alignment of PEX5 proteins (ClustalW2, www.ebi.ac.uk). The long isoform of human (Hs) PEX5 is aligned with homolog proteins from *Danio rerio* (Dr), *Caenorhabditis elegans* (Ce), *Arabidopsis thaliana* (At) and *Saccharomyces cerevisiae* (Sc). Red colour marks the conserved cysteine residue located at PEX5 N termini that is target of ubiquitination (for further details see sections 3.1.2.2 and 4.2, *Introduction*).

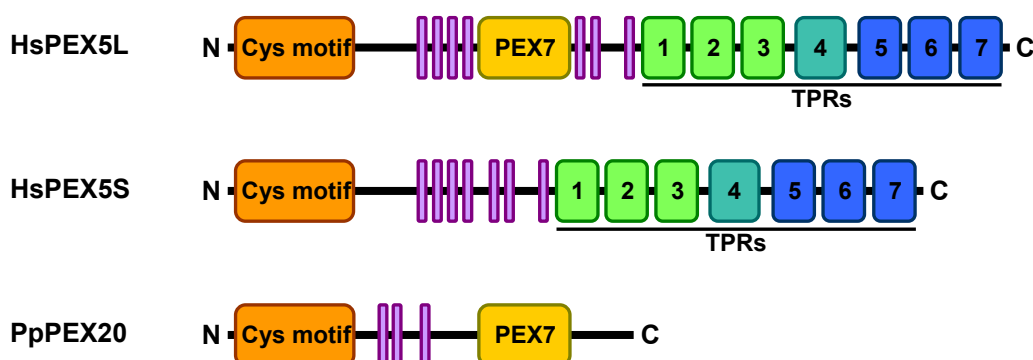


Figure 3 - Schematic representation of mammalian PEX5 and a PEX5-like protein from a lower eukaryote. Mammals synthesize two PEX5 isoforms, the large (PEX5L) and the short (PEX5S) one. Human (Hs) PEX5L and PEX5S and *Pichia pastoris* (Pp) PEX20, an example of a PEX5-like protein, are depicted. The N-terminal cysteine-containing motif (orange Cys motif box) is evolutionary conserved, as are the WXXXF/Y repeats (violet boxes). The number and distance of these repeats vary from organism to organism. PEX13 and PEX14 interact with at least one of the repeats. PEX5 recognize directly PTS1-containing proteins via its TPRs motifs (TPRs 1 to 7: coloured light green to blue) located at its C-terminal half. PEX5L and PEX20-like proteins (includes PEX20 and *S. cerevisiae* PEX18/PEX21), also known as PTS2 co-receptors, possess a PEX7-binding domain (yellow PEX7 box) absent in PEX5S, which provides them a role in the sorting of PTS2-containing proteins to the peroxisomal matrix.

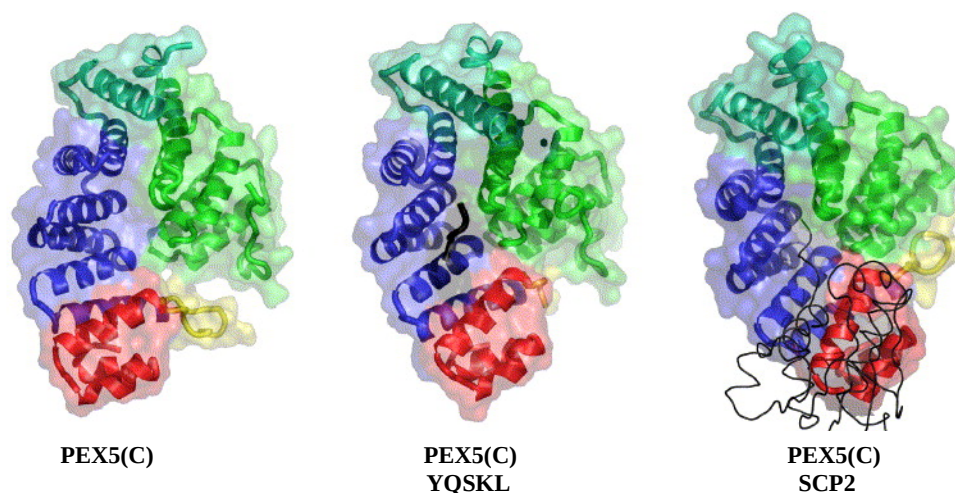


Figure 4 - Structures available for the C-terminal half of PEX5 (PEX5(C)). The structures were obtained in the absence of a ligand (left, [114]), in the presence of the consensus PTS1 peptide YQSKL (Protein Data Bank accession number 1FCH) (central, [113]), and in the presence of the functional PTS1 cargo protein SCP2 (right, [114]). Colour coding: TPR segments 1–3, light green; TPR4 segment, dark green; TPR segments 5–7, blue; loop connecting the TPR segment and the C-terminal helical bundle, yellow; C-terminal α-helical bundle, red; PTS1 ligands, black (adapted from [114]).

3.2.1.2 - The Cycling Receptor Model

PEX5 is predominantly cytosolic but a small percentage is peroxisome-associated [69,79,125]. Its bimodal distribution as well as its ability to recognize cargo proteins suggested that PEX5 acted as cycling receptor. Dodt and Gould provided the first experimental evidence supporting this hypothesis. PEX5 was found to accumulate in a reversible manner at the peroxisomal membrane by manipulating temperature and ATP-levels in cultured human fibroblasts [126]. Additionally, Dammai and Subramani provided some data suggesting that PEX5 is able to undergo multiple cycles between the cytosol and peroxisomes [127].

Presently available data point to a tight coupling between PEX5 cycling and cargo-sorting and -translocation processes. In line with this, PEX5 interacts with and is part of a transient protein complex at the peroxisomal membrane. The characterization of peroxin-peroxin interactions [yeast two-hybrid, coimmunoprecipitation assays (see Ref. 128 and references cited therein), isolation of complexes from the peroxisomal membrane in yeast and mammals [129-131]] and analysis of steady-state levels of PEX5 in *PEX* mutants [132] have helped to define the dynamic architecture of the peroxisomal import machinery (PIM) (Fig. 5). The mammalian PIM comprises ten peroxins. More recently elements of the ubiquitin-conjugating cascade (UCC) were shown to be part of this machinery as well: ubiquitin, ubiquitin-activating enzyme (E1) and an ubiquitin-activating enzyme (E2). Most of the PIM components are arranged into two structural/functional units: a membrane-embedded docking/translocation machinery (DTM) that includes PEX13, PEX14 and the really interesting new gene (RING)-finger peroxins PEX2, PEX10 and PEX12, and a receptor export module (REM) comprising the ATPases associated with diverse cellular activities (AAA) peroxins, PEX1 and PEX6, and their membrane anchor PEX26.

During the last years our laboratory has been characterizing the mammalian PEX5-mediated peroxisomal protein import pathway using a rat liver-based *in vitro* import system, particularly suited to follow radiolabeled PEX5 trafficking. The data gathered until now are depicted in the model shown in Fig. 6 (see also Ref. 133). The model presented comprises five main stages (numbered 0-4). In the first step, free/soluble PEX5 (stage 0) binds cargo proteins in the cytosol. This interaction induces conformational changes on PEX5, enabling it to interact with the DTM [113,114,134,135]. PEX13 and PEX14 are suggested to comprise the docking platform at the peroxisomal membrane for cargo-loaded PEX5, but the precise contribution of these two peroxins remains a matter of debate (see Refs. 136 and 137 for recent reviews). Following this initial interaction PEX5 becomes inserted into the DTM and acquires a transmembrane topology, exposing a small N-terminal domain to the cytosol (and thus protease accessible; stage 2), while the

bulky part of its polypeptide chain faces the organelle matrix [138]. *In vivo* data supporting a transient exposure of PEX5 into the peroxisomal matrix is also available [127]. The stage 1-to-stage 2 cargo-dependent transition is an ATP-independent, essentially irreversible, process [122,135,139]. This finding is at the basis that translocation of PEX5-enfolded cargo proteins is driven by interactions established between the N-terminal of the receptor and DTM components. The strong interactions (K_d values in the nanomolar range) between the (N-terminal) diaromatic motifs of PEX5 and the N-terminal region of PEX14 [119] that is deeply embedded into the DTM or even exposed into the organelle lumen [140] favours this view. Next, DTM-embedded PEX5 is monoubiquitinated at a conserved N-terminal cysteine residue (at position 6 and 11 in yeast [141] and mammals [142], respectively; see Fig. 4) (Ub-PEX5, stage 3, completely protease-resistant). Deletion of the 17 amino acid domain containing this cysteine, its alkylation or its substitution by a serine results in PEX5 proteins still functional in the docking and membrane insertion steps but incompetent in the export step [122,134,142]. Thus, monoubiquitination is a mandatory event for the ATP-dependent export step [142]. This event is promoted by the mechanoenzyme couple PEX1/PEX6 that extract Ub-PEX5 conjugates from the peroxisomal membrane to the cytosol yielding soluble Ub-PEX5 species (stage 4) [143,144]. Mechanistic details on the mode of action of the REM are still missing. Finally, the ubiquitin moiety is presumably removed from the soluble Ub-PEX5 conjugate (stage 4), thus regenerating stage 0 PEX5 by a still unknown mechanism.

The model described above contains few data on the transported cargo proteins. When are cargoes inserted into the DTM and released into the organelle lumen? Until very recently there was no evidence on how these events happened, only hypotheses [133,145]. Using a PTS2-centered *in vitro* import system Alencastre *et al.* have clarified these questions [146]. The authors provide for the first time evidence suggesting that cargo insertion into the DTM occurs at stage 1b-to-stage 2 transition as previously hypothesized [145]. Furthermore, this same study showed that the release of the reporter protein into the organelle lumen occurs prior to (and not upon) ubiquitination of the N-terminal conserved cysteine residue of PEX5 (*i.e.* at stage 2-to-stage 3 transition).

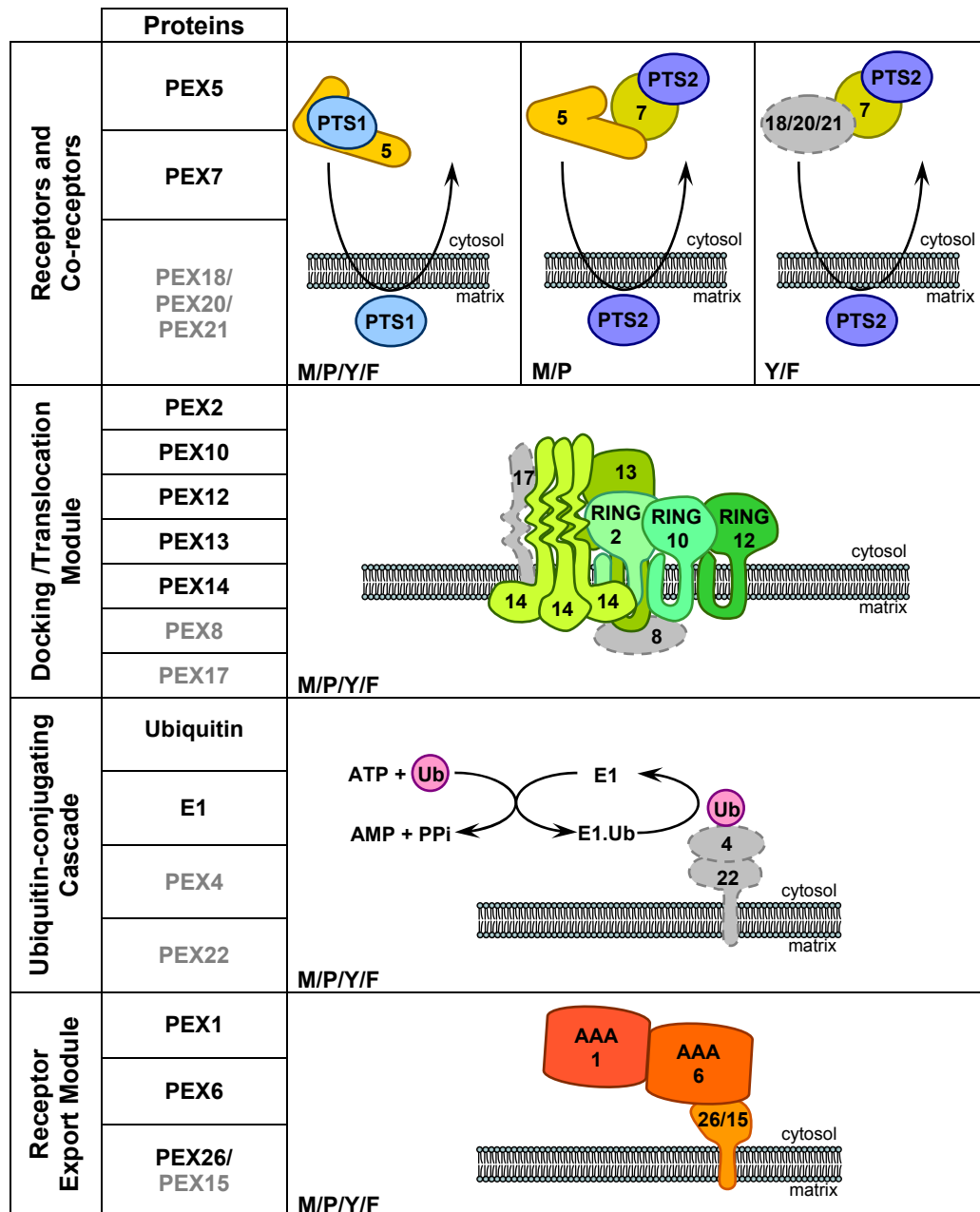


Figure 5 - Components of the peroxisomal import machinery (PIM). Peroxins are the main components of the PIM. Peroxins absent in mammals (M) are depicted in gray. Receptors and co-receptors of cargo proteins are represented (further details on cargo specificity on section 3.2, *Introduction*). The docking/translocation module comprises mostly transmembrane proteins. PEX8 and PEX17 have been found only in lower eukaryotes [40,41]. The first two reactions of the ubiquitin-conjugating cascade involved in the peroxisomal protein import pathway are shown. In plants (P), yeasts (Y) and fungi (F) the E2 PEX4 is bound to the organelle membrane via PEX22, whereas their mammalian homologs have not been identified yet. The identity of the ubiquitin ligase acting at the end of the cascade remains speculative. The receptor export module comprises AAA peroxins PEX1 and PEX6 and one intrinsic membrane protein anchor [143,144], PEX26 in mammals and other organisms and PEX15 in some yeasts. PEX26 and PEX15 are unrelated at the primary structure level [40]. Their plant functional counterpart is presently unknown [22].

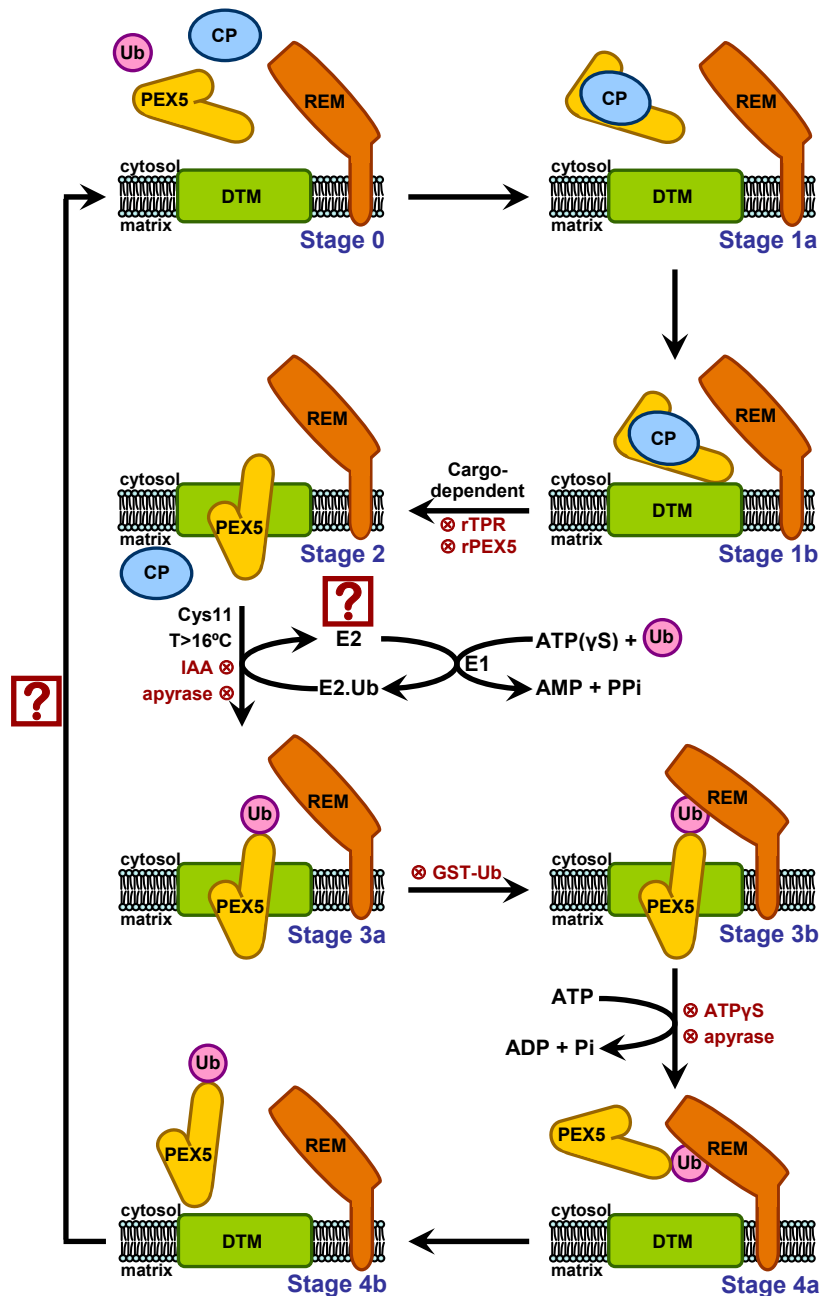


Figure 6 - PEX5-mediated peroxisomal protein import pathway in mammals. This protein sorting pathway comprises five major stages (numbered 0–4). The sub-steps are mostly of conceptual nature. The different stages have been characterized with the *in vitro* system developed in our laboratory, applying several strategies to block (⊗) the pathway at different steps. *Stage 0*, cytosolic cargo-free PEX5 (protease accessible). *Stage 1*, cytosolic PEX5-cargo protein complex (protease accessible). *Stage 2*, PEX5 embedded in the peroxisomal docking/translocation machinery (DTM) (only 2 kDa of PEX5 N terminus are accessible to exogenously added proteinase K). *Stage 3*, DTM-embedded monoubiquitinated PEX5 at Cys11 (protease protected). *Stage 4*, protease accessible monoubiquitinated PEX5. *Question mark*, indicate the steps that were addressed in this work, namely the identity of the mammalian E2(s) mediating PEX5 monoubiquitination at Cys11 and the deubiquitination mechanisms acting at stage 4-to-stage 0 transition. CP, cargo protein; Ub, ubiquitin; IAA, iodoacetamide; rTPR, recombinant protein comprising the PTS1-binding domain of PEX5; rPEX5, recombinant full-length PEX5.

4 - Ubiquitination

4.1 - Brief description

Ubiquitination is a reversible and versatile posttranslational modification involved in various biological processes [147,148]. Substrates can be modified either by the attachment of a single ubiquitin (monoubiquitination) or ubiquitin chains (polyubiquitination). The number of ubiquitin molecules, as well as the lysine within the ubiquitin molecule used for ubiquitin-chain growth (often Lys48 and Lys63), determines both the fate and function of the modified protein. For instance, polyubiquitin chains of at least four ubiquitin moieties linked through Lys48 comprise the canonical signal for protein degradation at the proteasome [149]. In contrast, monoubiquitination and Lys63-based polyubiquitination are used as transient modifications modulating the activity of proteins. Examples of such modifications have been found in proteins involved in DNA repair, signal transduction, endocytosis and ribosomal protein synthesis [150-153].

Regardless of the final outcome, there is a common mechanism underlying protein ubiquitination (reviewed in [154]). Proteins are ubiquitinated through the concerted action of an (thiolester-based) enzymatic cascade (Fig. 7). The so-called ubiquitin-conjugating cascade (UCC) comprises three enzymes: an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an ubiquitin ligase (E3). First, the E1 activates ubiquitin C-terminal carboxyl group in an ATP-requiring step yielding a high energy thiolester intermediate (E1-S~Ub) [155]. Subsequently the E2 receives the activated ubiquitin moiety on its active center cysteine (E2-S~Ub). Finally, the transfer of ubiquitin to the substrate-bound E3 depends on the nature of the ligase that primarily dictates ubiquitination specificity. Two main classes of E3s are commonly distinguished: the RING type and the Homologous to the E6-AP carboxyl terminus (HECT) type. RING E3 ligases act as scaffolds that bring into close proximity the ubiquitin-charged E2 and the substrate, facilitating the direct transfer of ubiquitin to the substrate [156]. HECT-domain E3 ligases accept ubiquitin through a thiolester bond (E3-S~Ub) prior to ubiquitin transfer to a protein target [157]. Successive rounds of ubiquitin conjugation result in the formation of polyubiquitin chains. In some cases an additional conjugation factor, an ubiquitin-chain elongation enzyme (E4), has been reported to be required for the efficient formation of polyubiquitin chains comprising more than four ubiquitins [158,159].

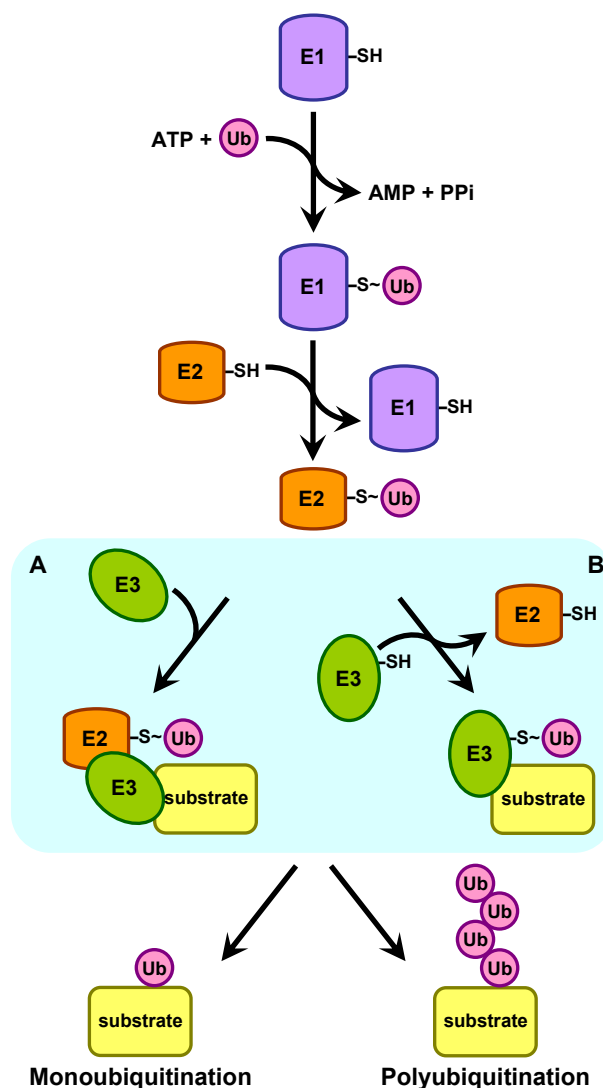


Figure 7 – Ubiquitin-conjugation pathway. Activation of ubiquitin C terminus by E1 proceeds in two steps: adenylation (not shown) followed by attack by a cysteine side chain to form a thiolester bond between the E1 and ubiquitin (E1-S~Ub). Ubiquitin is then passed to a cysteine of an E2. Ligation of the ubiquitin to a substrate, usually to a lysine side chain, follows either A) directly with the aid of a RING-bearing E3 or B) after an intermediate transthiolation to a cysteine side chain of a HECT-domain E3. Both E3 types interact with their substrates that can acquire just a single ubiquitin or a polyubiquitin chain.

Conventionally, ubiquitin is attached via an isopeptidic bond between its C terminus [160] and the ϵ -amine group of lysine residues [161] or the α -amine group of the N-terminal residue of the substrate [162]. Remarkably amine groups appear not be the sole targets of ubiquitination. Indeed, in recent years ubiquitination of cysteines, threonines and serines has been uncovered [141,142,163-166]. PEX5, one of the peroxisomal cycling receptors, was shown to be modified in this manner, specifically at a cysteine residue [141,142]. Protein ubiquitination at cysteine residues is an intriguing phenomenon. Thermodynamically it is not the most favorable event when compared to

ubiquitination of other residues [167,168]. In addition, ubiquitin-protein thiolester conjugates seem to be easily disrupted by nucleophiles such as GSH [169]. Hence, the chemical lability of thiolesters also argues against this type of covalent interaction, as proteins modified in this manner may, to some extent, be subjected to futile ubiquitination/deubiquitination cycles. Thus, apparently thiolester bonds are not the most efficient way to attach ubiquitin to a protein, except if the aim is to generate an activated (easily transferable) form of ubiquitin as it is illustrated by the high energy ubiquitin thiolester intermediates at the UCC.

4.2 - Ubiquitination at peroxisomes

Several studies have documented ubiquitination of the peroxisomal receptors PEX5 [141,142,170-173], PEX18/PEX21 [174] and PEX20 [175] at the peroxisomal membrane.

The detection of polyubiquitinated receptor species at peroxisomal membranes in *PEX* mutants (*PEX4*, *PEX22*, *PEX1*, *PEX6*, *PEX15*) led several authors to propose the existence of a quality control mechanism, acting at membrane-trapped receptor molecules [170,173-175].

Noteworthy monoubiquitinated PEX5 species have also been detected in wild-type *S. cerevisiae* [141,171] and in mammals [142].

Williams *et al.* [141] and Carvalho *et al.* [142] revealed a(nother) remarkable feature of yeast and mammalian PEX5, respectively: during its transient passage through the peroxisomal membrane it is monoubiquitinated not at a lysine residue but at an N-terminal conserved cysteine residue instead, yielding a thiolester conjugate. Monoubiquitination of PEX5 does not target the protein for proteasomal degradation, rather it is required for the export of PEX5 [142] by the AAA ATPases, PEX1 and PEX6 [143,144] to the cytosol to promote further cycles of protein transportation.

In yeast polyubiquitination of PEX5 depends on the multipurpose E2 enzyme UBC4 [170,173], whereas monoubiquitination of PEX5 is PEX4 (UBC10)-dependent [141].

PEX4 is a peripheral peroxisomal membrane protein in yeasts, and probably in plants [176-180]. This peroxin is thought to comprise a structural/functional unit together with PEX22, its peroxisomal membrane anchor [177,180]. Despite several attempts no PEX4 and PEX22 homologs have ever been found in mammals (see Table 3). Proteomic studies aiming at establishing the peroxisomal protein repertoire [181-183] and *in silico* sequence analysis [37,40,41,180] have all failed to identify the mammalian homologs of yeast/plant PEX4 and PEX22.

The RING peroxins PEX2, PEX10 and PEX12 are the obvious candidates to be the E3(s) component(s) of the UCC acting on PEX5. Two studies support this hypothesis. Yeast PEX10 interacts with PEX4 in the split-ubiquitin system [184], suggesting that PEX10 acts as the E3 ligase for PEX4. However more recently, PEX10 was proposed to be the E3 ligase for the UBC4-dependent ubiquitination of PEX5 [185]. The reason why there are three putative E3s at the peroxisomal membrane remains elusive. Some authors have speculated that it might be related with substrate/residue specificity and/or ubiquitination purpose [133,185,186].

II. AIMS

PEX5 monoubiquitination in yeast is mediated by the ubiquitin-conjugating enzyme PEX4, a peroxin anchored to the peroxisomal membrane through PEX22. However, despite several attempts (proteomic approaches, *in silico* analysis of sequences), PEX4 and PEX22 mammalian homologs were never found. One of the aims of this work was to purify and identify the mammalian PEX4 functional counterpart.

Ubiquitination at cysteine residues seems to be a particularly rare event. PEX5 is one of the few proteins outside the UCC reported to be modified in this manner. Why a cysteine, and not a lysine residue, is used in this ubiquitination event is presently unknown. The work presented also sought to characterize the properties of Ub-PEX5 thiolester conjugate with the aim of unveiling the reasons behind this unconventional ubiquitination.

III. EXPERIMENTAL PROCEDURES

1 - Expression and purification of recombinant proteins

Both expression and purification of the recombinant proteins GST-LKS, GST-SKL [138], GST-Ub [142], His₆-PEX5 [124] and His₆-TPR-PEX5 [123] have been already described. Proteins were concentrated and the buffer exchanged using Vivaspin 500 columns (10,000 MWCO PES; Vivascience).

2 - Rat liver subcellular fractionation

Male Wistar rats approximately six weeks old were fasted, anesthetized with CO₂ and sacrificed by cervical dislocation. Livers were rapidly removed, minced and homogenized in the proper ice-cold homogenizing buffer (see below). All centrifugation steps were performed at 4 °C. Protein on subcellular fractions was determined by the Bradford method using bovine IgGs as protein standard [187].

2.1 - Preparation of postnuclear supernatants (PNS)

Rat liver PNS for *in vitro* assays was prepared in SEM buffer (0.25 M sucrose, 20 mM MOPS-KOH, pH 7.2, 1 mM EDTA-NaOH, pH 7.2) supplemented with 2 µg/ml E-64, as described before [138]. The PNS fractions were obtained after centrifuging the homogenates twice at 600 x *g* for 10 min, using the SS-34 rotor in a Sorvall® (DuPont Instruments) centrifuge.

For the steady-state analysis of rat liver PEX5 an SEM buffer supplemented with 20 mM NEM, 10 µg/ml PMSF and 1:300 (v/v) mammalian protease inhibitor cocktail (Sigma) was used.

2.2 - Preparation of peroxisomes

Isolation of highly pure peroxisomes from rat liver by differential centrifugation and Nycodenz gradient purification was performed as described [188], with minor modifications. Briefly, rat livers were homogenized in SIE buffer (0.25 M sucrose, 5 mM imidazole-HCl, pH 7.4, 1 mM EDTA-NaOH, pH 7.4) supplemented with 0.1 mg/ml PMSF and 1:500 (v/v) mammalian protease inhibitor cocktail. The homogenate was centrifuged at 600 x *g* for 10 min. The resulting supernatant was then subjected to two other centrifugation steps: 10 min at 2,350 x *g* followed by 15 min at 12,300 x *g*. The obtained pellet was resuspended in SIE-supplemented buffer and centrifuged at 12,300 x *g* for additional 15 min. The resulting pellet was again resuspended in supplemented SIE buffer

and applied (1.5 ml/gradient) onto the top of a discontinuous Nycodenz gradient (0.5 ml of 25% (w/v) Nycodenz, 0.5 ml of 28% (w/v) Nycodenz and 7 ml of 33% (w/v) Nycodenz in 5 mM imidazole-HCl, pH 7.4, 1 mM EDTA-NaOH, pH 7.4). The gradient was centrifuged for 20 min at 2,350 x *g*, time after which the speed was increased to 60,000 x *g* and the centrifugation continued for additional 30 min. Purified peroxisomes were carefully resuspended in SEM buffer containing 2 µg/ml E-64, divided into single-use aliquots, frozen in liquid N₂, and stored at -70 °C.

2.3 - Cytosol preparation and fractionation

Livers were homogenized in 5 volumes of ice-cold 0.25 M sucrose, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA-NaOH, pH 7.5, supplemented with 2 µg/ml E-64. The total homogenate was subjected to sequential centrifugation steps as follows: 10 min at 600 x *g*, 20 min at 12,300 x *g*, and 1 h at 100,000 x *g*. The cytosolic fraction (last supernatant) was aliquoted, frozen in liquid N₂, stored at -70 °C, and used within 5-10 days.

Alternatively, freshly prepared cytosol was further fractionated into Fraction I and Fraction II according to Ciechanover *et al.* [189]. Briefly, a cytosolic fraction (1.35 g of cytosolic protein at 30 mg/ml) was applied onto a 30 ml-DEAE-cellulose (DE52, Whatman) column. The flowthrough (unbound protein fraction, Fraction I) was collected, the column washed with 3 volumes of 20 mM Tris-HCl, pH 7.5 and the adsorbed proteins (Fraction II) eluted with 2.5 volumes of high salt buffer (20 mM Tris-HCl, 0.5 M KCl, 0.5 mM DTT) at 2 ml/min. Both fractions were precipitated with NH₄(SO₄)₂ (at 90% saturation), followed by overnight dialysis at 4 °C against 4 L of 25 mM Tris-HCl, pH 7.5, supplemented with 0.5 mM DTT.

3 - Partial purification of E2s

One hundred and fifty milligrams of cytosolic proteins (at 30 mg/ml) were precipitated with 16% (w/v) PEG 4000 (added from a 50% (w/v) stock solution in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl) for 20 min on ice. The pellet obtained after centrifugation at 15,000 x *g* for 20 min was resuspended in 5 ml of 50 mM Tris-HCl, pH 7.5, 5 mM ATP, 10 mM MgCl₂, and 0.2 mM DTT. The clarified sample was applied at a 0.1 ml/min flow rate to a 0.8-ml ubiquitin-agarose column (10 mg/ml; Biomol), previously equilibrated with 5 volumes of buffer A (50 mM Tris-HCl, pH 7.5, 2 mM ATP, 5 mM MgCl₂, 0.2 mM DTT). The column was washed at 0.5 ml/min as follows: 3 volumes of buffer A, 6 volumes of 50 mM Tris-HCl, pH 7.5, and 3 volumes of 50 mM Tris-HCl, pH 7.5, 1 M KCl. Elution was performed at 0.1 ml/min flow rate with 3 volumes of a high pH/DTT buffer (50 mM Tris-

HCl, pH 9.0, 20 mM DTT). The eluate was immediately neutralized with a 100 mM Tris-HCl, pH 7.5, 33 µg/ml BSA solution. The wash and elution fractions were subjected to three cycles of concentration/dilution with 20 mM Tris-HCl, pH 7.5, supplemented with 0.2 mM DTT, using Vivaspin 500 columns (3,000 MWCO PES), pretreated for 30 min at room temperature with 600 µl of 0.1% (w/v) BSA to minimize protein loss by adsorption. In the subsequent steps, only Eppendorf® protein LoBind microcentrifuge tubes were used. Concentrated eluates (100-150 µl) were incubated with 30 µl (bed volume) of DE52 for 30 min at 18 °C. The beads were washed two times for 10 sec with 100 µl of 20 mM Tris-HCl, pH 7.5, and the bound proteins were eluted twice with 50 µl of 20 mM Tris-HCl, pH 7.5, 0.5 M KCl, for 5 min.

Throughout the entire purification procedure, aliquots of all fractions were withdrawn for SDS-PAGE analysis and *in vitro* import experiments to test for E2 activity. With the exception of the cytosolic fraction which was kept frozen at -70 °C, the entire procedure, activity assays included, was always performed in a single day.

4 - Plasmids and synthesis of ³⁵S-labeled PEX5 proteins

The cloning strategy for the first 324 amino acids of the large isoform of human PEX5 (ΔC1-PEX5) into pET28a (Novagen) has been described elsewhere [190]. cDNAs encoding the large isoform of human PEX5 [80,109,110] possessing an alanine (PEX5(C11A)), an arginine (PEX5(C11R)), a lysine (PEX5(C11K)) at position 11 were obtained with the QuickChange® Site-directed Mutagenesis Kit (Stratagene), using pGEM4-PEX5 [138] as template. The primers used are listed in Table 4. The same strategy had been previously used to obtain pGEM4-PEX5(C11S) [142].

Table 4 - List of primers used to generate PEX5 mutants at position 11

pGEM4-based construct	Primer	Sequence
PEX5(C11A)	P5C11A Fw	5'-GGTGGAGGCCGAAGCCGGGGGTGCCAAC-3'
	P5C11A Rv	5'-GTTGGCACCCCCGGCTTCGGCCTCCACC-3'
PEX5(C11R)	P5C11R Fw	5'-GGTGGAGGCCGAACGCGGGGGGTGCCAAC-3'
	P5C11R Rv	5'-GTTGGCACCCCCGCGTTCGGCCTCCACC-3'
PEX5(C11K)	P5C11K Fw	5'-GGTGGAGGCCGAAAAGGGGGGTGCCAAC-3'
	P5C11K Rv	5'-GTTGGCACCCCCCTTTTCGGCCTCCACC-3'

Fw, forward primer; Rv, reverse primer

^{35}S - ΔC1 -PEX5 was synthesized in an *E. coli* lysate using the *E. coli* T7 S30 Extract System for Circular DNA kit (Promega), whereas ^{35}S -labeled full-length PEX5 versions were synthesized using the TNT[®] Quick Coupled Transcription/Translation System (Promega) in the presence of EasyTag[™] L- ^{35}S -Methionine (specific activity >1000 Ci/mmol; Perkin-Elmer Life Sciences) following the manufacturer's instructions.

5 - *In vitro* reactions

In vitro import assays using PNS or purified peroxisomes were performed in import buffer (0.25 M sucrose, 50 mM KCl, 20 mM MOPS-KOH, pH 7.2, 3 mM MgCl_2 , 20 μM methionine, 2 $\mu\text{g/ml}$ E-64) in a final volume of 100 μl . NEM treatment of import reactions was done on ice for 5 min by adding 25 μl of a freshly made 0.1 M solution per *in vitro* reaction (20 mM final concentration). To separate organelles from soluble proteins *in vitro* reactions were diluted with SEM buffer (typically 900 μl) and centrifuged at 15,000 x *g* for 20 min at 4 °C. Organelle pellets and the soluble phase of the reactions were analyzed by SDS-PAGE and autoradiography.

5.1 - Peroxisome-based *in vitro* assay

In vitro import experiments containing 50 μg of purified peroxisomes and 1 μl of radiolabeled ΔC1 -PEX5 were performed in import buffer supplemented with 10 μM ZnCl_2 , 5 mM ATP and 25 μg GST-Ub. When specified 500 μg of cytosolic protein and 250 ng of recombinant human E1 (Biomol) were used per import reaction. The reactions were incubated for 20 min at 37 °C, treated with NEM and the organelles were isolated by centrifugation and analyzed by SDS-PAGE and autoradiography.

5.2 - PNS-based *in vitro* assays

Four hundred micrograms of PNS protein and 1 μl of the relevant ^{35}S -labeled full-length PEX5 (see section 4, *Experimental Procedures*) were incubated in import buffer for 30 min at 37 °C, in the presence of 5 mM ATP and 10 μM bovine ubiquitin, unless otherwise indicated. Ubiquitin aldehyde (Ubal; Biomol), an inhibitor of deubiquitinating enzymes (DUBs), was used at a final concentration of 3 μM . Proteasome inhibitors MG132 and clastolactacystin β -lactone (Biomol), each at 10 μM final concentration, were included in some of the initial experiments but similar results were obtained in their absence. When specified, 20 units/ml apyrase (grade VII, Sigma) and 2 μg of recombinant PEX5 were added to the reactions.

To determine the lability of the soluble Ub-PEX5 thiolester conjugate (stage 4) in the presence of GSH a standard *in vitro* reaction containing bovine ubiquitin, Ubal and proteasome inhibitors was performed. At the end of the 37 °C incubation, the reaction was diluted with 400 µl of SEM supplemented with Ubal and proteasome inhibitors (but lacking NEM) and centrifuged. The supernatant was then incubated for the indicated time periods at 37 °C, pH 7.2, with 5 mM GSH (added from a freshly prepared 0.1 M solution in 20 mM MOPS-KOH, 1 mM EDTA, pH 7.2 adjusted at 37 °C). After NEM treatment the samples were subjected to TCA precipitation and processed for SDS-PAGE. To determine the half-life of the membrane-bound Ub-PEX5 conjugate (stage 3) in the presence of GSH, a total organelle pellet enriched in stage 3 PEX5 was prepared as follows. An *in vitro* reaction containing bovine ubiquitin, Ubal and proteasome inhibitors was first incubated for 5 min at 37 °C in the presence of 0.5 mM ATP [to release the DTM from the endogenous PEX5 [139]] followed by a second 25 min incubation in the presence of 7 mM ATPγS to accumulate stage 3 PEX5. A total organelle pellet was obtained by centrifugation and resuspended in 500 µl of SEM buffer supplemented with Ubal and proteasome inhibitors. Treatment with 5 mM GSH and processing of the samples for SDS-PAGE was done as above.

Cargo protein-dependent import experiments using IAA-treated substrates were done as follows. Two reactions (tubes A and B, respectively) containing 1 mg of PNS protein each in 250 µl of import buffer supplemented with ATP, 1 µM bovine ubiquitin and Ubal were assembled on ice. Twelve microliters of ³⁵S-labeled PEX5 were then added to reaction A and both tubes were incubated at 37 °C for 30 min. At the end of the incubation, the tubes were centrifuged, the supernatants were recovered, placed on ice and 12 µl of ³⁵S-labeled PEX5 were then added to supernatant B. Both supernatants were treated with 12 mM IAA (final concentration) on ice for 15 min. In parallel, a tube containing 4.8 mg of PNS protein in 720 µl of import buffer supplemented with 1 mM ATP and Ubal was incubated at 37 °C for 3 min (to release the DTM from endogenous PEX5). This suspension was placed at 16 °C, supplemented with 12 µM GST-Ub and 120 µl aliquots were pipetted into two sets of three tubes (tubes 1-3). Tubes "1" contained no addition; tubes "2" contained a recombinant protein comprising the PTS1-binding domain of PEX5 (TPR-PEX5) plus a PTS1-containing peptide (CRYHLKPLQSKL) [135]; and tubes "3" contained this recombinant protein plus a control peptide (CRYHLKPLQLKS). The final concentration of recombinant TPR-PEX5 and peptides were 1.7 µM and 33 µM, respectively. Each set of tubes was then programmed with 80 µl/tube of supernatant A or B and the incubation continued for 30 min at 16 °C. At the end of the incubation one half of each reaction was treated with NEM and the organelles were isolated by centrifugation.

The other half was treated with proteinase K for 30 min on ice as described before [142], but using a lower protease concentration (250 µg/ml) to minimize degradation of the ubiquitin moiety in the stage 3 PEX5 species, followed by protease inactivation with 50 µg/ml PMSF (2 min on ice) and organelle isolation. Organelle pellets were subjected to TCA precipitation (10% (w/v)), washed with acetone and analyzed by nonreducing SDS-PAGE/autoradiography.

6 - GST-pull down assay

An organelle pellet from a 4-fold scale-up of an *in vitro* reaction containing peroxisomes and cytosol (see section 5.1, *Experimental Procedures*) was resuspended in solubilization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA-NaOH, pH 8.0, 1% (w/v) Triton X-100, 5 mM NEM, 50 µg/ml PMSF and 1:100 (v/v) mammalian protease inhibitor cocktail). After 2 h at 4 °C, the insoluble material was removed by centrifugation, and the supernatants were incubated at 4 °C for 3 h with glutathione-Sepharose beads (50-µl bed volume). The Sepharose beads were then washed three times with 1 ml of solubilization buffer lacking detergent and incubated with Laemmli gel loading buffer (lacking reducing agents) supplemented with 10 mM NEM at 37 °C for 30 min. Where indicated, samples received 100 mM DTT and were then heated at 65 °C for 15 min.

7 - Sucrose gradient density centrifugation

Eighty microliters of a supernatant from a PNS-derived *in vitro* reaction performed in the presence of Ubal (see section 5.2, *Experimental Procedures*) were treated with 12 mM IAA (15 min on ice) and incubated on ice for 5 min with 3 µg of recombinant PEX5. The solution was brought to 400 µl with buffer B (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA-NaOH) and halved. One half received GST-SKL, a GST containing a PTS1 signal at the C terminus; the other received GST-LKS, a GST ending with a non-functional PTS1-like sequence [135], both at 2 µM final concentration. After 10 min at room temperature, 30 µg each of bovine IgGs (6.9 s), BSA (4.3 s) and STI (2.3 s) were added to the samples as internal standards (numbers in parenthesis represent the sedimentation coefficients). These mixtures were then applied onto the top of continuous 5-30% (w/v) sucrose gradients in buffer B supplemented with 0.25 µM of the corresponding GST-fusion protein, GST-SKL or GST-LKS, respectively. After centrifugation at 260,000 x g for 29 h at 4 °C (SW41 rotor, Beckman), 13 fractions of 0.8 ml were collected from the bottom of the tube, immediately precipitated with TCA and analyzed by SDS-PAGE under

nonreducing conditions. All recombinant proteins and sedimentation standards were pretreated with 10 mM IAA before use.

8 - Miscellaneous

Ub-PEX5 thiolester conjugates were analyzed under nonreducing conditions. Samples were heated for 30 min at 37 °C in Laemmli gel loading buffer supplemented with 10 mM NEM and centrifuged for 2 min at 11,000 x *g* at room temperature to remove insoluble material, before being loaded onto the gel. Nonreducing SDS-PAGE was done at 4 °C. Samples analyzed under reducing conditions were heated in Laemmli gel loading buffer supplemented with 100 mM DTT for 20 min at 65 °C instead.

Gels containing protein fractions resultant from the purification procedure of E2(s) were stained with Colloidal Coomassie Blue G-250. Gels containing ³⁵S-labeled proteins were transferred onto a nitrocellulose membrane and exposed to an x-ray film. When indicated the membranes were probed with antibodies.

PEX5 was detected on Western blots using an antibody directed to human PEX5 [130] or by blot overlay using human ³⁵S-labeled PEX14, as described before [140]. The anti-PMP70 antibody [191] was kindly provided by Dr. Wilhelm J. Just (University of Heidelberg, Heidelberg, Germany). The anti- α -tubulin was purchased from Sigma. Rabbit and mouse antibodies were detected on Western blots using alkaline phosphatase-conjugated anti-rabbit and anti-mouse IgGs (Sigma), respectively.

Densitometric analysis of x-ray films were performed using the UN-SCAN-IT automated system.

IV. RESULTS

1- Partial purification of the mammalian PEX4 functional counterpart

Our group has developed two versions of an *in vitro* system specially suited to study the PEX5-mediated peroxisomal import pathway. In one version a PNS fraction is used as organelle source and it is possible to reconstitute/monitor all steps of the cycle of PEX5 (see Fig. 6): cargo-dependent but ATP-independent insertion into the DTM, monoubiquitination at the conserved N-terminal cysteine (the ATPyS-insensitive stage 2-to-stage 3 transition) and ATP-dependent (and ATPyS-sensitive) export into the cytosol. When highly purified peroxisomes are used instead, although the insertion of PEX5 into the DTM (in a cargo-dependent and ATP-independent manner) is still possible, the stage 2-to-stage 3 transition, *i.e.* the thiol-sensitive monoubiquitination, is impaired. Consequently PEX5 accumulates at the peroxisomal membrane as it cannot be recognized and therefore not exported by the REM. This is in agreement with the inexistence of ubiquitin and/or some other component(s) of the UCC in adequate amounts in this incomplete version of the *in vitro* import system. In order to identify the rat liver E2 enzyme(s) that mediates ubiquitination of the conserved cysteine residue of PEX5 (at position 11 in mammals), we have exploited the properties of the peroxisome-based incomplete *in vitro* system.

³⁵S-labeled ΔC1-PEX5 synthesized in an *E. coli* transcription/translation system was used in *in vitro* reactions containing peroxisomes. The aim was two-fold. First, ΔC1-PEX5 (a truncated version of the large isoform of human PEX5 comprising its first 324 amino acids) was chosen instead of the full-length protein because Cys11 is the only cysteine residue present in this truncated PEX5 version. Moreover, despite lacking the C-terminal cargo-binding domains, this protein is completely competent in the insertion, ubiquitination and export steps. Second, an *E. coli*, and not a rabbit reticulocyte, transcription/translation system was chosen to synthesize this protein because bacteria do not possess an UCC. *In vitro* reactions were also supplemented with GST-Ub. This ubiquitin analogue is efficiently used by the UCC acting on Cys11 of PEX5. However, probably due to its bulkiness, GST-Ub-PEX5 is not a substrate for the ATP-dependent REM [142]. As a result GST-Ub-³⁵S-(ΔC1-)PEX5 conjugates accumulate at the peroxisomal membrane, thereby simplifying the ubiquitination assays as only organelle pellets need to be analyzed.

It is important to highlight that the isolation/purification process was monitored by SDS-PAGE profile analysis and by ubiquitination activity assays of the protein fractions.

1.1 - PEX5 monoubiquitination at Cys11 requires both peroxisomal and cytosolic components

In order to purify the E2(s) mediating the thiol-sensitive ubiquitination of PEX5, we first asked whether E1-supplemented purified peroxisomes could carry out ^{35}S - ΔC1 -PEX5 ubiquitination. As shown in Fig. 8A, no ubiquitinated ^{35}S - ΔC1 -PEX5 was detected under this condition (*lane Per+E1*). Incubation of ^{35}S - ΔC1 -PEX5 with a cytosolic fraction did not result in GST-Ub- ^{35}S - ΔC1 -PEX5 conjugates either (*lane Cyt*). Generation of thiol-sensitive GST-Ub- ^{35}S - ΔC1 -PEX5 species was only detected when both peroxisomes and the cytosolic fraction were included in the assay (*lane Cyt+Per*). The radiolabeled protein band detected under these conditions (*arrow a*) was affinity-purified with immobilized GSH and thiol-sensitive, confirming that this protein band corresponds indeed to GST-Ub- ^{35}S - ΔC1 -PEX5 thiolester conjugates (Fig. 1B). Thus, the results obtained suggested that besides the E1 enzyme, the cytosolic fraction contained other component(s) of the UCC required to ubiquitinate Cys11 of PEX5. The most probable candidate(s) would be the E2 enzyme(s).

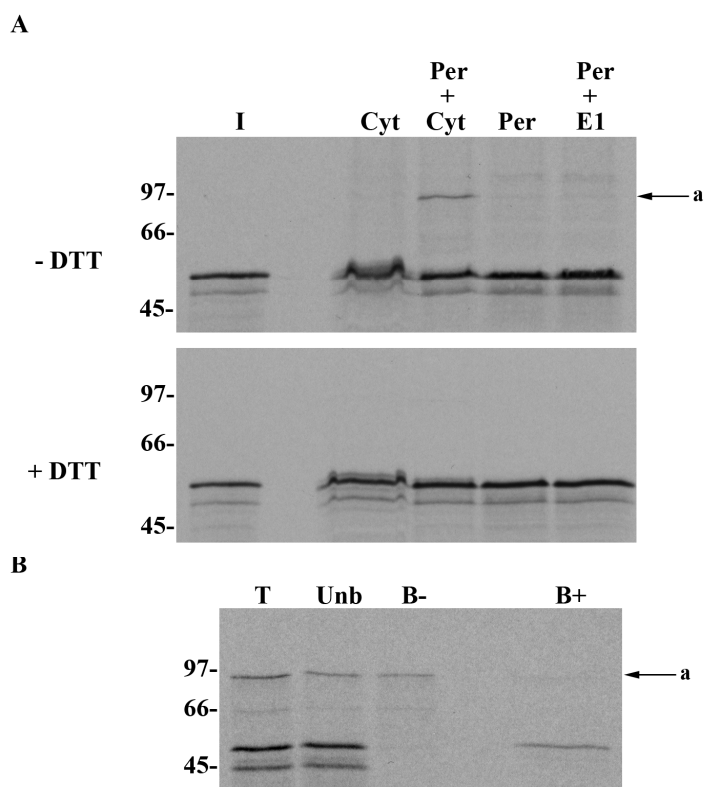


Figure 8 - Ubiquitination of ΔC1 -PEX5 requires both cytosolic and peroxisomal components. A, ^{35}S -labeled ΔC1 -PEX5 was incubated in import medium containing ATP and GST-Ub with cytosol alone (Cyt; 500 μg of protein), cytosol and peroxisomes (Per+Cyt), or peroxisomes in the absence (Per) or presence of recombinant E1 (Per+E1). Organelle pellets and 1/4 of the Cyt sample were analyzed by SDS-PAGE under nonreducing (-DTT), and reducing conditions (+DTT). Lane I, 10% of the *E. coli* lysate containing ^{35}S -labeled ΔC1 -PEX5 used in each lane. a, GST-Ub- ΔC1 -PEX5. Numbers to the left indicate the molecular masses of reduced protein standards in kDa. B, ^{35}S -labeled ΔC1 -PEX5 was incubated in

import buffer containing cytosol, peroxisomes, GST-Ub and ATP. The organelles were isolated by centrifugation, solubilized with detergent, and subjected to a pull-down assay using glutathione-Sepharose beads. The total (T), unbound (Unb), and bound fractions (in this case, treated (B-) or not (B+) with DTT) were subjected to SDS-PAGE. Three equivalents of the bound fraction in relation to the total and unbound samples were loaded onto the gel.

1.2 - The E2(s) acting on PEX5 Cys11 do(es) not interact with a DE52 matrix

Based on the hypothesis that the E2(s) acting on Cys11 of PEX5 is cytosolic, we moved forward on its isolation from rat liver cytosol. We applied the two-step procedure previously developed and used by Ciehanover *et al.* [189] to purify E2 enzymes from rabbit reticulocyte lysates. In the first step the cytosolic fraction is subjected to anion-exchange chromatography using a DE52-column. Two protein fractions are obtained: Fraction I (unadsorbed proteins; includes ubiquitin) [192] and Fraction II (adsorbed proteins eluted with a high salt buffer; contains E1 and E2 enzymes among other proteins). In the second step, the ubiquitin-depleted Fraction II is subjected to covalent affinity chromatography using an ubiquitin-derivatized matrix. Surprisingly, when Fraction II was tested in our *in vitro* assay no GST-Ub-³⁵S-ΔC1-PEX5 conjugates were detected. Two hypotheses were raised to explain the absence of ubiquitinating activity of Fraction II: 1) the fractionation procedure destroyed the relevant E2(s); or 2) the E2(s) of interest is found in Fraction I and due to the lack of the E1 enzyme it can no longer generate ubiquitinated PEX5 conjugates. We decided to test this second hypothesis. Indeed, when Fraction I was supplemented with E1, the ubiquitinating activity on ³⁵S-ΔC1-PEX5 was recovered, in contrast to the results obtained with the E1-supplemented Fraction II (Fig. 9).

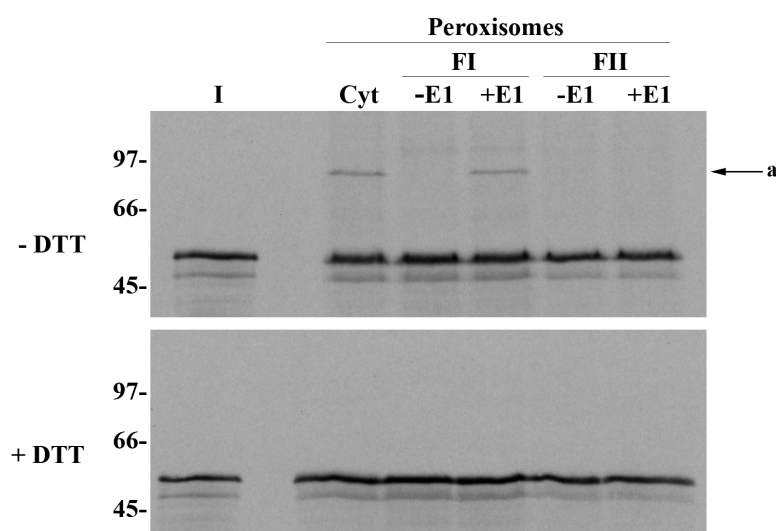


Figure 9 - Cys11 of ΔC1-PEX5 is ubiquitinated by E2(s) that do(es) not bind to DE52. ³⁵S-labeled ΔC1-PEX5 was subjected to import reactions containing peroxisomes and GST-Ub supplemented either with 500 μg cytosolic protein or the corresponding amounts of Fraction I (FI) or Fraction II (FII) in the absence (-E1) or presence of recombinant E1 (+E1). The samples were then supplemented with NEM, the organelles were isolated by centrifugation, and analyzed by SDS-PAGE under nonreducing (-DTT) or reducing conditions (+DTT). a, GST-Ub-ΔC1-PEX5. Lane I, 10% of the *E. coli* lysate containing ³⁵S-labeled ΔC1-PEX5 used in each lane. Numbers to the left indicate the molecular masses of reduced protein standards in kDa.

Altogether, and contrary to our expectations, the E2(s) mediating thiol-sensitive ubiquitination of PEX5 was present in Fraction I and not in Fraction II. If on one hand the fact that Fraction I contains just a few E2 enzymes [193,194] greatly reduces the number of putative E2 candidates acting on Cys11 of PEX5, on the other, the presence of ubiquitin hampers an affinity chromatography step. Nevertheless, the finding that the cytosolic protein fraction precipitated with 16% (w/v) PEG conserved the majority of the ubiquitination activity, whereas ubiquitin was mostly found in the supernatant, allowed us to move forward to the affinity chromatography step. The results concerning this purification step are presented in Fig. 10. Virtually all the ubiquitinating activity of the PEG-precipitated fraction is recovered in the high pH/DTT eluate of the ubiquitin-agarose column (*lane E, lower panel*, Fig. 10A). As the complexity of this fraction was still considerably high (*lane E, upper panel*, Fig. 10A), we took advantage of the fact that the relevant E2(s) do(es) not interact with DE52 to perform an extra purification step. As expected, GST-Ub-³⁵S-ΔC1-PEX5 conjugates were detected mainly when using the unbound-DE52 protein fraction (*lane Unb, lower panel*, Fig. 10B). SDS-PAGE analysis of this fraction revealed two protein bands (*lane Unb, upper panel*, Fig. 10B). Mass spectrometry analysis performed by the group of Bettina Warscheid (Medizinisches Proteom-Center, Ruhr-Universitaet Bochum, Bochum, Germany) identified E2L3 (UbchH7) as the main protein present in slower migrating band (*band 1*). This same protein was present in the faster migrating band (*band 2*) together with E2N (UbchH13) and the three closely related E2 enzymes E2D1 (UbchH5a), E2D2 (UbchH5b) and E2D3 (UbchH5c).

In an attempt to identify which of the five E2s were involved in the ubiquitination of Cys11 of PEX5, Andreia Carvalho screened a commercially available collection of human recombinant E2 enzymes containing the human orthologs of the five rat liver E2s candidates. Using the peroxisome-dependent PEX5-ubiquitination assay it was possible to demonstrate that all the three members of the UbchH5 E2 family are able to promote thiol-sensitive ubiquitination of PEX5 as long as they are catalytically active. Thus, UbchH5 proteins are the functional counterparts of yeast PEX4. UbchH5-mediated ubiquitination of PEX5 was further characterized by Andreia Carvalho and it is discussed elsewhere ([190]; see also *Discussion*).

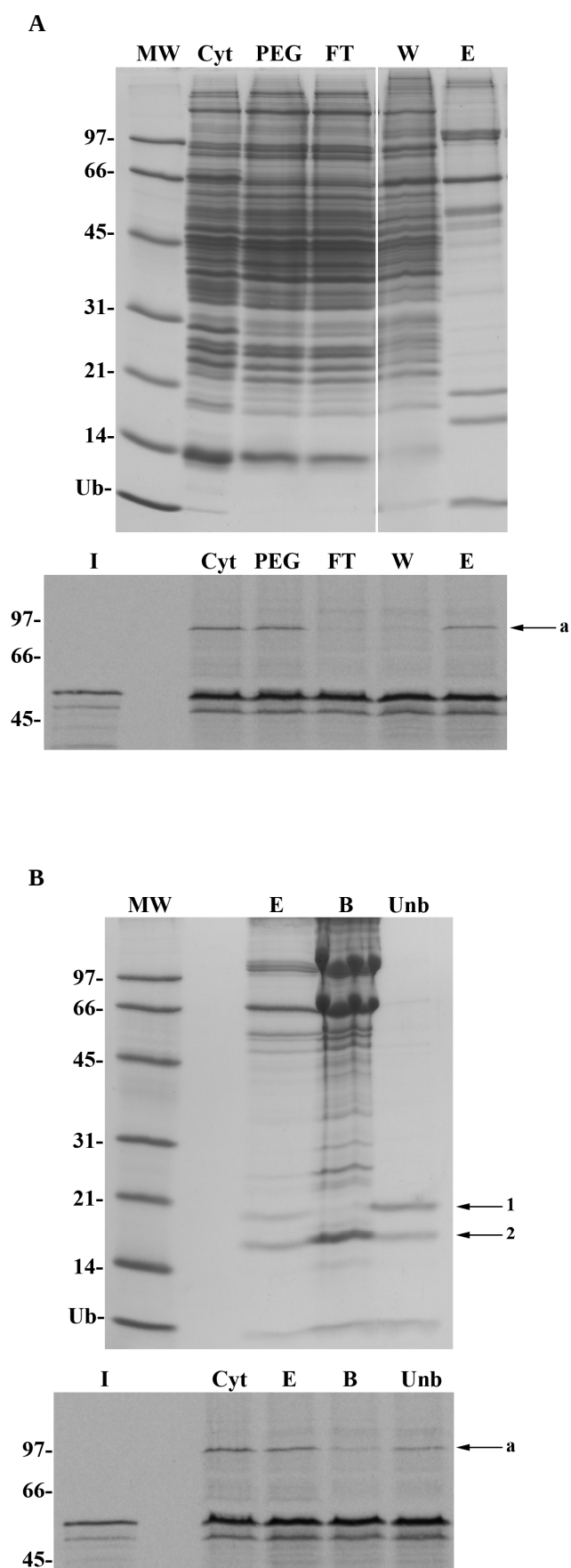


Figure 10 - Partial purification of the E2(s) mediating the ubiquitination of Cys11 of Δ C1-PEX5. A, protein fractions obtained during the purification procedure were analyzed by SDS-PAGE/Coomassie Blue staining (*upper panel*) and assayed for peroxisome-dependent ubiquitination activity on Δ C1-PEX5 (*lower panel*). In the *upper panel*, 100 μ g of cytosolic protein (Cyt) were loaded onto the gel; the PEG-precipitated fraction (PEG) and the affinity chromatography flow-through (FT), wash (W), and eluate (E) fractions shown were derived from 0.2, 0.2, 15, and 15 mg of cytosolic protein, respectively. In the ubiquitination assay, cytosolic protein (500 μ g) and PEG-precipitated, flow-through, wash, and eluate fractions derived from 500 μ g of cytosolic protein were used. a, GST-Ub- Δ C1-PEX5. Lanes I, 10% of the *E. coli* lysate containing ^{35} S-labeled Δ C1-PEX5 used in each lane. Numbers to the left indicate the molecular masses of reduced protein standards in kDa (*lanes MW*); Ub, 2 μ g of bovine ubiquitin. B, the affinity chromatography eluate and the DE52-bound (B) and unbound (Unb) fractions derived from 8, 25, and 25 mg of cytosolic protein, respectively, were analyzed by SDS-PAGE and stained with colloidal Coomassie Brilliant Blue (*upper panel*). Protein bands 1 and 2 were subjected to mass spectrometry. The activity assays (*lower panel*) were performed as described above.

2 - Characterization of the Ub-PEX5 thiolester conjugate

Monoubiquitination of mammalian PEX5 at the conserved N-terminal cysteine while inserted into the DTM was shown to be part of the normal PEX5 transit through the peroxisomal membrane [142]. Although never demonstrated it is suggested that in the cytosol (thus soluble and already exported) Ub-PEX5 is converted into PEX5. As ubiquitination of proteins at cysteine residues seems to be a quite uncommon event, we were puzzled why a cysteine and not a lysine was the chosen residue in this ubiquitination reaction. In the following sections (2.1-2.3) the results of the experiments performed to clarify this issue are presented.

2.1 - PEX5(C11K) is a competent protein in all steps of the PEX5-mediated peroxisomal import pathway

As our initial question concerned the identity of the ubiquitinated residue (cysteine *versus* lysine) and thus the chemical nature of the ubiquitinated PEX5 conjugate (thiolester *versus* isopeptidic bond, respectively), we decided to replace the conserved cysteine at position 11 of PEX5 by a lysine (PEX5(C11K)), a serine (PEX5(C11S)), an arginine (PEX5(C11R)) or an alanine (PEX5(C11A)) residue. These PEX5 versions were then tested in the complete version of our *in vitro* import system. The results are depicted in Fig. 11. When incubated with a rat liver PNS fraction in the presence of ATP, the wild-type peroxin continuously enters the DTM, being monoubiquitinated and rapidly exported back into the soluble phase of the reaction in an ATP-dependent manner (*lanes 1 and 2*) [122,138,140,142]. In contrast, although PEX5(C11S) becomes inserted into the DTM, it is a poor substrate for the UCC acting on PEX5, thereby accumulating at the peroxisomal membrane (compare *lanes 1 and 2* with *5 and 6*, respectively) [134,142]. The same distribution pattern was obtained for PEX5(C11R) (*lanes 5 and 6, lower panel*) and PEX5(C11A) (*lanes 7 and 8, lower panel*). Neither PEX5(C11R) nor PEX5(C11A) can be ubiquitinated at the residue at position 11. To our surprise, not only PEX5(C11K) was still a substrate for the peroxisomal E3 ligase(s) catalyzing this ubiquitination event, but also its distribution between organelle pellet and soluble phase suggests that like the wild-type peroxin this mutant is promptly removed from the peroxisomal membrane by the REM (compare *lanes 1 and 9, upper panel*). It should be noted that the presence of monoubiquitinated species of PEX5 and PEX5(C11K) in the soluble phase of the reactions (*lanes 2 and 10*) show that a fraction of both proteins has indeed passed through the DTM, because only DTM-embedded PEX5 molecules are monoubiquitinated in this *in vitro* system [190].

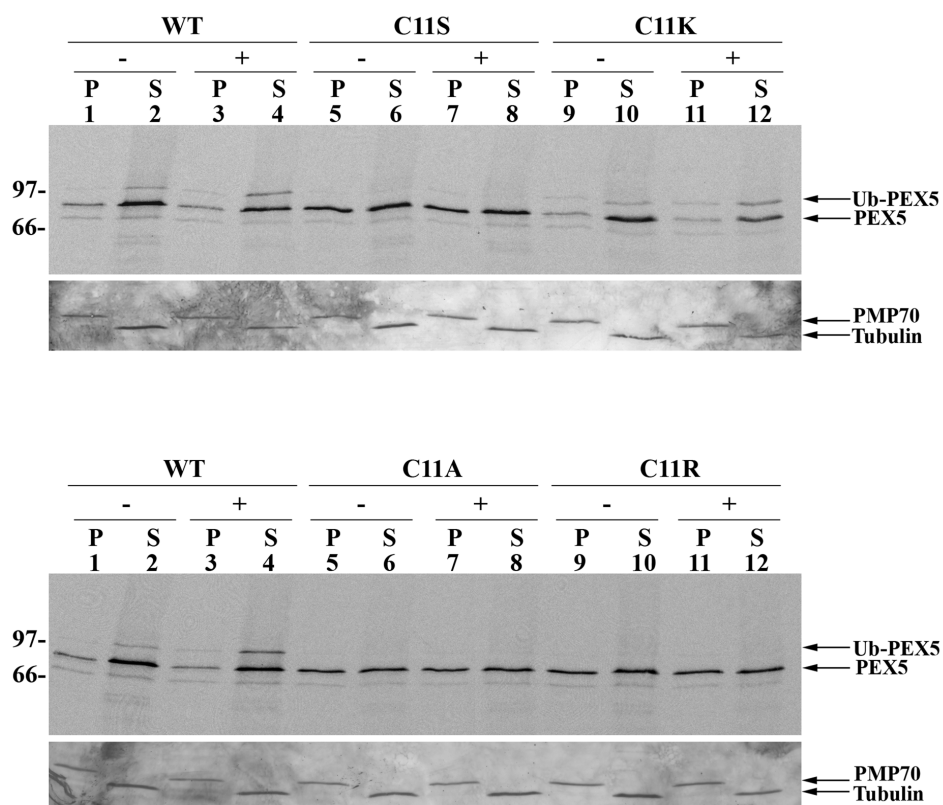


Figure 11 - PEX5(C11K) is a substrate for the peroxisomal import and export machinery. Import reactions containing 400 μ g of PNS protein were programmed with ^{35} S-labeled PEX5 (WT) or with mutant versions in which Cys11 was replaced by a serine (C11S), a lysine (C11K), an alanine (C11A), or an arginine (C11R). The samples were incubated for 30 min at 37 °C in the absence (–) or presence (+) of Ubal. After treatment with NEM, the samples were separated into an organelle pellet (P) and a supernatant (S) by centrifugation, subjected to SDS-PAGE under nonreducing conditions, and blotted onto a nitrocellulose. The membrane was exposed to an x-ray film and afterward probed sequentially with antibodies against PMP70 and α -tubulin. Note that there is always a large fraction of ^{35} S-labeled PEX5 that never passes through the DTM. Competition with endogenous rat liver PEX5 surely contributes to the low yields of Ub-PEX5 in these assays. *Numbers to the left* indicate the molecular masses of reduced protein standards in kDa.

In an attempt to confirm and eventually strengthen the results obtained with our *in vitro* system, Sofie Huybrechts from Dr. Marc Fransen laboratory (KU Leuven, Leuven, Belgium) conducted *in vivo* studies with wild-type PEX5 and the mutant versions PEX5(C11S) and PEX5(C11K) whose results are presented in Fig. 12. *PEX5*^{-/-} mouse embryonic fibroblasts [195] were transiently transfected with a bicistronic plasmid encoding both the PEX5 version of interest and a reporter cargo protein recognized by PEX5, EGFP-PTS1, and 48 h after transfection processed for (immuno)fluorescence (for experimental details see Ref. 196). In *PEX5*-KO mice fibroblasts expressing PEX5, EGFP-PTS1 is mostly peroxisomal as indicated by its co-localization with PEX14, a peroxisomal membrane protein marker (punctate distribution pattern). PEX5(C11K) was found to display the same effect as the wild-type peroxin, *i.e.* to restore peroxisomal biogenesis in these cells. A different scenario is observed in PEX5(C11S)-transfected *PEX5*-KO cells where the reporter protein remains in the cytosol. The observations reported for *PEX5*-KO fibroblasts were further extended in CHO cells transfected with these same plasmids. The experiment performed with CHO cells is particularly important since it illustrated the dominant negative effect of PEX5(C11S) expected from our *in vitro* observations. As the serine residue at position 11 is a very poor substrate for the UCC acting on PEX5, PEX5(C11S) becomes trapped at the DTM and thus blocking it, impairing the endogenous PEX5-promoted import of peroxisomal matrix proteins. In agreement with our expectations, PEX5 and PEX5(C11K) did not interfere with the proper peroxisomal targeting of EGFP-PTS1 in CHO cells.

In conclusion, the *in vivo* observations corroborate the *in vitro* results, thus reinforcing the validity of the *in vitro* system used to address questions related with the PEX5-mediated peroxisomal protein targeting.

All in all, PEX5(C11K) is a functional protein in all the steps of PEX5 cycle, differing from the wild-type protein only in the chemical nature of the bond linking ubiquitin to the residue at position 11 in ubiquitinated PEX5 conjugates. Therefore PEX5(C11K) constitutes the appropriate control to address the lability of ubiquitinated PEX5 species.

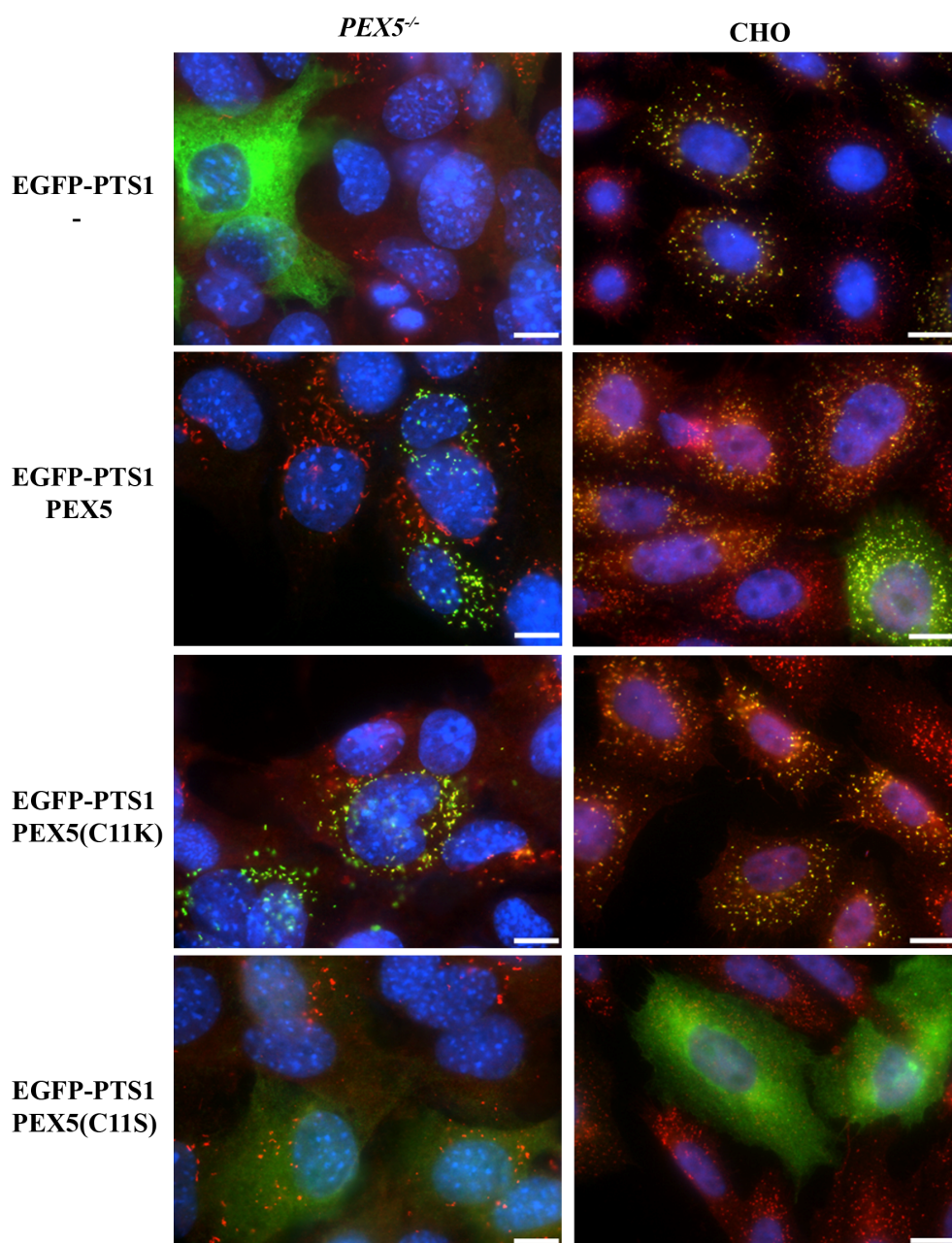


Figure 12 - Localization of EGFP-PTS1 in *PEX5*^{-/-} and CHO cells upon co-expression with PEX5, PEX5(C11K), or PEX5(C11S). PEX5-deficient mouse fibroblasts (*PEX5*^{-/-}) or CHO cells were transiently transfected with a bicistronic plasmid encoding EGFP-PTS1 and no other protein (–) or one of the indicated PEX5 variants. After 48 h, the cells were fixed and processed for fluorescence microscopy by using anti-PEX14 antibodies (red). The nuclei were counterstained with 4',6'-diamino-2-phenylindole (blue). Scale bar, 10 μ m.

2.2 - Stage 4 PEX5 is more susceptible to both DUB- and GSH-promoted deubiquitination than stage 3 PEX5

Deubiquitinating enzymes (DUBs) balance the action of the UCC removing ubiquitin from modified substrates [197,198].

The *in vitro* reactions shown in Fig. 11 were performed in the absence and in the presence of Ubal, an inhibitor of DUBs [199]. If one compares the amount of soluble Ub-PEX5 and UbPEX5(C11K) detected in the absence (*lanes 2 and 10, upper panel*) and in the presence (*lanes 4 and 12, upper panel*) of the DUB-inhibitor, we note that slightly higher yields of these species are recovered in the presence of Ubal (30%). To obtain a clearer result, we tried to circumvent the unavoidable dilution of rat liver components (UCC components, DUBs, etc) in our *in vitro* assay, by increasing the concentration of PNS by a factor of two. The results are shown in Fig. 13. In this assay, after generation of Ub-PEX5 species (DTM-embedded, stage 3 PEX5; and soluble already exported, stage 4 PEX5), apyrase and a vast excess of recombinant PEX5 were added. Recombinant PEX5 prevents *de novo* insertion of radiolabeled PEX5 molecules in a cargo-dependent mode into the DTM by competing with ³⁵S-PEX5 for the binding of cargoes. Apyrase depletes ATP thereby preventing *de novo* ubiquitination (stage 2-to-stage 3 transition) and export of Ub-PEX5 back into the soluble phase of the reactions (stage 3-to-stage 4 transition), by blocking the ATP-dependent UCC cascade and export machinery, respectively (see Fig. 6). Then deubiquitination was allowed to proceed for an additional 20 min. When this procedure was performed in the presence of Ubal, a large amount of stage 4 PEX5 was detected (compare *lanes 2 and 4*). In contrast, no stage 4 PEX5 could be detected in the absence of Ubal. Interestingly, no significant differences were registered for stage 3 PEX5 between the two conditions tested (absence and presence of Ubal; *lanes 1 and 3*, respectively). Thus, besides confirming the existence of an enzymatic Ubal-sensitive deubiquitination mechanism acting on stage 4 PEX5, this experiment also revealed that stage 3 PEX5, contrary to stage 4, is not a substrate for DUBs.

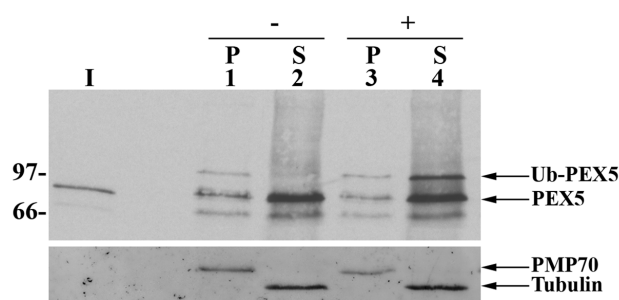


Figure 13 - Stage 3 and stage 4 PEX5 display a different susceptibility to DUBs. ^{35}S -labeled PEX5 was subjected to *in vitro* import reactions containing 800 μg of PNS in 100 μl , in the absence (-) or presence (+) of Ubal. After 20 min at 37 $^{\circ}\text{C}$, apyrase was added, the reactions were incubated for 3 min and then supplemented with recombinant PEX5. Incubation proceeded for an additional 20 min. After NEM treatment, the reactions were separated into organelle pellets (P) and supernatants (S), subjected to SDS-PAGE under nonreducing conditions, and blotted onto a nitrocellulose membrane. The membrane was exposed to an x-ray film and afterward probed sequentially with antibodies against PMP70 and α -tubulin. Lane I, 10% of the ^{35}S -labeled PEX5 reticulocyte lysate used in each lane. Numbers to the left indicate the molecular masses of reduced protein standards in kDa.

In addition to the classical enzymatic mechanism of deubiquitination, the fact that PEX5 is ubiquitinated at a cysteine residue, thereby yielding a thiolester conjugate, raises the possibility that a fraction of stage 4 PEX5 might be deubiquitinated by a nonenzymatic deubiquitinating mechanism promoted by physiological relevant nucleophilic compounds, as for example GSH. In fact Rose and Warms [169] had previously shown that GSH can disrupt the E1-S~Ub thiolester conjugate quite efficiently. To determine if the same could be true for Ub-PEX5 thiolester conjugates, stage 3 and stage 4 PEX5 were generated *in vitro* and incubated with physiological concentrations of GSH (5 mM in rat liver, [200]) (see Fig. 14, *upper panels*). We noticed that stage 4 PEX5 is highly susceptible to GSH-promoted deubiquitination (half-life, 2.3 min), whereas stage 3 PEX5 is more resistant (half-life, 10 min). Nevertheless, if incubated in the presence of DTT (a smaller and stronger nucleophile), stage 3 PEX5 regenerates stage 2 PEX5 (DTM-embedded nonmodified PEX5), indicating the disruption of the thiolester bond. Ubiquitinated PEX5(C11K) species were also tested for this mode of deubiquitination (Fig. 14, *lower panels*). As expected the isopeptidic bond linking ubiquitin to PEX5(C11K) cannot be disrupted either by GSH-, DTT- or heat-treatment in the presence of 100 mM DTT and 2% SDS, and thus the amount of Ub-PEX5(C11K) species (stage 3 and stage 4) remains unaltered.

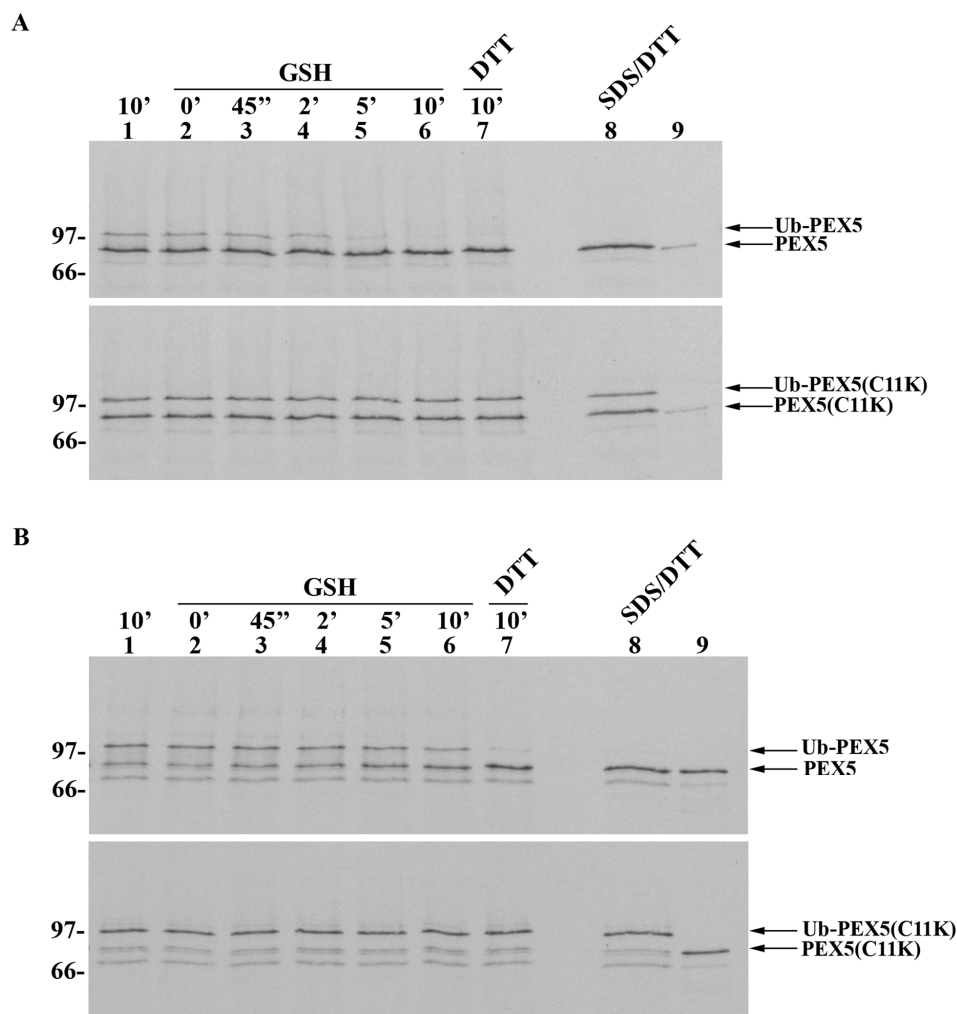


Figure 14 - The susceptibility of the Ub-PEX5 thiolester bond to GSH is context-dependent. A, soluble Ub-PEX5 (*upper panel*) and Ub-PEX5(C11K) (*lower panel*) were generated as described under *Experimental Procedures*, incubated with 5 mM GSH (*lanes 3–6*) or 5 mM DTT (*lane 7*) for the indicated periods of time, and treated with 20 mM NEM for 5 min on ice. The samples in *lanes 1* and *2* received the same amount of NEM-neutralized GSH after 10 and 0 min of incubation at 37 °C, respectively. All of the samples were subjected to TCA precipitation and analyzed by SDS-PAGE under nonreducing (*lanes 1–7*) or reducing conditions (*lanes 8*; 100 mM DTT, 2% (w/v) SDS, 15 min at 65 °C). *Lanes 9*, rabbit reticulocyte lysates containing the ^{35}S -labeled PEX5 and PEX5(C11K) proteins. B, the same as in A) with the exception that membrane bound Ub-PEX5 (*upper panel*) and Ub-PEX5(C11K) (*lower panel*) were used. Autoradiographs are shown. Numbers to the left indicate the molecular masses of reduced protein standards in kDa.

To gain more insight into the susceptibility of stage 3 and stage 4 PEX5 to deubiquitination we have analyzed the steady-state levels of rat liver PEX5 and Ub-PEX5 in a PNS freshly prepared in the presence of NEM (an alkylating reagent that inhibits DUBs and blocks the thiol group of endogenous GSH, thus preventing deubiquitination) (Fig. 15). When analyzed under nonreducing conditions, a small portion of rat liver PEX5 can be detected as a 100-kDa species that corresponds to Ub-PEX5 as judged by its molecular weight and DTT sensitivity (compare lanes 1 and 4). Ub-PEX5 is only detected in the organelle pellet (*lane 2*). On the other hand, in the soluble phase of the PNS fraction, obtained upon a centrifugation step, only nonmodified PEX5 (*lane 3*) is detected, suggesting that *in vivo* stage 4 PEX5 is promptly deubiquitinated.

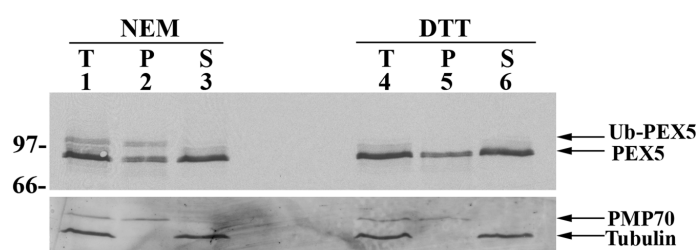


Figure 15 – Steady-state analysis of rat liver PEX5 species. Rat liver PNS prepared in the presence of NEM (T) was separated into an organelle pellet (P) and supernatant (S) by centrifugation. Equivalents to 80 µg of PNS protein were analyzed by SDS-PAGE under nonreducing (NEM; lanes 1–3) or reducing conditions (DTT; lanes 4–6). Endogenous rat PEX5, Ub-PEX5, PMP70, and α-tubulin are indicated. Numbers to the left indicate the molecular masses of reduced protein standards in kDa.

This set of results allows us to conclude that Ub-PEX5 conjugates can be disrupted by a combination of enzymatic and nonenzymatic mechanisms. In addition, another important conclusion can be drawn. Ub-PEX5 exhibits context-dependent susceptibilities to both deubiquitination mechanisms: stage 4 (soluble already exported Ub-) PEX5, in contrast to stage 3 (DTM-embedded Ub-) PEX5, is a substrate for DUBs and is more susceptible to GSH-promoted nucleophilic attack. The resistance of stage 3 PEX5 to both enzymatic and nonenzymatic deubiquitination should avoid futile ubiquitination/deubiquitination cycles at the DTM.

2.3 - Deubiquitination is not a mandatory step for the cargo-dependent insertion of PEX5 into the DTM

It had been assumed that, upon ATP-dependent release of Ub-PEX5 from the DTM back into the cytosol, the ubiquitin moiety is removed thus regenerating nonmodified PEX5. The data presented in the previous *Results* section (2.2) validates this hypothesis.

Deubiquitination of PEX5 could be related with the need to have nonubiquitinated PEX5 available to promote further cycles of protein transportation. However, the ratio of unconjugated to ubiquitinated PEX5 is similar in the organelle pellet and in the soluble phase of the import reactions presented in Fig. 11. This observation suggests that both Ub-PEX5 and Ub-PEX5(C11K) may have the capacity to reenter the DTM.

In order to clarify this matter, a cargo-dependence assay was performed. Before describing the results obtained (Fig. 16), a brief explanation on the fundamentals of this assay is provided. Insertion of soluble and nonmodified PEX5 into the DTM occurs only when the cycling receptor is in complex with cargo proteins. If PTS1-containing proteins are sequestered the import is inhibited. The addition of recombinant TPRs-PEX5 (PTS1-binding domain) has this effect. Besides PEX5 monoubiquitination at Cys11, cargo-dependent PEX5 insertion into the peroxisomal membrane can also be assessed by protease protection assays. While soluble (Ub-)PEX5 is completely sensitive to exogenously added protease, membrane-bound PEX5 can be either partly protease accessible (an N-terminal segment of ~2 kDa is clipped) - PEX5 at stage 2 - or completely protease-resistant - PEX5 at stage 3 level (see Fig. 6). We applied this strategy to address the putative insertion into the DTM of Ub-PEX5 (stage 4). Two *in vitro* reactions were performed. In the first reaction the aim was to generate soluble Ub-PEX5. The supernatant of this *in vitro* assay, containing a mixture of Ub-PEX5 and PEX5, was treated with IAA and then subjected to the cargo-dependence assay. This second reaction contained PNS and was performed at 16 °C to inhibit *de novo* ubiquitination. In addition, this reaction was supplemented with a vast excess of GST-Ub to evaluate the efficiency of the inhibitory strategy for *de novo* ubiquitination. In parallel, a control experiment was carried out. The first reaction occurred in the absence of ³⁵S-labeled PEX5. Radiolabeled PEX5 was mixed with the supernatant of the first reaction, IAA-treated and used in the cargo-dependence assay. The acquisition of a protease-resistant status by Ub-PEX5 indicates that this protein is truly inserted into the peroxisomal membrane DTM (*lane 1*). Moreover, the insertion step is shown to occur in a cargo-dependent manner, as the PTS1-containing peptide (*lanes 3*) reverts the inhibitory effect caused by the addition of recombinant TPRs (*lanes 2*) that sequester endogenous cargoes thereby preventing this step.

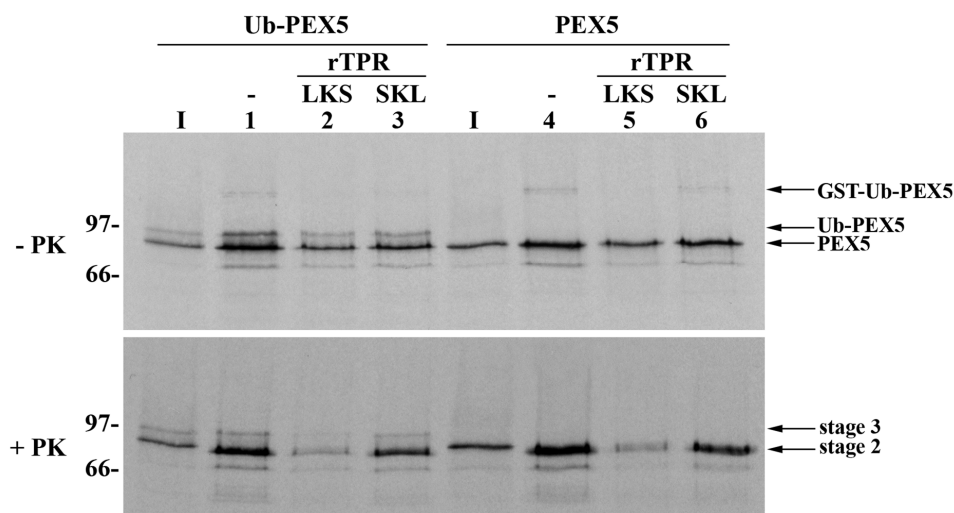


Figure 16 - Stage 4 PEX5 interacts with the DTM in a cargo-dependent manner. The supernatant of an *in vitro* reaction containing a mixture of ^{35}S -labeled PEX5 and Ub-PEX5 or a similar supernatant containing only the ^{35}S -labeled PEX5 were treated with IAA and subjected to an *in vitro* reaction at 16 °C for 30 min (see *Experimental Procedures* for details). A vast excess of GST-Ub was added to the reaction medium to assess the inhibition of *de novo* ubiquitination. The reactions in lanes 2 and 5 also contained a recombinant protein comprising the PTS1-binding domain of PEX5 (*rTPR*) plus a control peptide (*LKS*). The reactions in lanes 3 and 6 contained this inhibitory recombinant protein neutralized with a vast molar excess of PTS1-containing peptide (*SKL*). At the end of the incubation half of each sample was subjected to proteinase K treatment. Protease-treated (+PK) and untreated (–PK) halves were incubated on ice with 20 mM NEM, diluted with SEM buffer, and subjected to centrifugation. Organelle pellets were analyzed under nonreducing conditions by SDS-PAGE/autoradiography. Note the near absence of *de novo* ubiquitination of PEX5 (*GST-Ub-PEX5*) in these reactions. Lanes 1, 2.5% of the IAA-treated Ub-PEX5 and PEX5 radiolabeled proteins used in each lane. Numbers to the left indicate the molecular masses of reduced protein standards in kDa.

Thus, deubiquitination of PEX5 is not a mandatory event for the cargo-dependent insertion of PEX5 into the DTM.

This conclusion also suggests that ubiquitination does not alter significantly the cargo-binding properties of PEX5. To validate this supposition we subjected a mixture containing stage 0 (soluble and nonmodified) and stage 4 (soluble and already exported monoubiquitinated) PEX5 to a sucrose density centrifugation either in the absence or in the presence of a PTS1-containing cargo protein (GST-SKL) (Fig. 17). As shown previously by Costa-Rodrigues *et al.* [124] in the absence of GST-SKL the majority of PEX5 sediments as a monomeric protein above BSA. The abnormal sedimentation behaviour of PEX5 is due to the natively unfolded nature of its N-terminal segment as mentioned in the *Introduction* (see section 3.2.1.1). However, in the presence of GST-SKL, a complex between the cargo protein and PEX5 of stoichiometry 1:1 is formed. The PEX5-cargo complex sediments slightly below BSA. Most importantly, the distribution

profiles of Ub-PEX5 either in the presence or in the absence of GST-SKL completely match the corresponding ones displayed by unconjugated PEX5. Thus, PEX5 and Ub-PEX5 do have similar cargo-binding properties.

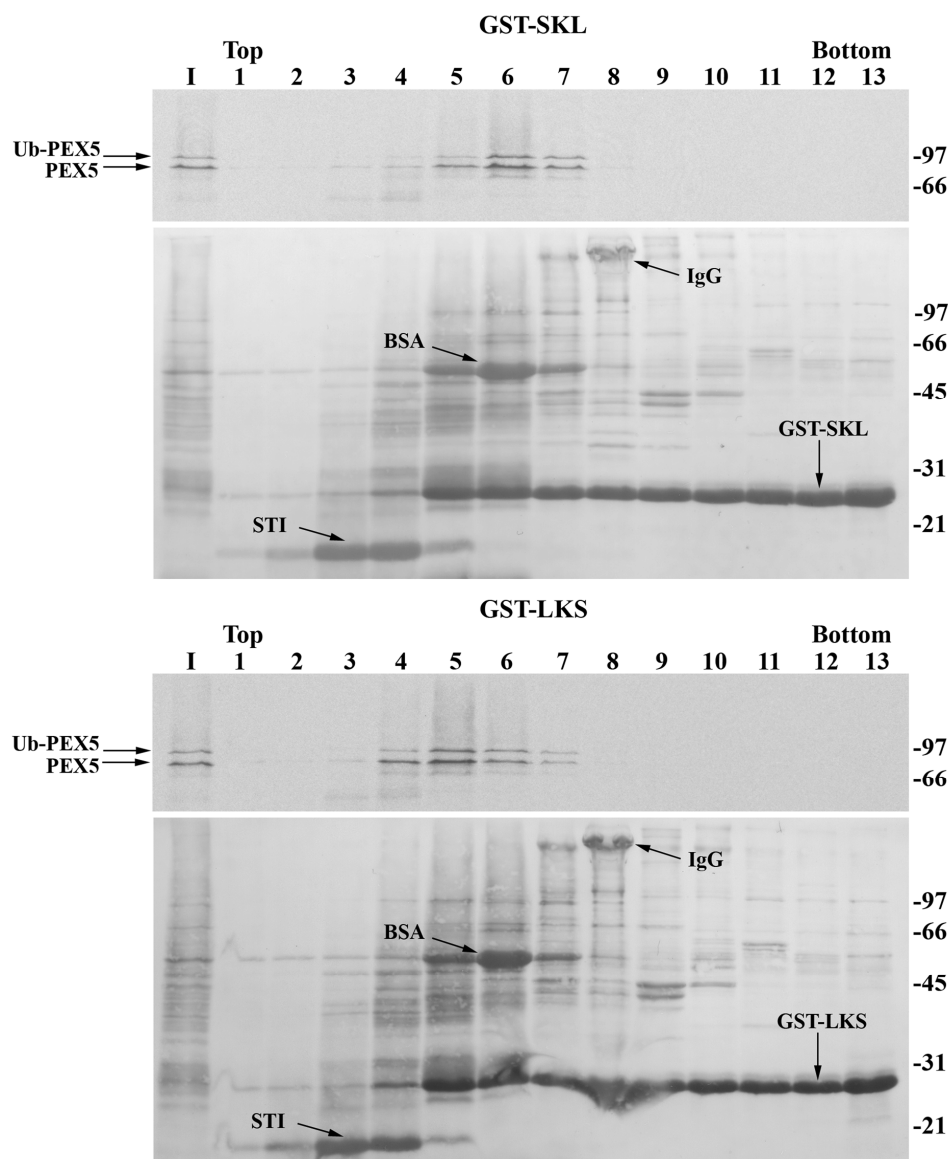


Figure 17 - Sucrose gradient sedimentation analysis of stage 4 PEX5. A mixture of soluble IAA-treated ^{35}S -labeled PEX5 and Ub-PEX5 was preincubated with either GST-SKL or negative control protein, GST-LKS. A mixture of proteins with known sedimentation coefficients was added to the samples immediately before loading onto the top of sucrose gradients containing either GST-SKL or GST-LKS, respectively. After centrifugation, the fractions were collected from the bottom of the tube, subjected to SDS-PAGE under nonreducing conditions, and blotted onto nitrocellulose membranes. Autoradiographs and the corresponding Ponceau S-stained membranes are shown. The internal sedimentation standards (soybean trypsin inhibitor (*STI*), bovine serum albumin (*BSA*), and immunoglobulins (*IgG*)) as well as GST-SKL and GST-LKS are indicated. *Lanes 1*, 25% of the IAA-treated Ub-PEX5 and PEX5 radiolabeled proteins used in each lane. *Numbers to the right* indicate the molecular masses of reduced protein standards in kDa.

V. DISCUSSION

Despite being a remarkable finding *per se*, ubiquitination of mammalian PEX5 uncovered other details on the PEX5-mediated peroxisomal protein import pathway. This ubiquitination event is related to PEX5 normal cycling rather than to a quality control process [142]. It is mandatory that DTM-embedded PEX5 is monoubiquitinated at its conserved N-terminal cysteine (Cys11) in order to be recycled back to the cytosol. These findings prompted us to further characterize this notable and unique pathway.

The identity of the E2(s) mediating this ubiquitination event was an immediate question. In *S. cerevisiae* the occurrence of PEX4-dependent monoubiquitination at the conserved N-terminal cysteine of PEX5 had been previously reported [141]. A mammalian ortholog of yeast/plant PEX4 would be the most probable candidate. PEX4 is an ubiquitin-conjugating enzyme anchored to the cytosolic face of the peroxisomal membrane through PEX22. The two proteins are suggested to constitute a structural/functional unit required for the peroxisomal matrix protein import pathway [177,180]. However, their mammalian orthologs remained to be found. The first aim of the work here presented was to identify the mammalian functional counterpart of yeast PEX4. For this purpose, we set up a protein purification procedure from rat liver cytosol coupled with a previously established peroxisome-dependent PEX5-ubiquitination assay to monitor the distribution of the relevant E2 throughout the process. Using a modified version of the method described by Ciechanover *et al.* [189], we obtained a low complexity protein fraction. In the two protein bands detected by SDS-PAGE analysis, five E2(s) were identified by mass spectrometry. Afterward Andreia Carvalho demonstrated that only the three members of the E2D family were able to promote thiol-sensitive monoubiquitination of PEX5. The E2D-mediated ubiquitination of PEX5 generates the appropriate substrate for the REM. Furthermore, it is possible that these conclusions are also extendable to humans as the same results were obtained with PNS from human skin fibroblasts [190]. The identification of rat liver E2D (UbcH5, human homologs) family members as the mammalian counterparts of yeast PEX4 is a major breakthrough. First, it provides a plausible explanation for the fact that several proteomic studies were not able to identify a peroxisome-associated E2. Indeed, it was demonstrated that the simple centrifugation of a PNS fraction was enough to deplete the PEX4-like activity from organelles [190]. On the other hand the inexistence of a peroxisomal-specialized E2 provides the biochemical explanation to why no functional counterparts of yeast/plant PEX22 were ever detected in *in silico* studies performed in mammals and in other organisms (see Refs. 40 and 41). Moreover the involvement of the E2D E2 family in the PEX5-mediated import pathway also explains why *PEX4*- and *PEX22*-based peroxisome biogenesis disorders were never identified in human patients up to now. The E2D/UbcH5 family comprises a promiscuous evolutionary conserved group of proteins that participate in a wide variety of biological processes [201-204]. In

addition, not only the three proteins are encoded by three different genes, but they also share a high sequence similarity [205,206]. Overall, and as it has been already suggested, it seems unlikely that the malfunction or the absence of these proteins will ever be associated with a specific peroxisomal biogenesis defect in humans [190].

As previously stated, ubiquitination of PEX5 at a cysteine residue, yielding a thiolester conjugate, is another interesting property of the peroxisomal cycling receptor. Although thiolesters are at the heart of the UCC chemistry, ubiquitination of proteins at cysteines and other nonlysine residues (threonines and serines, yielding oxyesters) was just recently uncovered [141,142,163-166].

No doubts remain about mammalian PEX5 being truly ubiquitinated at the conserved N-terminal cysteine residue. Several criteria allow us to state it: 1) the ubiquitinated PEX5 species generated are thiol-sensitive [142]; 2) PEX5 treated with IAA (-SH blocking agent) is no longer ubiquitinated [122]; 3) the replacement of the cysteine residue at position 11 by a serine residue does not yield thiol-sensitive ubiquitinated conjugates [142]; and 4) Δ C1-PEX5, a C-terminal truncated version of PEX5 which is as competent as the wild-type protein in all the steps of the import pathway, contains only one cysteine residue (the conserved N-terminal one) and is monoubiquitinated, yielding conjugates disrupted by reducing agents (section 1.1, *Results*). However the reason why a cysteine and not a lysine residue is used in this ubiquitination event is not presently known. Accordingly, the second aim of this work was to clarify this point.

One of the most surprising findings achieved during this work was the observation that the substitution of the conserved cysteine residue at position 11 of PEX5 by a lysine residue yielded a protein as functional as the wild-type protein. Consequently some of the hypotheses anticipatively advanced to justify the nontraditional ubiquitination of PEX5 were excluded. Early on, it was suggested that PEX5 could be an HECT-like E3 ligase. If this was the case, PEX5 would then transfer the ubiquitin attached through its N-terminal cysteine residue to a protein substrate. However the stability of the isopeptide bond linking Ub to PEX5(C11K) strongly argues against it, as it would make any PEX5(C11K)-promoted ubiquitination extremely unfavorable from the thermodynamic point of view. This same reasoning suggests that ubiquitination at the DTM can be made irreversible without a deleterious effect. Indeed, and in perfect agreement with this conclusion, assays using wild-type PEX5 have been unable to demonstrate the reversibility of the ubiquitination step (data not shown). Most probably, after the ubiquitination reaction a kinetic barrier is raised, thus obviating its reversibility. Another possible explanation could be related with the nucleophilic character of the target residues. Thiol groups of cysteines are better nucleophiles than ϵ -amine group of lysines. In line with this, the use of a cysteine instead

of a lysine could provide a kinetic advantage for the ubiquitination of PEX5 at the DTM. Nevertheless, this is not the case, otherwise PEX5(C11K) would display a dominant negative effect at the DTM as PEX5(C11S) does, accumulating and blocking it. Taken together neither the conserved cysteine nor the thiolester are important at any step occurring at DTM. Yet advantages in the last step of the PEX5-mediated import pathway *i.e.* its deubiquitination, can be envisaged. Not surprisingly deubiquitination of PEX5 may occur by a combination of nonenzymatic and enzymatic mechanisms. Strikingly the two mechanisms act in a context-dependent manner: stage 3 PEX5 is more resistant to both, thus avoiding futile cycles of ubiquitination/deubiquitination at the DTM. The data obtained for stage 3, indicates that Ub-PEX5 thiolester conjugate can exist in an environment protected from GSH. Thus, it illustrates that a thiolester bond in ubiquitin-protein conjugates have the potential to be as stable and durable as the isopeptidic bonds formed in classical lysine-based ubiquitination, provided that they are protected, either by an interacting protein or a membrane, from small nucleophiles.

Alternatively the labile nature of thiolester could result in a rapid stage 4-to-stage 0 transition using for this purpose the nonenzymatic cleavage in addition to the DUB-mediated one. Actually the half-life of 2.3 min determined for stage 4 PEX5 in the presence of GSH (5 mM, pH 7.2) is in perfect agreement with the one estimated for ubiquitin-E1 thiolester (half-life 1.4 min, 5 mM GSH, pH 7.2; based on the data in Ref. 169 and assuming that the glutathiolate anion, $pK_a = \sim 9.0$ [207], is the attacking species).

The need for a rapid regeneration of nonmodified PEX5 could be related to a putative requirement of the PEX5-mediated import cycle: only nonmodified PEX5 could be capable of promoting protein transportation. However, this hypothesis turned out to be false. Deubiquitination seems not to be mandatory step in this pathway. Indeed, soluble and already exported Ub-PEX5 can still bind cargo proteins, interact with and be inserted into the DTM efficiently, thus acquiring a protease-protected status. Nevertheless steady-state analysis of rat liver (Ub-)PEX5 levels indicate that *in vivo* a bypass of the stage 4-to-stage 0 transition might not occur, as we were not able to detect stage 4 PEX5. Williams *et al.* [141] reported similar steady-state levels for yeast PEX5. Thus it seems that stage 4-to-stage 0 transition occurs at a faster rate than stage 4 generation. Although cargo-dependent insertion of Ub-PEX5 species into the DTM might not be physiologically relevant, the *in vitro* assays and sedimentation analysis performed with these conjugates clearly show that upon ubiquitination PEX5 cargo-binding properties remain unchanged. If these properties are extrapolated to DTM-embedded PEX5 species (stage 2), it could be inferred that the ubiquitination event, *i.e.* the stage 2-to-stage 3 transition, is not coupled with cargo release into the organelle lumen as speculated by Williams *et al.* [141]. In fact this hypothesis has been confirmed recently by Alencastre *et al.* [146]. In this study the

authors developed a PTS2-centered *in vitro* system and showed that cargo release into the peroxisomal matrix occurs before stage 3a.

Another hypothetical advantage centered on the lability of the thiolester bond in stage 4 PEX5 can be forwarded. Although monoubiquitinated proteins are not generally considered substrates for the proteasomal degradation, the odds of ending in the proteasome might not be zero. In fact, monoubiquitin can be directly recognized by the proteasome itself [208-210]. Furthermore, modification by a single ubiquitin has been shown to be also sufficient for proteasomal processing of the p105 NF- κ B precursor [211]. Alternatively, an E4 component of the UCC can recognize monoubiquitinated proteins and promote the addition of further ubiquitin moieties, thus targeting the protein for proteasomal degradation [158,212]. The fast disruption of the thiolester bond in stage 4 PEX5 shortens its half-life and thereby could decrease its chances of being degraded. Attempts were made to validate this possibility. For this purpose PNS-based *in vitro* assays and transfection experiments with CHO cells stably expressing PEX5 and PEX5(C11K) were performed. According to our hypothesis, PEX5(C11K) would be less stable than PEX5 as the ubiquitin moiety cannot be removed by nucleophilic attack. However, no differences were observed between the levels of both proteins (data not shown). Nevertheless, it should be mentioned that this negative result might be consequence of the limitations underlying the experimental approaches adopted. If on one hand ubiquitin-proteasome system components are (unavoidably) highly diluted in our *in vitro* assays, on the other high nonphysiological levels of PEX5 and PEX5(C11K) in the transfection experiments are attained. Consequently, differences in the stabilities of the two proteins might have been easily missed.

The biological properties of cysteine residues might also provide an explanation for the evolutionary conservation of the cysteine residue at the N terminus of PEX5. Cysteine residues can be modified in numerous manners (*e.g.* nitrosylation, oxidation, glutathionylation, acylation, etc) in order to modulate protein activities [213,214]. In the specific case of PEX5, modification of its conserved N-terminal cysteine residue would block the DTM. Consequently peroxisomal matrix proteins would remain at the cytosol. If properly coupled with an appropriate transcription regulation of peroxisomal protein-encoding genes, such hypothetical mechanism could result in an advantageous mislocalization of some peroxisomal enzymes into the cytosol (*e.g.* catalase in an oxidative stress situation). According to this view the cysteine residue would function as a regulatory switch.

Clearly, further work is necessary to understand the importance of the conserved N-terminal cysteine residue of PEX5.

VI. CONCLUDING REMARKS

The work described here adds new mechanistic details to the mammalian PEX5-mediated peroxisomal protein import pathway. First, the partial purification of the mammalian PEX4 functional counterpart allowed subsequent identification of the E2D family members as the E2s mediating PEX5 monoubiquitination at its N-terminal conserved cysteine in mammals. Second, it is demonstrated that deubiquitination of Ub-PEX5 may occur by a combination of context-dependent nonenzymatic (GSH-mediated) and enzymatic (DUB-mediated) mechanisms, thus generating nonmodified PEX5 (stage 4-to-stage 0 transition). An updated version of the model described in *Introduction* (section 3.2.1.2, Fig. 6) for this pathway is presented in Fig. 18.

Although not included in the new version of the model for the mammalian PEX5-mediated peroxisomal protein import pathway, other relevant findings were made during the progression of this work.

PEX5 is one of the few proteins outside the UCC shown to be ubiquitinated at a cysteine residue. On one hand, its ubiquitination is a prerequisite for the recognition and export from the peroxisomal membrane to the cytosol by the ATPases PEX1/PEX6. On the other, the modification of PEX5 in this unconventional manner does not seem to be relevant *per se* to the PEX5-mediated peroxisomal protein import pathway, as the conserved cysteine can be replaced by a lysine residue without causing deleterious effects. It was also shown that after being exported, soluble Ub-PEX5 does not remain in complex with the AAA peroxins. Furthermore, this species retains the same cargo-binding properties as nonmodified soluble PEX5 and can be again inserted into the membrane-embedded DTM in a cargo-dependent manner. This led us to suggest that cargo release and ubiquitination of PEX5 are two independent events. A recent study has confirmed our proposal [146].

In addition to the reason for this unconventional type of ubiquitination at this pathway, there are still many other interesting aspects that remain obscure and demand further investigation. Here are presented some examples:

- which are the RING peroxins that promote PEX5 monoubiquitination at the conserved cysteine in mammals?
- what are the mechanistic details underlying the recognition and export of monoubiquitinated PEX5 back into the cytosol? Do PEX1/PEX6 require an adaptor protein or do they interact directly with monoubiquitinated PEX5?
- what is the identity of the DUB(s) acting on PEX5 thiolester-attached ubiquitin?

Besides contributing with new relevant insights for the comprehension of peroxisomal biogenesis, this work also provides interesting data for the ubiquitin research field. Studies on ubiquitin-protein thiolester conjugates are almost inexistent and this study describes some of the properties of this class of conjugates. Ub-PEX5 species constitute most probably the first example of how ubiquitination at a cysteine residue can yield durable and stable conjugates, as the ones originated by the conventional lysine-based ubiquitination. However, if the thiolester bond linking ubiquitin to the target cysteine residue is exposed it can be easily disrupted and thus particularly suited for transient events. This mode of ubiquitination appears to be a quite simple and versatile mechanism to be used just for one protein. We do believe that there are many more proteins modified in this manner. Nevertheless, the discovery of new proteins ubiquitinated at cysteine residues has been hampered by the use of reducing agents (e.g. β -mercaptoethanol, DTT), that cleave thiolesters, in the protocols aiming at identifying and characterizing ubiquitinated proteins (for an example see [215]). It is thus crucial that when planning assays and protocols with the purpose of analyzing ubiquitinated proteins at cysteine residues, the lability of these conjugations is taken into consideration. Worth mentioning the study performed by Fratelli *et al.* [216] where the identification of proteins undergoing glutathionylation is reported. Like ubiquitin-protein thiolesters, GSH-protein conjugates are labile and can be easily disrupted by nucleophilic compounds and treatments at alkaline pH. In the aforementioned report, care was taken during sample preparation and mass spectrometry analysis in order to avoid disruption of the mixed disulfides between protein cysteines and the cysteine residue in GSH. Nevertheless, the authors stress the limitations of the applied procedure and highlight the fact that although they were able to identify new glutathionylated proteins, it was not possible to identify the modified cysteine and thus to directly demonstrate their glutathionylation.

It is thus clear that the identification of proteins ubiquitinated at cysteine residues will be challenging. Ubiquitination of nonlysine residues and their physiological relevance will surely be a topic of great interest in the near future.

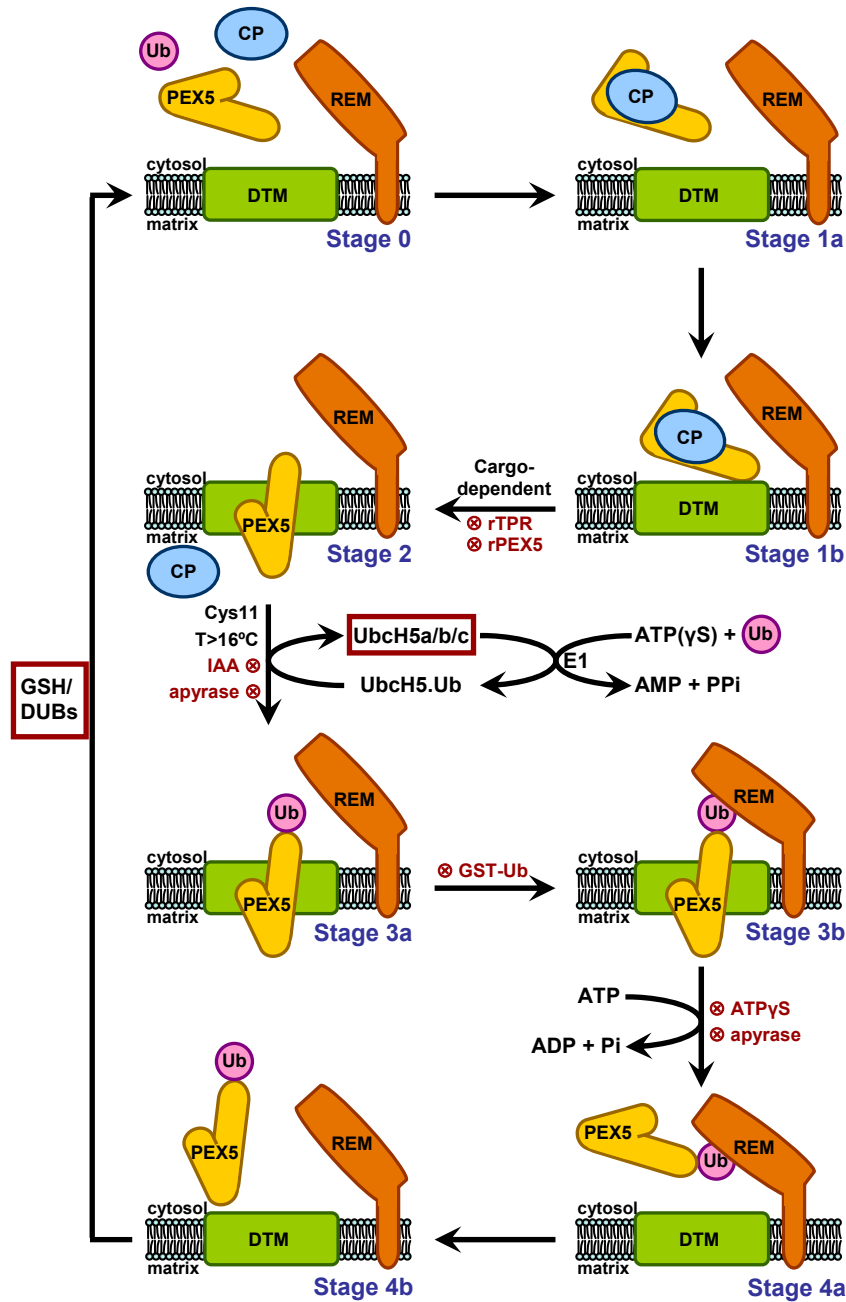


Figure 18 – The PEX5-mediated peroxisomal protein import pathway in mammals. This is an updated version of the model presented in Fig. 6 (*Introduction*), in which the question mark at stage 2-to-stage 3 and stage 4-to-stage 0 transitions steps has been replaced by the E2 mediating PEX5 monoubiquitination at Cys11 (*UbcH5a/b/c*) and by the deubiquitination (DUBs- and GSH-promoted) mechanisms acting on soluble and already exported Ub-PEX5. *Stage 0*, cytosolic cargo-free PEX5 (protease accessible). *Stage 1*, cytosolic PEX5-cargo protein complex (protease accessible). *Stage 2*, PEX5 embedded in the peroxisomal docking/translocation machinery (DTM) (only 2 kDa of PEX5 N terminus are accessible to exogenously added proteinase K). *Stage 3*, DTM-embedded monoubiquitinated PEX5 at Cys11 (protease protected). *Stage 4*, protease accessible monoubiquitinated PEX5. $\otimes$, blocking strategies; CP, cargo protein; Ub, ubiquitin; IAA, iodoacetamide; rTPR, recombinant protein comprising the PTS1-binding domain of PEX5; rPEX5, recombinant full-length PEX5.

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