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BIOGENESIS OF PEROXISOMAL INTRINSIC MEMBRANE PROTEINS: UNVEILING THE ROLE OF PEX19P

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Dissertação de doutoramento em Ciências Biomédicas

2008

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

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PRECEITOS LEGAIS

De acordo com o disposto no Decreto-Lei nº 216/92 de 13 de Outubro, esclarece-se serem da nossa responsabilidade a execução das experiências que estiveram na origem dos resultados apresentados neste trabalho, assim como a sua interpretação, discussão e redacção.

Nesta tese foram apresentados os resultados contidos no artigo já publicado e seguidamente discriminado:

Pinto, M. P., Grou, C. P., Alencastre, I. S., Oliveira, M. E., Sá-Miranda, C., Fransen, M. and Azevedo, J. E. (2006) The import competence of a peroxisomal membrane protein is determined by Pex19p before the docking step. J. Biol. Chem. 281, 34492-34502.

AGRADECIMENTOS/ACKNOWLEDGEMENTS

Aproveito para agradecer a todas as pessoas que me apoiaram ao longo destes anos e que de alguma forma contribuíram para este trabalho.

Agradeço ao Prof. Doutor Jorge Azevedo pela excelente orientação que prestou e pela disponibilidade e incentivos constantes. As suas discussões científicas foram sempre muito enriquecedoras e a sua motivação e entusiasmo contagiantes. O seu acompanhamento permanente foi essencial para a realização deste trabalho.

Agradeço também à Doutora Clara Sá Miranda por todo o seu apoio e preocupação em proporcionar as melhores condições de trabalho possíveis.

Aos elementos que fizeram ou fazem parte deste laboratório, um agradecimento pelo bom ambiente e pelo apoio e ajuda que recebi. Em especial, um profundo obrigado à Cláudia que trabalhou directamente comigo, pela sua preciosa ajuda e dedicação.

I am very grateful to Dr. Marc Fransen for giving me the opportunity to work in his laboratory at the Katholieke Universiteit Leuven, and for receiving me so well in Belgium. His teaching and support were extremely helpful. I am also thankful to Chantal for helping me in the laboratory and to Dr. Paul Van Veldhoven. Many thanks to the people I met in the lab's PhD student room that made my stay in Leuven a wonderful experience.

Agradeço ainda à Fundação para a Ciência e Tecnologia pela bolsa de doutoramento atribuída (SFRH/BD/18089/2004), e à Fundação Calouste Gulbenkian pelo apoio financeiro para participação em congressos científicos.

Por fim, muito obrigado à minha família que sempre me apoiou e zelou pelo meu bem-estar.

ABSTRACT

Biogenesis of the peroxisome membrane is still a poorly understood process with many of the fundamental aspects being disputed. Paradoxically, such process seems to depend on a quite simple machinery comprising only three proteins: Pex3p, Pex16p and Pex19p. Pex3p and Pex16p are both integral proteins of the peroxisomal membrane whereas Pex19p is a soluble protein apparently distributed between the cytosol and the organelle membrane. Pex19p displays the capacity to interact with all the peroxisomal membrane proteins investigated thus far. However, it remains unclear whether Pex19p acts as a cycling import receptor for peroxisomal membrane proteins or as a membrane chaperone assisting the assembly/disassembly of these proteins at the organelle surface.

In this work, an *in vitro* system was developed to characterize the import pathway of peroxisomal membrane proteins. For this purpose, a PMP24-based reporter protein was first produced. Using a post-nuclear supernatant from rat liver, this reporter protein (GFP-P24) was shown to be specifically inserted into the peroxisomal membrane despite the presence of all the other cellular organelles in the *in vitro* import reactions. Indeed, GFP-P24 was detected in mature peroxisomes after only 7.5 min of import suggesting that it is directly inserted from the cytosol into the peroxisomal membrane without any passage through the endoplasmic reticulum. Furthermore, the import process shows a strong dependence on both time and temperature but does not require ATP or GTP hydrolysis.

The insertion of GFP-P24 into the peroxisome membrane is blocked by IgGs directed to Pex3p and by the presence of very low concentrations of a recombinant version of its cytosolic domain, indicating that such mechanism is Pex3p-dependent. Importantly, in experiments where different recombinant versions of Pex19p were added either to the *in vitro* synthesis of the reporter protein or to the organelle suspensions, it was demonstrated that import-competent Pex19p-cargo complexes are formed during or shortly after the translation step in the cytosol and afterwards recognized by the peroxisomal docking and insertion machinery.

In addition, the binding properties of Pex19p for Pex3p were analyzed by native polyacrylamide gel electrophoresis. In these gels, dimeric complexes between Pex19p and GFP-P24 and trimeric complexes composed of Pex19p, GFP-P24 and Pex3p were detected. The trimeric complex is likely to reflect the docking of cargo-loaded Pex19p to peroxisomal Pex3p. Moreover, the binding of a cargo to Pex19p was shown to markedly increase its affinity towards Pex3p. The results presented here support a role for Pex19p in the sorting of newly synthesized peroxisomal membrane proteins to the organelle membrane and thus strongly favour the cycling receptor model for the biogenesis of this class of proteins.

RESUMO

A biogénese da membrana peroxissomal é ainda pouco conhecida e muitos dos seus aspectos fundamentais são controversos. Paradoxalmente, este processo parece depender de uma maquinaria proteica bastante simples, envolvendo apenas três proteínas: Pex3p, Pex16p e Pex19p. A Pex3p e a Pex16p são ambas proteínas intrínsecas da membrana peroxissomal enquanto que a Pex19p é uma proteína solúvel aparentemente distribuída entre o citosol e a membrana do organelo. A Pex19p possui a capacidade de interactuar com todas as proteínas da membrana peroxissomal investigadas até ao momento. Contudo, não é claro se a Pex19p actua como um receptor cíclico na importação das proteínas da membrana peroxissomal, ou como um chaperone membranar promovendo a formação de complexos destas proteínas na superfície do organelo.

Neste trabalho, foi desenvolvido um sistema de importação *in vitro* para caracterizar os mecanismos de endereçamento das proteínas da membrana peroxissomal. Com esta finalidade, começou-se por produzir uma proteína da membrana peroxissomal repórter a partir da PMP24. Usando um sobrenadante pós-nuclear de fígado de rato, demonstrou-se que esta proteína repórter (GFP-P24) é inserida especificamente na membrana do peroxissoma apesar da presença de todos os organelos restantes nas reacções de importação *in vitro*. De facto, a GFP-P24 foi detectada em peroxissomas maduros após 7,5 min de importação apenas, sugerindo que é inserida directamente na membrana peroxissomal sem qualquer passagem pelo retículo endoplasmático. O processo de importação apresenta ainda uma grande dependência tanto do tempo como da temperatura, mas não requer a hidrólise de ATP ou GTP.

A inserção da GFP-P24 na membrana peroxissomal é bloqueada por IgGs dirigidas para a Pex3p e pela presença de concentrações muito baixas de uma versão recombinante do seu domínio citosólico, indicando que o mecanismo é dependente de Pex3p. Em experiências onde diferentes versões recombinantes da Pex19p foram adicionadas às reacções de síntese *in vitro* da proteína repórter ou às suspensões organelares, demonstrou-se que os complexos Pex19p-carga competentes para importação se formam durante ou imediatamente após o passo de tradução no citosol e são posteriormente reconhecidos pela maquinaria peroxissomal de recrutamento e inserção.

As propriedades de ligação da Pex19p à Pex3p foram também analisadas recorrendo a electroforese em gel nativo de poliacrilamida. Esta técnica permitiu a detecção de complexos diméricos entre a Pex19p e a GFP-P24 e de complexos

triméricos compostos pela Pex19p, GFP-P24 e Pex3p. O complexo trimérico corresponde provavelmente ao passo de reconhecimento da Pex19p carregada pela Pex3p peroxissomal. Demonstrou-se ainda que a ligação de uma proteína da membrana peroxissomal à Pex19p resulta num aumento significativo da sua afinidade para a Pex3p. Os resultados apresentados neste trabalho indicam que a Pex19p actua no endereçamento para o organelo das proteínas da membrana peroxissomal recém-sintetizadas, deste modo favorecendo fortemente o modelo do receptor cíclico na biogénese desta classe de proteínas.

RÉSUMÉ

La biogenèse de la membrane du peroxysome est un processus encore peu connu, des nombreux aspects fondamentaux étant encore controversés. Paradoxalement, ce processus semble être dépendant d'une machinerie protéique très simple, qui comprend seulement trois protéines: Pex3p, Pex16p et Pex19p. Les protéines Pex3p et Pex16p sont toutes les deux des protéines intrinsèques de la membrane peroxysomiale, par contre Pex19p est une protéine soluble apparemment distribuée entre le cytosol et la membrane de l'organelle. Pex19p a la capacité d'interagir avec toutes les protéines de la membrane peroxysomiale étudiées jusqu'à présent. Cependant, il reste toujours à déterminer si Pex19p agit comme récepteur de recyclage dans l'importation des protéines de la membrane peroxysomiale, ou comme une chaperonne de la membrane assistant l'assemblage/désassemblage de ces protéines à la surface de l'organelle.

Au cours de ce travail nous avons développé un système *in vitro* dans le but de caractériser les mécanismes d'importation des protéines de la membrane du peroxysome. Pour cela nous avons tout d'abord produit une protéine rapporteuse basée sur la PMP24. En utilisant un surnageant post-nucléaire d'extrait de foie de rat, nous avons pu démontrer que cette protéine rapporteuse (GFP-P24) s'insère spécifiquement dans la membrane du peroxysome, en dépit de la présence de l'ensemble des autres organelles cellulaires durant ces réactions d'importation *in vitro*. En effet, GFP-P24 a été détectée dans des peroxysomes matures, seulement 7,5 minutes après l'importation, suggérant que GFP-P24 soit insérée directement dans la membrane du peroxysome sans passage à travers le réticulum endoplasmique. Le processus d'importation apparaît fortement dépendant de la température et du temps, mais indépendant de l'hydrolyse de l'ATP ou du GTP.

L'insertion de GFP-P24 dans la membrane du peroxysome est bloquée par des IGgs dirigées contre Pex3p et par une forme recombinante de son domaine cytosolique en concentrations très basses, indiquant que ce mécanisme est dépendant de Pex3p. Notamment, dans des expériences dans lesquelles différentes formes recombinantes de Pex19p ont été rajoutées aux réactions de synthèse *in vitro* de la protéine rapporteuse ou à des suspensions d'organelles, il a été démontré que des complexes Pex19p-cargo compétents pour l'importation se forment pendant où immédiatement après l'étape de traduction dans le cytosol et qu'ils sont reconnus postérieurement par la machinerie peroxysomiale de recrutement et d'insertion.

De plus, les propriétés de liaison de Pex19p à Pex3p ont été analysées par électrophorèse sur gel de polyacrylamide en conditions natives. Cette technique a permis la détection de complexes dimériques composés de Pex19p et GPF-P24, ainsi que de complexes trimériques composés de Pex19p, GFP-P24 et Pex3p. Le complexe trimérique

doit correspondre à l'étape de reconnaissance par la Pex3p du peroxysome de Pex19p chargée. De plus, il a été démontré que la liaison d'une protéine de la membrane du peroxysome à Pex19p augmente significativement son affinité pour Pex3p. Les résultats présentés ici montrent que la Pex19p joue un rôle important dans le cheminement des protéines membranaires du peroxysome synthétisées *de novo* vers la membrane de l'organelle et supportent donc le modèle du récepteur de recyclage pour la biogenèse de cette classe de protéines.

ABBREVIATIONS

AAA	ATPases associated with diverse cellular activities
A. thaliana	*Arabidopsis thaliana*
ATPγS	Adenosine 5'-*O*-(thiotriphosphate)
CAAX	Farnesylation motif where C is a cysteine, A is an aliphatic amino acid and X is glutamine, cysteine, serine, alanine or methionine
CAT	Catalase
CG	Complementation group
CHO	Chinese hamster ovary
CoA	Coenzyme A
COP	Coat protein complex
DHAP	Dihydroxyacetone phosphate
DLP	Dynamin-like protein
DTT	Dithiothreitol
E2	Ubiquitin-conjugating enzyme
E-64	*N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
GAL1	Galactokinase
GFP	Green fluorescent protein
GTPγS	Guanosine 5'-*O*-(thiotriphosphate)
HcRed	Far-red fluorescent protein from *Heteractis crispa*
His_6	Hexahistidine
Hs	*Homo sapiens*
IC_{50}	Inhibitory concentration 50%
IgG	Immunoglobulin G
IPTG	Isopropyl 1-thio-β-D-galactopyranoside
IRD	Infantile Refsum disease
K_D	Dissociation constant
MOPS	4-Morpholinepropanesulfonic acid
mPTS	Peroxisomal membrane protein targeting signal
NALD	Neonatal adrenoleukodystrophy
PAGE	Polyacrylamide gel electrophoresis
PBD	Peroxisome biogenesis disorders
PCR	Polymerase chain reaction

Pex	Peroxin
PMP	Peroxisomal membrane protein
PMSF	Phenylmethylsulfonyl fluoride
PNS	Post-nuclear supernatant
PTS1	Peroxisomal targeting signal type 1
PTS2	Peroxisomal targeting signal type 2
RCDP1	Rhizomelic chondrodysplasia punctata type 1
RING	Really interesting new gene
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium dodecylsulfate
SH3	Src homology domain 3
TCP1	Tailless complex polypeptide 1
TEV	Tobacco etch virus protease cleavage site
TPR	Tetratricopeptide repeats
TRiC	TCP1 ring complex
Tris	Tris(hydroxymethyl)aminomethane
WD	Domain containing the motif tryptophan-aspartate
YFP	Yellow fluorescent protein
Y. lipolytica	*Yarrowia lipolytica*
ZS	Zellweger syndrome

1. INTRODUCTION

1. INTRODUCTION

1.1. Structure and function of peroxisomes

Peroxisomes are a class of intracellular organelles nearly ubiquitous within eukaryotic cells [1]. These structures were first described by Rhodin in 1954 as microbodies [2] and later named peroxisomes by de Duve and Baudhuin in 1966 [3] after its conserved hydrogen peroxide-based respiration. These organelles are surrounded by a single membrane and display specialized functions and a diverse morphology among different tissues and organisms [1, 4]. Peroxisome specializations include the glyoxysomes in plants (responsible for the glyoxylate cycle) [5], the glycosomes in trypanosomes (contain part of the glycolysis pathway) [6] and the Woronin body in *Neurospora crassa*, which helps maintaining cellular integrity by preventing cytoplasmic bleeding [7]. Although they can display tubular and branched shapes, peroxisomes are typically spherical and range from 0.1-1 μm in diameter, presenting a very electron-dense matrix with a paracrystalline core observed in rat liver cells [4] (Figure 1).

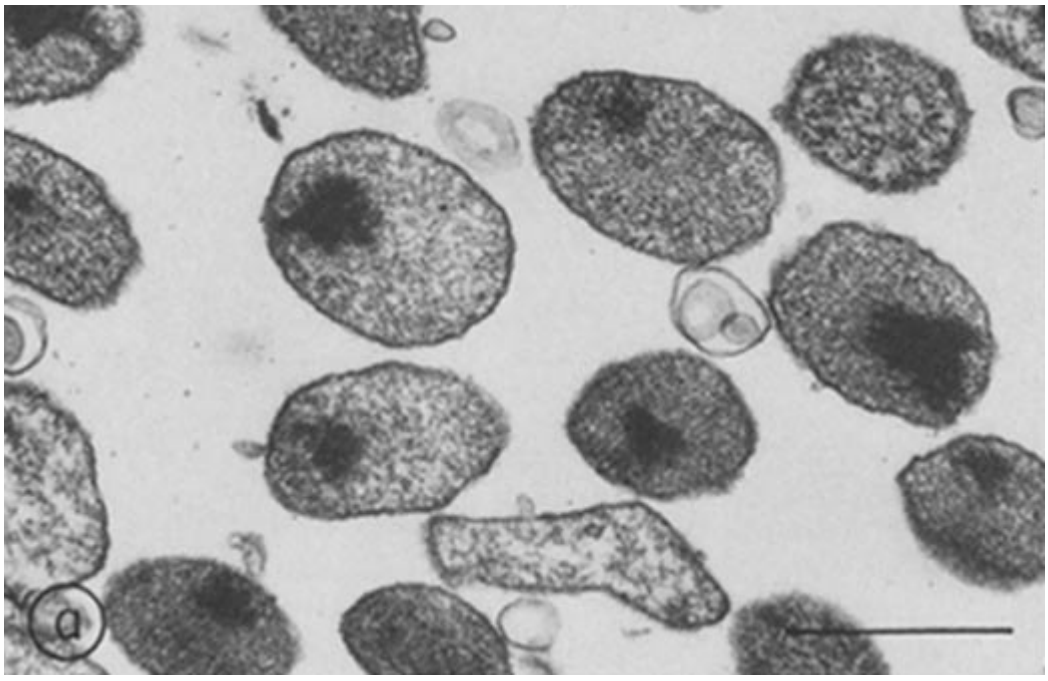

Figure 1. Purified rat liver peroxisomes observed under the electron microscope.
Bar, 0.5 μm x 38,000 (extracted from [8]).

Besides presenting a hydrogen peroxide-based respiration, another general feature of peroxisomal biochemistry regards fatty acid β-oxidation [1]. Indeed, in plants and yeasts peroxisomes are the sole site of fatty acid β-oxidation, whereas in mammals

this function is accomplished both by peroxisomes and mitochondria [9]. In higher eukaryotes, the organelle also catalyzes a set of additional metabolic functions which are not present in lower eukaryotes. Mammalian peroxisomes comprise around 50 different enzymes that participate in multiple metabolic pathways. Most of these enzymatic activities are exclusively peroxisomal but some are shared with the mitochondria and the cytosol (reviewed in [10]). Many of the peroxisomal enzymes are oxidases that generate hydrogen peroxide which is afterwards disposed by other enzymes of the organelle, most notably catalase [11]. Furthermore, other reactive oxygen and nitrogen species are also produced and detoxified within peroxisomes [10, 11].

Mammalian peroxisomes are able to promote β-oxidation of several substrates. While medium-chain fatty acids are only degraded in mitochondria and long-chain fatty acids are β-oxidized in both organelles, very-long-chain fatty acids are solely metabolized in peroxisomes [12]. Other substrates that also undergo β-oxidation only in peroxisomes include pristanic acid, bile acid intermediates, long-chain dicarboxylic acids, some polyunsaturated fatty acids, certain prostaglandins and leukotrienes, a few xenobiotics and vitamins E and K [10, 13]. Fatty acids that hold a methyl group at position 3 cannot undergo β-oxidation and need to be α-oxidized first. In contrast to β-oxidation, fatty acid α-oxidation is restricted to peroxisomes [14]. In mammals, peroxisomes are also involved in the oxidation of D-amino acids, L-lysine and its metabolite L-pipecolate, uric acid (nonprimates) and polyamines (spermine and spermidine) [10]. The toxic metabolite glyoxylate is converted into glycine by the alanine:glyoxylate aminotransferase which localizes exclusively to peroxisomes in humans [15]. The biosynthesis of ether-phospholipids is initiated in peroxisomes, where the ether linkage is introduced, while the final steps are performed in the endoplasmic reticulum [16, 17]. Although the participation of peroxisomes in isoprenoid and cholesterol biosynthesis was generally accepted for several years, recent data suggest no direct involvement of the organelle in these pathways [18-20].

1.2. Peroxisomal disorders

The importance of peroxisomes in human development and its essential role in metabolism is well illustrated by a group of inherited diseases in which one or more peroxisomal functions are impaired. The peroxisomal disorders are classified in two groups, comprising the peroxisomal biogenesis disorders and the single enzyme deficiencies [21-23]. The latter group of disorders currently includes ten different diseases and can be divided into subgroups on the basis of the metabolic pathway affected by the

mutated enzyme (Table 1). The archetype and most common single enzyme disease is X-linked adrenoleukodystrophy (X-ALD) with an incidence of about 1:20,000 newborn males [24, 25]. This disorder affects peroxisomal β-oxidation and is generally characterized by a progressive degeneration of the central nervous system and the adrenal gland [26].

Table 1. Peroxisomal single enzyme deficiencies

Metabolic pathway affected	Peroxisomal disease	Enzyme defect
Ether phospholipid synthesis	Rhizomelic chondrodysplasia punctata Type 2	DHAP acyltransferase (DHAPAT)
	Rhizomelic chondrodysplasia punctata Type 3	Alkyl-DHAP synthase (ADHAPS)
Peroxisomal β-oxidation	X-linked adrenoleukodystrophy	Adrenoleukodystrophy protein (ALDP)
	Acyl-CoA oxidase deficiency	Acyl-CoA oxidase-1 (ACOX1)
	D-bifunctional protein deficiency	D-bifunctional protein (DBP)
	2-Methylacyl-CoA racemase deficiency	2-Methylacyl-CoA racemase (AMACR)
	Sterol carrier protein X deficiency	Sterol carrier protein X (SCPx)
Peroxisomal α-oxidation	Refsum disease	Phytanoyl-CoA hydroxylase (PAHX)
Glyoxylate detoxification	Hyperoxaluria Type 1	Alanine:glyoxylate aminotransferase (AGT)
H_2O_2-metabolism	Acatalasaemia	Catalase (CAT)

DHAP, dihydroxyacetone phosphate (adapted from [22]).

The peroxisomal biogenesis disorders (PBD) are a genetically diverse group of diseases wherein peroxisome assembly and multiple metabolic functions are impaired [23]. This group of diseases occurs approximately in 1:50,000 live births and consists of four clinical phenotypes: the Zellweger syndrome (cerebro-hepato-renal syndrome) (ZS), neonatal adrenoleukodystrophy (NALD), infantile Refsum disease (IRD) and rhizomelic chondrodysplasia punctata type 1 (RCDP1) [21, 27]. The first three syndromes were included in the so-called Zellweger spectrum after it became clear that mutations in the same gene can lead to any of the disorders. Indeed, these overlapping phenotypes represent three degrees of severity, with the ZS as the most severe form, followed by NALD, and IRD at the milder end of the spectrum [21, 23]. Typical phenotypic features within Zellweger spectrum patients include facial dysmorphism, ocular abnormalities, liver and neurological diseases, hearing loss and premature death [28]. The fourth clinical phenotype, RCDP1, shows more specific characteristics [29]. In fact, this disorder involves mutations in one single gene, PEX7, which only affects the limited subset of PTS2 enzymes [30] (see section 1.3.1.). In contrast to RCDP1, the Zellweger spectrum is characterized by the impairment of most, if not all, peroxisomal functions and has been

associated with defects in 12 distinct genes [21]. Complementation studies with PBD patient fibroblasts together with transfection experiments using peroxin-encoding plasmids (see section below) have been used to identify the affected genes (PEX genes). The presently known complementation groups (CG) are shown in Table 2 [21, 27].

Table 2. PEX gene defects and complementation groups in PBD

Gene	CG-Dutch	CG-Japan	CG-KKI	Clinical phenotypes	Proportion of ZSS*
PEX1	2	E	1	ZS NALD IRD	70%
PEX2	5	F	10	ZS IRD	3%
PEX3		G	12	ZS	<1%
PEX5	4		2	ZS NALD	<2%
PEX6	3	C	4,6	ZS NALD IRD	10%
PEX7	1	R	11	RCDP1	-
PEX10		B	7	ZS NALD	3%
PEX12			3	ZS NALD IRD	5%
PEX13		H	13	ZS NALD	<1%
PEX14		K		ZS	<1%
PEX16		D	9	ZS	<1%
PEX19		J	14	ZS	<1%
PEX26		A	8	ZS NALD IRD	5%

*Estimates derived from KKI data; CG, complementation group; Dutch, group at University of Amsterdam; Japan, group at Gifu University School of Medicine; KKI, Kennedy Krieger Institute; ZSS, Zellweger syndrome spectrum; ZS, Zellweger syndrome; NALD, neonatal adrenoleukodystrophy; IRD, infantile Refsum disease; RCDP1, rhizomelic chondrodysplasia punctata type 1 (adapted from [21]).

1.3. Peroxisome biogenesis

The proteins encoded by PEX genes are required for peroxisome biogenesis and are collectively designated peroxins. These proteins are represented by the acronym Pex followed by a number reflecting its order of discovery [31]. At present, over 30 different peroxins are known, many of which are conserved from lower to higher eukaryotes, whereas for some others no mammal homologs were found [32, 33] (Table 3). Among the lower eukaryotes, most of the additional peroxins are specific to one species and seem to be functional redundant. This is specially the case for peroxins involved in peroxisome proliferation in fungi [32]. According to their roles in peroxisomal biogenesis, peroxins are usually divided into three classes: the ones required for the peroxisomal membrane assembly, those responsible for the import of matrix proteins into the organelle and the ones that regulate peroxisomal proliferation.

Table 3. Proteins implicated in the biogenesis of peroxisomes (Peroxins)

Peroxin	Organisms	Localization	Domains	Proposed function
Pex1p	m, p, f, y	cytosol/membrane	AAA ATPase	Matrix protein import, export of Pex5p
Pex2p	m, p, f, y	membrane	Zinc RING finger	Matrix protein import, translocation
Pex3p	m, p, f, y	membrane		Membrane biogenesis, PMP import
Pex4p[a]	p, f, y	cytosol/membrane	E2 Ubiquitin-conjugating enzyme	Matrix protein import , Pex5p ubiquitination
Pex5p[b]	m, p, f, y	cytosol/membrane	TPRs	Matrix protein import, PTS1 (and PTS2 in m, p) receptor
Pex6p	m, p, f, y	cytosol/membrane	AAA ATPase	Matrix protein import, export of Pex5p
Pex7p	m, p, f, y	cytosol/membrane	WD repeats	Matrix protein import, PTS2 receptor
Pex8p	f, y	matrix/membrane		Matrix protein import
Pex9p	*Yl*	(ORF wrongly identified, antisense sequence of Pex26p)		
Pex10p	m, p, f, y	membrane	Zinc RING finger	Matrix protein import, translocation
Pex11p[c]	m, p, f, y	membrane		Division and proliferation
Pex12p	m, p, f, y	membrane	Zinc RING finger	Matrix protein import, translocation
Pex13p	m, p, f, y	membrane	SH3	Matrix protein import, docking
Pex14p	m, p, f, y	membrane	Coiled-coil	Matrix protein import, docking
Pex15p	*Sc*	membrane		Matrix protein import, Pex1p/Pex6p anchor
Pex16p	m, p, f, *Yl*	membrane		Membrane biogenesis
Pex17p	y	membrane	Coiled-coil	Matrix protein import, docking
Pex18p	*Sc*	cytosol/membrane		Matrix protein import, PTS2 import
Pex19p	m, p, f, y	cytosol/membrane	Farnesylation motif	Membrane biogenesis, PMP import
Pex20p	f, y	cytosol/membrane		Matrix protein import, PTS2 import
Pex21p	*Sc*	cytosol/membrane		Matrix protein import, PTS2 import
Pex22p	p, f, y	membrane		Matrix protein import, Pex4p anchor
Pex23p	f, y	membrane	Dysferlin	Proliferation
Pex24p	f, y	membrane		Proliferation
Pex25p	y	membrane		Proliferation
Pex26p	m, f, y[d]	membrane		Matrix protein import, Pex1p/Pex6p anchor
Pex27p	*Sc*	membrane		Proliferation
Pex28p	*Sc*	membrane		Proliferation (Pex24p ortholog)
Pex29p	y	membrane		Proliferation
Pex30p	*Sc*	membrane	Dysferlin	Proliferation (Pex23p ortholog)
Pex31p	*Sc*	membrane	Dysferlin	Proliferation
Pex32p	y	membrane	Dysferlin	Proliferation

m, mammals; p, plants; f, filamentous fungi; y, yeasts; *Yl*, *Yarrowia lipolytica* only; *Sc*, *Saccharomyces cerevisiae* only; [a]In mammals the E2 acting on Pex5p is the multipurpose UbcH5a/b/c [34]; [b]Mammals contain two isoforms, Pex5pS and Pex5pL, the latter harbouring a Pex7p-binding site; [c]Mammalian cells contain three PEX11 genes encoding Pex11pα, Pex11pβ and Pex11pγ; [d]Pex26p is absent in *Sc* and related yeasts [1, 23, 32, 33, 35].

1.3.1. Matrix protein import

Peroxisomal matrix proteins are synthesized on cytosolic ribosomes and post-translationally imported into the lumen of the organelle [1, 36, 37]. Interestingly, peroxisomes are able to import proteins that are already folded and even oligomerized [38]. Newly synthesized matrix proteins are directed to the organelle through their peroxisomal targeting signal (PTS1 or PTS2). Most of these proteins contain a PTS1 signal which consists of the C-terminal tripeptide S-K-L, or a conserved variant, and that is recognized by the cycling receptor Pex5p in the cytosol [39-41]. A small fraction of matrix proteins contains an N-terminal PTS2 targeting signal comprising the consensus sequence (R/K)-(L/I/V)-X_5-(H/Q)-(L/A/F) which interacts with Pex7p [42-44]. In mammals, this peroxin binds to the long isoform of Pex5p (Pex5pL), an interaction that is essential for targeting PTS2-containing proteins into the organelle [45, 46]. Thus, in these organisms Pex5p is in charge of sorting both PTS1- and PTS2-containing proteins.

The binding of a cargo to Pex5p is believed to induce conformational alterations at its N terminus, triggering the interaction of the receptor-cargo complex with the docking/translocation machinery at the peroxisomal membrane [36, 47]. The core components of this machinery are Pex13p, Pex14p and the three Zinc RING finger proteins Pex2p, Pex10p and Pex12p [48-51]. The interaction of the Pex5p-cargo complex with this apparatus ultimately results in Pex5p insertion into the peroxisomal membrane and release of the cargo protein into the organelle matrix [36, 52, 53]. Strikingly, the docking/translocation steps do not require ATP hydrolysis, suggesting that its driving force is provided solely by the strong protein-protein interactions involving Pex5p and some components of the docking/translocation machinery, such as Pex14p [36, 54].

In order to be exported from the membrane, Pex5p has to undergo monoubiquitination at cysteine 11, an unusual type of ubiquitination [55]. Both plants and yeasts/fungi harbour Pex4p, a specialized ubiquitin-conjugating enzyme (E2) acting on Pex5p [56-58]. In contrast, mammals do not contain such a peroxisomal-specialized E2 and Pex5p is instead ubiquitinated by the promiscuous UbcH5 family, specifically UbcH5a/b/c (E2D1/2/3) [34]. The identity of the ubiquitin-ligase(s) (E3) involved in the ubiquitin transfer remains unknown, although the RING finger peroxins are the most obvious candidates. Finally, ubiquitinated Pex5p is dislocated back to the cytosol in an energy-dependent process [54] by the action of the AAA ATPases Pex1p and Pex6p [59, 60], which are anchored to the peroxisomal membrane by Pex26p [61]. After the export step, Pex5p is once again available for promoting further cycles of protein transportation.

1.3.2. Peroxisome proliferation

Peroxisomes are highly dynamic organelles that increase in size and number in response to a wide variety of both internal and external stimuli [62, 63]. In mammals, three peroxins, the isoforms Pex11pα, Pex11pβ and Pex11pγ, have been implicated in peroxisomal proliferation [64-66]. Pex11pα expression is induced by peroxisome proliferating agents [67] whereas both Pex11pβ and Pex11pγ are constitutively expressed, though the latter seems to be tissue-specific [65, 66]. Therefore, Pex11pα is apparently involved in peroxisome proliferation following external stimuli while Pex11pβ is responsible for constitutive peroxisome biogenesis. In contrast, yeasts only have one Pex11p but contain several Pex11p-like proteins and other peroxins that regulate the size and abundance of the organelle [33, 62] (see Table 3). Pex11pβ promotes peroxisome growth and elongation but the division itself requires the dynamin-like protein DLP1 [68, 69]. Fis1p is a DLP1-interacting protein also involved in the fission process that recruits DLP1 to the peroxisomal membrane [70]. Interestingly, DLP1 and Fis1p participate in mitochondria division as well, representing shared components of the fission machineries of both organelles [71].

1.3.3. Peroxisomal membrane biogenesis

1.3.3.1. Biogenesis of peroxisomal membrane proteins

The biogenesis of peroxisomal membrane proteins involves a machinery which is distinct from the one required for matrix protein import. This partition is well illustrated by the existence of two cellular phenotypes within the CGs of peroxisome biogenesis disorders [27]. Most of these cell lines contain peroxisomal remnant structures lacking most or all of their matrix protein content but apparently retaining the normal peroxisomal membrane protein (PMP) repertoire [23, 72]. In merely three CGs comprising defects in Pex3p, Pex16p or Pex19p, no identifiable peroxisomal structures were found, suggesting that these peroxins are responsible for the organelle membrane biogenesis [73-77].

Strikingly, Pex16p is absent in yeasts with the exception of *Yarrowia lipolytica*, reducing the proteins required for peroxisome membrane biogenesis in those organisms to only Pex3p and Pex19p [32, 78]. Furthermore, *Y. lipolytica* Pex16p is thought to regulate the organelle proliferation rather than the assembly of the peroxisomal membrane [79]. In mammals, Pex16p is reported to have a role in the early steps of the peroxisomal membrane biogenesis (see section 1.3.3.2.), upstream of Pex3p [80, 81].

Nevertheless, the mechanistic role of Pex16p in this process remains very poorly understood. Taken together, it seems that biogenesis of PMPs depends on a very simple machinery, comprising as few as three proteins or even only two in the case of yeast species.

1.3.3.2. Origin of the peroxisomal membrane

The early stages of peroxisome biogenesis have been a subject of much controversy [82, 83]. A longstanding concept proposed that peroxisomes are autonomous organelles deriving from pre-existing ones by growth and division (see section 1.3.2.). This model was supported by a large amount of data indicating that both peroxisomal matrix proteins (see section 1.3.1.) and at least some PMPs [37, 84, 85] are synthesized on free cytosolic ribosomes and post-translationally imported into the organelle, through peroxisome-specific protein machineries. In addition, the source of phospholipids for the peroxisomal membrane was proposed to be the endoplasmic reticulum (ER), like in the case of mitochondria [37, 82]. The phospholipid transfer could occur either via small vesicles or in sites of close apposition between peroxisomes and the ER [82], previously observed in morphological studies [86].

However, cell lines defective in Pex3p, Pex16p or Pex19p lacking any detectable peroxisomal remnants are still able to restore peroxisome formation when the wild-type version of the gene is reintroduced [75, 77]. Some authors explained this reappearance by postulating the existence of undetected membrane remnants termed protoperoxisomes as membrane donors [82]. Conversely, this regeneration capacity led to the proposal of the ER involvement in *de novo* formation and maintenance of peroxisomes [83, 87]. In this regard, recent data on the intracellular trafficking of Pex3p in yeast cells have been collected and used to sustain this alternative model [88-90] (see Figure 2). Nonetheless, the evidence remains inconclusive and should be interpreted with great care. Most of these studies use engineered overexpressed membrane proteins, truncated or tagged versions, all of which are frequently associated with mistargeting events, generally to the ER [1, 91]. Moreover, PMPs are also known to mislocalize to other membrane systems in peroxisome-deficient cells [92, 93]. Since most of these studies use such mutant cell lines the attained conclusions may not apply to a normal cell. Another important aspect is related to the long time windows of the experiments. In fact, the fate of the protein is often followed several hours upon expression when steady-state levels are reached and therefore miss the early kinetics of the protein. For instance, PMP70 becomes associated with peroxisomes *in vivo* with a half-time of 3 minutes [85].

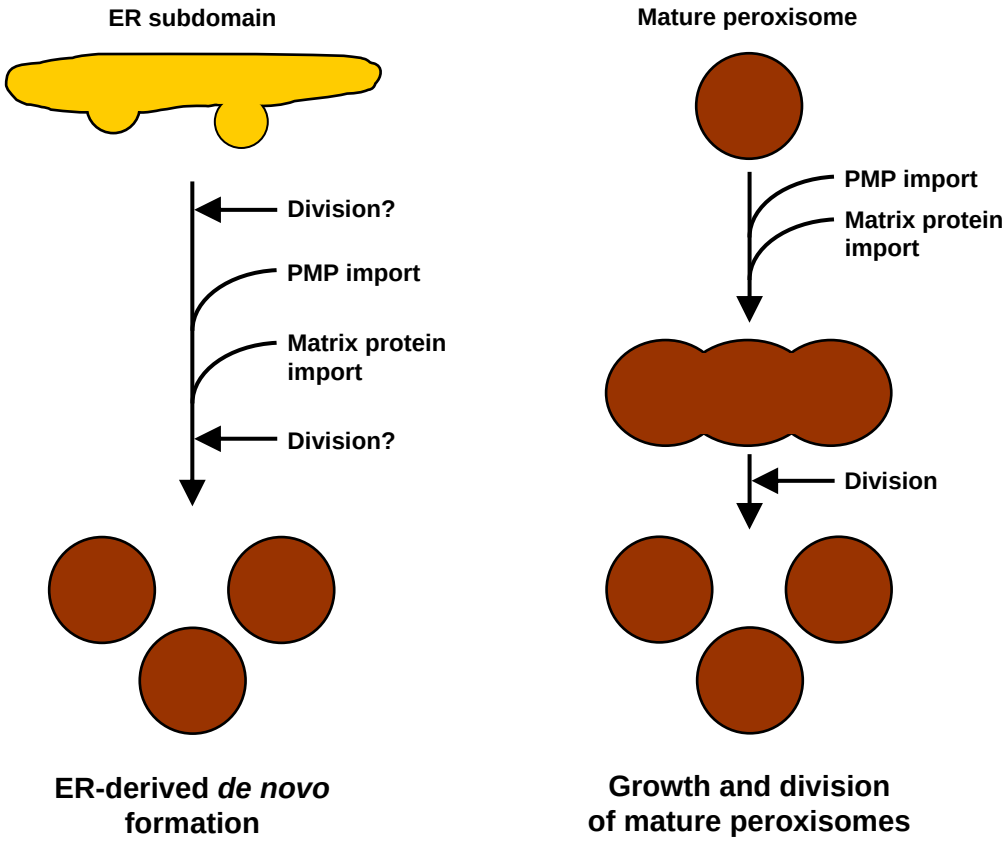

Figure 2. Models of peroxisome biogenesis and multiplication.

Conflicting data have given rise to different concepts regarding peroxisome formation and maintenance. According to some authors, peroxisomes derive *de novo* from the ER and mature into functional organelles through the import of PMPs followed by the import of the matrix content. Within this perspective, the division process has been suggested to occur either at an ER subdomain before the maturation pathways [83], or only after the ER-derived membrane structures mature into functional peroxisomes [63]. On the other hand, the longstanding view considers that peroxisomes arise from growth of pre-existing ones via PMP and matrix protein import and subsequent fission of the enlarged organelles. In such model, membrane components could be provided by the ER [82] (adapted from [62, 94]).

One of these studies tried to prevent artefacts from high expression by using the *GAL1* promoter to limit the induction of Pex3p to the steady-state levels of the endogenous peroxin, in *Saccharomyces cerevisiae* [88]. However, the authors did not consider the much higher rate of expression with this promoter. YFP-tagged Pex3p was shown to route from the ER to newly formed peroxisomes over a period of 120 minutes in a PEX3-deficient strain. However, when the authors repeated these experiments using wild-type cells no clear result could be obtained. Furthermore, the Pex3p version used in this study contains a bulky YFP tag at the N terminus next to its putative transmembrane domain (see section 1.3.3.3.), rendering it unlikely to cross a lipid bilayer and more prone

to mistargeting. In another study, an engineered Pex3p variant harbouring an ER signal peptide coupled to a FLAG epitope was used [89]. This signal peptide was shown to be cleaved in the ER and only the processed peroxin was found to rescue peroxisome formation in a PEX3-deficent strain. Despite trafficking through the ER, this tagged protein is less efficient and may be processed without actually being inserted, becoming later available for peroxisomal membranes.

In contrast to yeasts, mammalian Pex3p mistargets to mitochondria when overexpressed in both normal and PEX19-deficient cells [92, 95-97] and so far has not been found in the ER compartment. Indeed, data suggesting ER participation in mammalian peroxisome biogenesis are scarce. Early morphological observations showed peroxisomes juxtaposed to the ER with possible membrane continuities pointing to an ER origin [98]. More recently, these observations were extended in mouse dendritic cells which display unique elaborate peroxisomal structures in the vicinity of the ER [99]. The authors detected two PMPs (Pex13p and PMP70) apparently in specialized ER regions but the results and specificity of the antibodies used were unclear. Instead of tracking Pex3p, one study followed Pex16p travelling from the ER to peroxisomes, in both wild-type and PEX16-deficient mammalian cells, by using a photo-activatable GFP version [81]. However, the assays included highly expressed tagged versions and long incubation periods of many hours. In previous studies Pex16p was observed readily and exclusively in peroxisomes [77, 100]. Furthermore, the authors report that in wild-type cells most new peroxisomes are formed *de novo* [81]. Unfortunately, these experiments comprised again overexpression of tagged Pex16p which may cause peroxisome proliferation. Under these unbalanced conditions, it is possible that the *de novo* pathway is induced and contributes considerably to the peroxisomal figures, but remains uncertain whether this is also the case in normal cells. Both in this study and in the yeast ones, it was assumed that Pex3p or Pex16p generate new peroxisomes through vesicles budding out of the ER, although such process was never shown. In fact, peroxisome biogenesis does not depend on COPI- and COPII-mediated vesicle budding within the early secretory pathway [77, 93, 100] nor on the translocation factors Sec61p and Ssh1p which are required for protein import into the ER compartment [101]. Hence, these data support an ER independent biogenesis though its involvement through a thus far unknown pathway remains possible.

In a recent study, Motley and Hettema analyzed the contribution of *de novo* formation from the ER against growth and fission of pre-existing mature peroxisomes, in *S. cerevisiae* [94]. By using matting assays and pre-labelled peroxisomes with GFP-PTS1 and HcRed-PTS1, the authors demonstrated that in wild-type cells these organelles arise solely by division of pre-existing ones without any *de novo* formation. Another experiment showed that Pex3p-GFP mislocalized to the ER in a peroxisome-deficient strain redirects

to existing peroxisomes after cell fusion and does not generate *de novo* organelles. Therefore, these results indicate that if there is an ER to peroxisome pathway in wild-type cells, it provides existing organelles with membrane components rather than produce new ones. Nevertheless, in cells that lost their peroxisomes due to a segregation deficiency, the organelles did reappear [94]. Altogether, it seems more likely that *de novo* formation of peroxisomes represents a backup system of the cell to cope with a situation where the organelle is lost and may reflect its evolutionary origin.

1.3.3.3. General properties of Pex3p

As stated above, the biogenesis of PMPs is still quite elusive despite relying on a rather simple protein machinery with Pex3p and Pex19p as the core components. Over the past few years, several properties of these two proteins have been described [78]. Pex3p behaves as an intrinsic protein of the peroxisomal membrane displaying a number of predicted transmembrane segments and a topology that seems to vary among species [97, 102-104]. However, these differences should be regarded with care because epitope-tagged proteins were used in these experiments, which may affect the protein topology. In the case of human Pex3p, a 373-amino acid protein, it has been suggested that a single transmembrane domain at the N terminus anchors this cytosolic-exposed protein to the peroxisomal membrane [97, 105]. In addition, the targeting information of Pex3p is confined to the first 46 residues or less in all the studied organisms [90, 97, 102, 104, 106, 107]. Interestingly, this targeting domain does not interact with Pex19p suggesting that Pex3p is sorted to the peroxisomal membrane in a Pex19p-independent way [97, 108-110], differently from all the other PMPs (see section 1.3.3.5.). As discussed in section 1.3.3.2., some authors proposed that Pex3p transits through the ER mediating the budding of new immature peroxisomes, before the Pex19p-dependent assembly of the other PMPs. Nevertheless, Pex3p does bind to Pex19p being this peroxin its only known interacting partner. The Pex19p-binding site resides within the cytosolic domain and comprises amino acid stretches 120-136 and 148-307 [95, 108]. In addition, a domain containing tryptophan 104 of Pex3p was recently shown to be essential for Pex19p binding [111].

1.3.3.4. Pex19p domain architecture and its interactions with PMPs

Pex19p, the other central peroxin of peroxisome membrane biogenesis, exhibits many distinct features. It is a hydrophilic and acidic protein with a partially disordered structure that comprises 299 amino acid residues in humans [74, 112]. This peroxin is mostly cytosolic though it also localizes to a smaller extent to the peroxisomal membrane [92, 113, 114]. Additionally, human Pex19p disperses as a monomer in solution upon analytical ultracentrifugation [112]. On the other hand, in the plant *Arabidopsis thaliana* the protein was reported to form a dimer [115]. However, this dimeric species was detected only under nonreducing conditions and was susceptible to DTT. The true nature of this species remains to be determined.

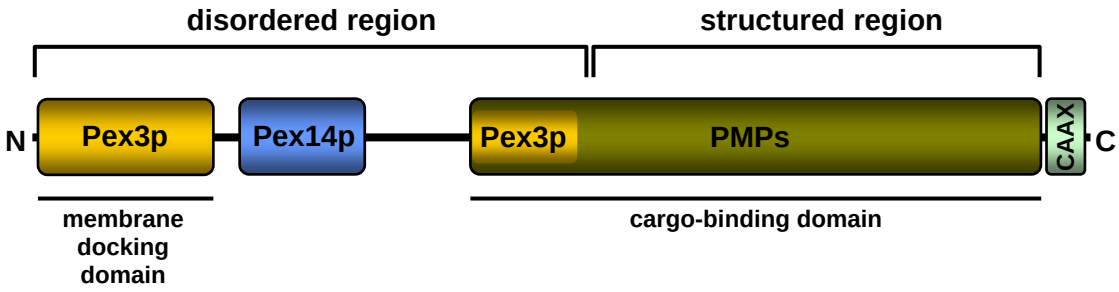

Figure 3. Domain organization of human Pex19p.

The domain structure of human Pex19p was basically predicted from protein-protein interaction studies such as two-hybrid and pull-down assays. The interaction domains depicted in boxes are in agreement with the domain sizes mapped in [116]. The box "PMPs" refers to the domain of Pex19p displaying the broad specificity of PMP binding. This cargo-binding region is indicated as well as the putative peroxisomal membrane docking domain. The second Pex3p-interacting site which seems to overlap with the N-terminal portion of the PMP-binding region is also shown. "CAAX" refers to the *CAAX* box farnesylation motif consisting of the C-terminal residues CLIM in the human peroxin. The disordered N-terminal half and the more structured region as determined by limited proteolysis [112] are indicated as well (adapted from [78, 116]).

Remarkably, Pex19p displays a multi-domain disposition with the ability to interact with all the PMPs investigated [92, 108, 110, 112, 116-120]. The structure of human Pex19p can be divided into a flexible and disordered N-terminal half (1-155) and a more compact C-terminal region (156-296), by limited proteolysis [112] (Figure 3). Furthermore, circular dichroism studies showed a low amount of secondary structure in the N-terminal half whereas a 55% content of α-helix was estimated for the C-terminal domain. In agreement, this 35-kDa protein elutes in gel filtration chromatography experiments with a retention time consistent with a molecular weight of 111 kDa, which is indicative of a non-globular conformation [112]. The N-terminal region further includes a Pex3p-binding site within the first 51 amino acids and a Pex14p-interacting region between residues 60 and

93 [116] (Figure 3). With respect to the C-terminal two-thirds, it harbours the broad binding specificity of Pex19p for all PMPs, except Pex14p, and a second binding site for Pex3p [116, 119]. Interestingly, this second Pex3p-interacting site appears to be weaker and is distinct from the one for the other PMPs, although these regions largely overlap and could not be physically separated. The PMP-binding domain encompasses amino acid residues 124-299 while the interaction with Pex3p seems to require only the initial portion of this domain [116] (Figure 3).

Finally, Pex19p also presents a *CAAX* box at the C terminus which has been shown to be a site of farnesylation [74, 113, 121]. This farnesylation consensus motif is found in most Pex19p sequences but is absent in the trypanosomatid homologs [122, 123]. In fact, the requisite of this post-translation modification for Pex19p function is controversial [74, 92, 108, 113, 114, 119]. Over the years, several studies have diverged on whether or not Pex19p farnesylation affects its affinity for PMPs [108, 113, 118, 119, 124]. Similarly, complementation assays of PEX19-deficient cell lines with variants holding a disrupted *CAAX* box have reached different conclusions regarding peroxisome-restoring activity [74, 92, 113, 118, 119]. Yet, it becomes apparent from those studies that such Pex19p versions are able to rescue peroxisome biogenesis whenever overexpressed. Recently, Vastiau and colleagues aimed at clarifying this subject and addressed Pex19p farnesylation in both yeasts and mammals [125]. The authors reported no significant differences between nonfarnesylated and farnesylated Pex19p proteins in the affinity for PMPs, restoration of biogenesis and peroxisome protein import. Therefore, the authors suggested that farnesylation of this peroxin is dispensable for its function and may play an ancillary role. Moreover, it was shown that this motif is not required for the peroxisomal localization of Pex19p [95, 118, 125].

1.3.3.5. PMP sorting to peroxisomes and its dependence on Pex19p

The trafficking of newly synthesized PMPs to the peroxisomal membrane is secured by the existence of *cis*-acting targeting signals, designated mPTSs [126]. In contrast to the targeting signals of peroxisomal matrix proteins (see section 1.3.1.), the mPTSs present a wide variety both in length and primary sequence [117, 126]. Indeed, the gathered evidence indicates that the peroxisomal sorting information of PMPs resides in physicochemical properties and structural elements which presently cannot be defined by an amino acid sequence. In general, the mPTS is composed of a targeting segment containing a cluster of basic amino acids and at least one transmembrane domain that anchors the protein to the peroxisomal membrane (reviewed in [126]). Notably, many

multispanning PMPs display several independent mPTSs that may function cooperatively [108, 127, 128]. In addition, the sorting of PMPs to the peroxisomal membrane is evolutionary conserved among distantly related species [126]. This feature is illustrated by the finding that some PMPs from yeasts and mammals are correctly targeted to peroxisomes when expressed in the other organism [129, 130].

Interestingly, most of the mPTSs are also involved in the interaction with Pex19p, directly implicating this peroxin in PMP targeting [117, 126, 129, 131]. Some authors suggested that these Pex19p-binding sites can be shortened to a helical motif of 11 amino acids comprising hydrophobic and positively charged residues [131]. Although the pursuit of a consensus sequence for the mPTS of PMPs has proven unsuccessful [126], those authors developed a yeast-based prediction matrix to detect Pex19p-binding sites in these proteins [131]. This algorithm was used to predict some Pex19p-interacting regions that were experimentally confirmed [117, 123, 129, 131, 132]. Nonetheless, this *in silico* approach should be regarded with care since it renders high numbers of false positive results.

The involvement of Pex19p in PMP sorting was further analyzed by transient inhibition of Pex19p expression using RNA interference [109]. The authors proposed the existence of two distinct PMP import mechanisms and two classes of mPTSs. The class 1 mPTSs, which are present in most PMPs, bind Pex19p and depend on this peroxin for targeting whereas class 2 mPTSs function independently of Pex19p [109]. Currently, Pex3p is the only identified class 2 PMP [109] which is supported by data showing that its mPTS does not interact with Pex19p (see section 1.3.3.3.). This distinct sorting pathway of Pex3p is consistent with an early role in peroxisome membrane biogenesis.

1.3.3.6. Pex19p interactions with Pex3p

The observations depicted above reflect a complex interaction mode of Pex3p towards Pex19p, different from all the other PMPs. In agreement, ternary complexes involving Pex3p, Pex19p and a PMP were detected *in vitro*, wherein Pex19p interacts with both Pex3p and the PMP [112, 133]. Furthermore, *in vivo* studies have shown that Pex3p recruits Pex19p to the peroxisomal membrane or even to other organelles when the former is artificially mistargeted [95, 96]. In experiments with transient inhibition of Pex3p expression, Pex19p is no longer detected on peroxisomes. Conversely, the targeting of the Pex3p mPTS is unaffected by this inhibition, further corroborating its distinct import mechanism [95]. Together with data from other studies, these results indicate that Pex3p is responsible for the docking of Pex19p at the peroxisome membrane [95, 96, 113, 116,

118]. As described in section 1.3.3.4., two separate Pex3p-binding sites have been mapped on Pex19p (see Figure 3). The N-terminal Pex3p-interacting region was reported to be essential and sufficient to dock Pex19p at the peroxisome surface [95, 116]. In contrast, the other Pex3p-binding site at the C-terminal domain does not appear to be involved in docking and probably serves a different purpose such as facilitating the release of the cargo PMP [116]. Altogether, the available data indicate that Pex19p and Pex3p compose, at least transiently, a structural and functional unit.

1.3.3.7. Models of Pex19p role in biogenesis

Taken as a whole, Pex19p emerges as a key peroxin participating in the PMP import pathway. However, despite all the data collected over the recent years, the role of this protein is still disputed [78]. At present, it is clear that Pex19p has the capacity to bind a broad range of newly synthesized PMPs. For instance, an overexpressed Pex19p version holding a nuclear localization signal led to the mislocalization of newly synthesized PMPs to the nucleus [92]. By using a cell-free translation system, it was suggested that this peroxin not only interacts with newly synthesized PMPs but also prevents them from aggregating [112]. In fact, *in vivo* studies have shown that PMPs are mistargeted or degraded in the absence of Pex19p [91, 92, 109, 118]. Thus, it is widely accepted that Pex19p behaves as a chaperone-like protein which binds and stabilizes PMPs by masking their hydrophobic transmembrane domains and preventing them from misfolding and aggregation [78, 108, 109, 112]. Nevertheless, divergence regarding the true physiological site of the Pex19p-PMP interaction (cytosol or peroxisomal membrane) raised two distinct models on the role of Pex19p in peroxisome membrane biogenesis (Figure 4).

Some authors have proposed that Pex19p binds newly synthesized PMPs in the cytosol in a chaperone-like function and transports them to the peroxisomal membrane acting as cycling receptor. At the organelle surface, Pex19p docks to Pex3p and mediates the PMP insertion into the lipid bilayer [92, 95, 109, 133]. This model is supported by several lines of evidence described above (sections 1.3.3.5. and 1.3.3.6). However, the cycling receptor hypothesis has still not met general acceptance and faces a few reservations. Indeed, this model is primarily based on the bimodal distribution of Pex19p which is not fully established [92, 134]. Moreover, it is assumed that the Pex19p-PMP complexes observed in the cytosol are in fact import-competent and recognized by the docking/insertion machinery. In addition, those studies cannot exclude the possibility that these observations represent an artefact from Pex19p overexpression. On the other hand, it was reported that for a few PMPs other than Pex3p, the mPTSs and the Pex19p-binding

sites are separate and nonoverlapping entities [108, 110, 120, 135]. Therefore, these authors suggested that Pex19p cannot function as a targeting receptor for PMPs and postulated that Pex19p interacts with pre-existing PMPs at the peroxisomal membrane, acting as membrane chaperone in the assembly or disassembly of PMP complexes [110, 120, 134, 136]. This second model is largely based on cross-linking experiments showing no difference between the amounts of Pex19p-PMP complexes in the presence or absence of new protein synthesis, together with the inability to find such complexes in the cytosol [110]. However, the assays were performed under steady-state conditions where the cytosolic pool of PMPs in transit is certainly much smaller than the steady-state PMP population residing at the peroxisomal membrane. In addition, the presented conclusions bear the problem of deriving solely from negative results. This concern also applies to the data suggesting that the mPTSs and the Pex19p-binding sites fall into distinct regions of the PMP. Indeed, the finding that Pex19p does not bind to some functional mPTSs arise from two-hybrid assays wherein negative results do not necessarily denote lack of interaction and should be interpreted with great care. Similarly, the Pex19p-binding sites that fail to target to peroxisomes *in vivo* may simply reflect the absence or disruption of the domain(s) within the mPTS required for membrane insertion. As mentioned in section 1.3.3.5., some PMPs appear to harbour multiple mPTSs, underlining the complexity of the receptor-cargo interaction and, ultimately, PMP insertion into the peroxisome membrane. Clearly, further work is necessary to clarify the role of Pex19p in peroxisome biogenesis.

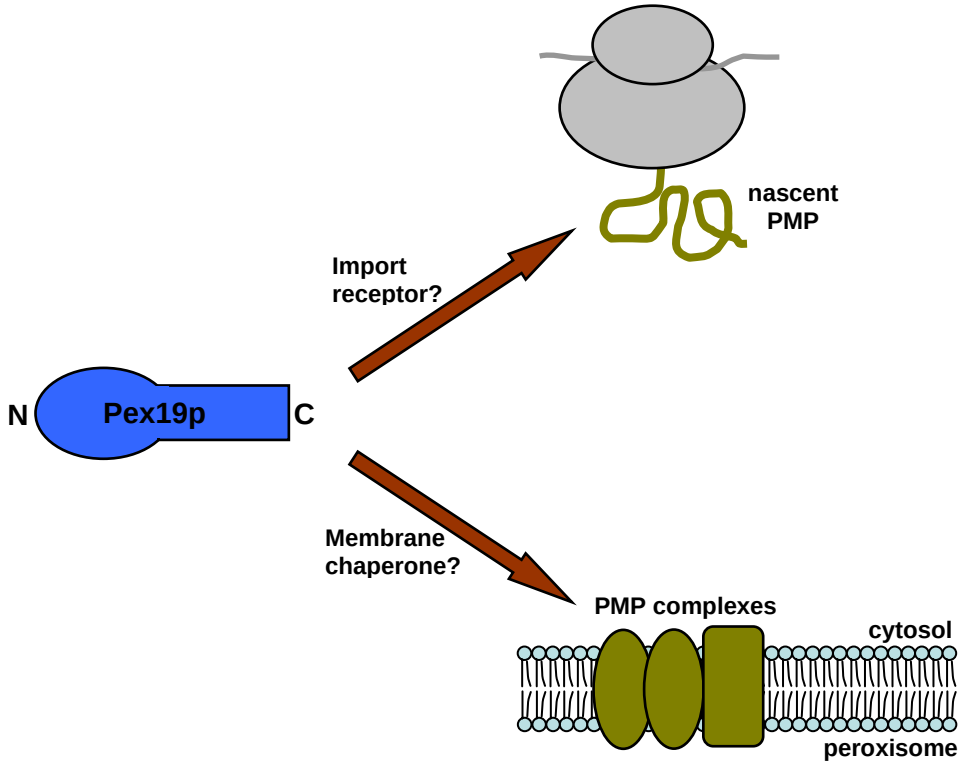

Figure 4. The role of Pex19p in the biogenesis of PMPs.

Two distinct models have been proposed regarding the role of Pex19p in PMP biogenesis. At the basis of such models is the dispute on whether the Pex19p-PMP interaction occurs primarily in the cytosol or at the peroxisomal membrane, under physiological conditions. According to the first model, Pex19p binds newly synthesized PMPs in the cytosol in a chaperone-like manner and transports them to the peroxisomal membrane acting as an import receptor. At the organelle surface Pex19p docks to Pex3p, delivers the PMP and recycles back to the cytosol. The second model states that Pex19p binds pre-existing PMPs at the peroxisome membrane, where it functions as a membrane chaperone assisting the assembly/disassembly of PMP complexes.

2. OBJECTIVES

2. OBJECTIVES

Biogenesis of the peroxisomal membrane remains a barely understood process and a matter of much debate. In this context, Pex19p appears as the key component involved in the PMP import pathway. Despite all the gathered data, it is still unclear whether this peroxin plays a role as a cytosolic chaperone and cycling receptor for PMPs, or as a membrane chaperone assisting the assembly or disassembly of PMP complexes at the peroxisomal surface.

Most of the available data on the role of Pex19p is derived from mutated cell lines and steady-state studies using overexpressed proteins. Alternatively, other studies have inferred Pex19p function from its protein-protein interactions determined essentially by two-hybrid, pull-down or immunoprecipitation assays. Therefore, a kinetic strategy should shed new light into these issues. The main objective of this study was the development of an *in vitro* import system to characterize the sorting pathway of a PMP into the peroxisomal membrane. *In vitro* import systems present two major advantages in relation to other assays. First and foremost, it provides a kinetic perspective because experimental time windows of minutes can be used. Secondly, these systems are inherently open which permits an easy addition or neutralization of components at any given time point.

In the past, this kind of strategy was employed and specific insertion of two PMPs, PMP22 and PMP70, into the peroxisomal membrane was shown [84, 85, 137]. However, when these studies were performed very little was known regarding peroxisome membrane biogenesis and the identity of the involved components. Currently, considerably more aspects about this subject are known and probably all the implicated peroxins have been identified. Thus, the aim of this work was to extend previous observations and further investigate the mechanisms of PMP import using other tools such as recombinant proteins and antibodies.

For this purpose, an appropriate reporter PMP was first constructed and validated *in vivo*. The *in vitro* import system was then developed using a post-nuclear supernatant (PNS) from rat liver. This *in vitro* system was next used to address the involvement of the central peroxins Pex3p and Pex19p in the PMP import pathway. In addition, the binding properties of Pex19p towards Pex3p and the reporter PMP were also analyzed.

3. EXPERIMENTAL PROCEDURES

3. EXPERIMENTAL PROCEDURES

3.1. Construction of plasmids encoding the reporter PMP

From the N to the C terminus, the reporter PMP used in this work comprises the green fluorescent protein (GFP), a linker of 12 amino acids (SGLRSRAQASNS), the first 175 amino acid residues of human PMP24 [138] and three c-Myc epitopes. In order to construct an expression plasmid encoding this fusion protein, the plasmid yf48b10.r1 (IMAGE Consortium ID 25369) was used as a template in a PCR reaction using the forward primer HsPMP24Fw and the reverse primer HsPMP24.175Rv. The amplified fragment, containing the PMP24 moiety, was digested with EcoRI and BglII and cloned into the EcoRI/BamHI sites of pEGFP-C1 (Clontech), originating pMP13. The latter plasmid was digested with BamHI and SalI and ligated to a DNA fragment obtained by annealing the primers 3xc-MycFw and 3xc-MycRv, producing pMP15. This plasmid encodes the desired PMP24-fusion protein and was used in transfection experiments. To generate a plasmid for expression of the reporter PMP in the *in vitro* transcription/translation reactions, pMP15 was subjected to a PCR reaction using the primer pair GFP-P24Fw and GFP-P24Rv. The resulting PCR product was digested with SalI and XmaI and inserted into the SalI/XmaI-digested pGEM-4 vector (Promega), yielding pMP17. In order to produce a DNA fragment encoding GFP along with the linker (see above) and preceded by the T7 RNA polymerase promoter, pMP17 was also used as a template in a PCR using the forward primer pGEM-4Fw and the reverse primer GFPlinkerRv.

Table 4. Oligonucleotides used in PCR reactions of the reporter PMP

Primer	Sequence
HsPMP24Fw	5'-GGGGGAATTCTATGGCAGCCCCGCCGCAG-3'
HsPMP24.175Rv	5'-CGGAGATCTCAGGTCGACGCGGCCGCGGATCCCCTTACGACCTCGGTGATACTC AAAGAGCCAC-3'
3xc-MycFw	5'-GATCATGGGACAGAAGCTGATCTCAGAGGAGGACCTGGAGCAGAAACTCATCTCT GAAGAAGATCTGGAACAAAAGTTGATTTCAGAAGAAGATCTG-3'
3xc-MycRv	5'-TCGACAGATCTTCTTCTGAAATCAACTTTTGTTCCAGATCTTCTTCAGAGATGAGTT TCTGCTCCAGGTCCTCCTCTGAGATCAGCTTCTGTCCCAT-3'
GFP-P24Fw	5'-GCGCGCGTCGACCACCATGGTGAGCAAG-3'
GFP-P24Rv	5'-TCCCCCCGGGCTACAGATCTTCTTCTGAAATC-3'
pGEM-4Fw	5'-AGTCAGTGAGCGAGGAAGCGGAAGAGC-3'
GFPlinkerRv	5'-CGCCACGCCTAAGAATTCGAAGCTTGAG-3'

3.2. Cell culture, transfections and immunofluorescence microscopy

Chinese hamster ovary (CHO) cells were cultured in alpha minimal essential medium supplemented with 10% (v/v) fetal calf serum, 100 µg/ml penicillin G, 100 µg/ml streptomycin sulfate and 0.25 µg/ml amphotericin B in a humidified incubator at 37°C in the presence of 5% CO_2. The cells were transferred to coverslips and afterwards transfected by employing the Magnetofection transfection technology (OZ Biosciences) or Lipofectamine Plus (Invitrogen). Indirect immunofluorescence analyses were performed as described [139], using the anti-Pex14p antibody. The subcellular localization of the GFP-fusion proteins was determined by co-localization with Pex14p. Fluorescence was observed under a Leica DMR microscope equipped with fluorescein isothiocyanate/RSGP/Bodipy/Fluo3/DIO and Texas Red filters. The membrane topologies of the GFP-fusion proteins were assessed by indirect immunofluorescence with antibodies directed to GFP or the c-Myc epitope, in the presence of streptolysin O to selectively permeabilize the plasma membrane or Triton X-100 to permeabilize all membranes, as described [140].

3.3. Expression and purification of recombinant proteins

The plasmids encoding the recombinant versions of human Pex19p, His_6HsPex19p (pMF119) [108], His_6HsPex19p(31-299) (pTW151) [108], His_6HsPex19p(1-124) (pMF956) [141] and His_6-TEV-HsPex3p(34-373) (pMF1259) [141], were provided by Dr. Marc Fransen (Katholieke Universiteit Leuven, Leuven, Belgium). His_6HsPex19p, His_6HsPex19p(31-299) and His_6HsPex19p(1-124) were expressed in the *Escherichia coli* strains M15, TOP10 and BL21 (DE3), respectively. Induction of 100-ml cultures with 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) (His_6HsPex19p and His_6HsPex19p(1-124)) or 2% (w/v) L-arabinose (His_6-HsPex19p(31-299)) was carried out for 3 h at 37 ºC. Bacterial pellets were cooled on ice and lysed by sonication in 1.5 ml of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM DTT, 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF) and 1:500 (v/v) mammalian protease inhibitor mixture (Sigma). Insoluble material and debris were removed by centrifugation at 10,000 *g* for 15 min, and the clarified supernatant was applied to 100 µl (bed volume) of HIS-Select™ nickel affinity gel (Sigma) and incubated for 2 h at 4 ºC. The nickel beads were washed three times with 1.5 ml of 50 mM sodium phosphate, pH 8.0, 150 mM NaCl and the His-tagged proteins eluted by three washes with 200 µl of 50 mM sodium phosphate, pH 8.0, 150 mM NaCl and 100 mM imidazole. The eluted proteins were concentrated to ~100 µl using 10,000-molecular weight cut-off

polyethersulfone Vivaspin concentrators (Vivascience), and the buffer was exchanged to SEM (0.25 M sucrose, 20 mM MOPS-KOH, pH 7.4, 1 mM EDTA-NaOH, pH 7.4), 1 mM DTT by three cycles of concentration/dilution. These Pex19p recombinant proteins were further purified by anion-exchange chromatography using a linear gradient of 25-500 mM NaCl in 50 mM Tris-HCl, pH 8.0 and 1-ml Econo-Pac® high capacity ion exchange cartridges (Bio-Rad). The fractions containing the purified proteins were pooled and subjected to five rounds of concentration/dilution with SEM, 1mM DTT, using the Vivaspin columns. Protein aliquots at 5-10 mg/ml were frozen in liquid N_2 and stored at -70 ºC.

His_6-TEV-HsPex3p(34-373) was expressed in the BL21 (DE3) strain of *E. coli*, as described [112]. Bacterial cells of 100-ml culture were induced with 1 mM IPTG for 24 h at 18 ºC. The recombinant protein was purified using the HIS-Select™ nickel affinity gel as described above except that the NaCl concentration was reduced to 75 mM in all buffers. His_6-TEV-HsPex3p(34-373) was concentrated and diluted with SEM, 1 mM DTT as described above.

3.4. *In vitro* synthesis of radiolabeled proteins

The ^{35}S-labeled proteins were synthesized using the TNT® Quick Coupled Transcription/Translation System (Promega) in the presence of Redivue™ L-[^{35}S]methionine (specific activity >1000 Ci/mmol; Amersham) according to the manufacturer's protocol. When specified, purified recombinant versions of Pex19p in SEM buffer were included in the transcription/translation reactions at 2.4 µM final concentrations. In experiments involving different reticulocyte lysates, the concentrations of the ^{35}S-labeled reporter protein were previously quantified by SDS-PAGE and autoradiography. Reticulocyte lysates showing a variation in the yield of the radioactive protein higher than 20% were not used in the experiments.

3.5. Preparation of rat liver post-nuclear supernatants

PNS fractions were prepared from the liver of male Wistar rats with 1-2 months of age, fasted overnight, essentially as described [52]. Briefly, the livers were quickly removed and homogenized in SEM buffer supplemented with 2 µg/ml *N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide (E-64). The PNS fractions were obtained after centrifuging the homogenates twice at 600 *g* for 10 min at 4 ºC, using the SS-34 rotor in a Sorvall® (DuPont Instruments) centrifuge. Aliquots at 40-60 mg/ml of PNS

protein were frozen in liquid N_2 and stored at -70 ºC. These PNS aliquots preserve their import competence for at least one month.

3.6. *In vitro* import experiments

In vitro import reactions were performed in a 100-µl final volume of import buffer (0.25 M sucrose, 50 mM KCl, 20 mM MOPS-KOH, pH 7.4, 3 mM $MgCl_2$, 0.2% (w/v) lipid-free bovine serum albumin, 80 µM methionine, 2 µg/ml E-64) using 450 µg of rat liver PNS protein and 0.15-0.25 µl of a rabbit reticulocyte lysate containing the ^{35}S-labeled reporter protein. Unless indicated otherwise, the reactions were carried out at 37 ºC for 30 min. Where specified, nucleotides were included at 5 mM (ATP or ATPγS) or 0.5 mM (GTP or GTPγS) final concentrations. Treatment of reticulocyte lysates and PNS fractions with apyrase (20 U/ml; grade VII; Sigma) or heat-inactivated apyrase (10 min at 95 ºC) were performed in import buffer for 5 min at 37 ºC. In antibody inhibition experiments, PNS fractions in import buffer were preincubated with 8 µg of purified IgGs in SEM buffer for 20 min on ice, before starting the import reaction by adding the ^{35}S-labeled reporter protein. At the end of the import reactions, the samples were processed as described [52, 53]. Accordingly, import reactions were treated with proteinase K at 400 µg/ml final concentration for 30 min on ice. Where indicated, the samples received detergents (1% (w/v) Triton X-100, 0.5% (w/v) sodium deoxycholate) before adding the protease. Proteinase K was inhibited with 0.5 mg/ml PMSF for 2 min on ice. The import reactions were diluted to 1 ml with SEM buffer and the organelles were isolated by centrifugation at 15,000 *g* for 15 min at 4 ºC. Protein in the samples was precipitated with 10% (w/v) trichloroacetic acid for 30 min on ice. The precipitated protein was pelleted at 15,000 *g* for 15 min at 4 ºC, washed with acetone and subjected to SDS-PAGE analysis. Typically, the gels were blotted onto a nitrocellulose membrane and the radioactive proteins detected by autoradiography. Upon exposure, the membrane was probed with the relevant antibodies. All *in vitro* import experiments were performed at least five times.

3.7. Extraction of membranes with alkali and high/low ionic strength solutions

After proteinase K treatment, organelle pellets from standard import reactions were extracted with alkaline carbonate [142] or with solutions of high or low ionic strengths [53], as described. In brief, the organelle pellets were either resuspended in 0.1 M Na_2CO_3, pH 11.5 and incubated for 30 min on ice, or resuspended in SEM buffer or 0.5 M NaCl in

SEM buffer followed by sonication (three times for 10 s). The samples were halved and one half was kept on ice as a control, whereas the other was separated into membrane and soluble fractions by centrifugation at 135,000 *g* for 1 h. The proteins in the samples were precipitated with 10% (w/v) trichloroacetic acid and analyzed by SDS-PAGE followed by autoradiography and immunoblotting, as described above (section 3.6.).

3.8. Density gradient centrifugation

Import reactions analyzed by density gradient centrifugation were prepared in a four-fold scale-up of the standard reactions. Proteinase K-treated import suspensions were diluted to 1.5 ml with SEM buffer and applied onto the top of step Nycodenz gradients (1.5 ml of 45% (w/v), 6 ml of 30% (w/v), 2 ml of 25% (w/v) and 2 ml of 20% (w/v) Nycodenz in 5 mM MOPS-KOH, pH 7.2 and 1 mM EDTA-NaOH, pH 7.2). The gradients were centrifuged at 48,000 *g* for 2 h at 4 ºC, using the vertical rotor STEPSAVER^{TM} 65V13 (Sorvall®). Fourteen equal fractions of ~920 µl were collected from the bottom to the top of the gradients and aliquots of 250 µl were withdrawn for trichloroacetic acid precipitation and SDS-PAGE analysis. The gradient distribution of the organelles was assessed by immunoblotting with marker antibodies and the radioactive proteins were detected by autoradiography.

3.9. Immunoprecipitation of the imported reporter protein

In the immunoprecipitation experiments of import reactions, a five-fold scale-up of the standard reaction was used. Protease-treated import reactions and reticulocyte lysates containing the ^{35}S-labeled reporter protein were diluted in immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA-NaOH, pH 8.0, 150 mM NaCl, 1% (w/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 500 µg/ml PMSF and 1:100 (v/v) mammalian protease inhibitor mixture (Sigma)) and incubated for 30 min at 4 ºC. After centrifuging the samples at 15,000 *g* for 15 min, the clarified supernatants were subjected to immunoprecipitation using Protein G-Sepharose 4 Fast Flow (Amersham) beads preincubated with the respective antibodies, as described [50]. After incubation for 3 h at 4 ºC, the beads were washed four times with immunoprecipitation buffer, and the immunoprecipitated proteins were eluted with Laemmli sample buffer [143] and subjected to SDS-PAGE analysis.

3.10. Native polyacrylamide gel electrophoresis

For native gel analysis, the recombinant proteins were incubated for 30 min at room temperature in 20 µl of 50 mM Tris-HCl, pH 8.0, 2 mM DTT. At the end of the incubation, the samples received 2 µl of 0.17% (w/v) bromphenol blue, 50% (w/v) sucrose and were centrifuged for 10 min at 15,000 *g*. The protein samples were subsequently loaded onto Tris nondenaturing discontinuous 9 or 10% polyacrylamide gels [144]. These gels were run at 250 V at 4 ºC and stained with Coomassie Brilliant Blue R-250. In experiments containing ^{35}S-labeled proteins, the gels were transferred to nitrocellulose membranes and exposed to an X-ray film or fixed, incubated with Amplify™ Fluorographic Reagent (Amersham) and dried following the manufacturer's instructions.

3.11. Antibodies

The rabbit antibodies directed to human Pex3p and Pex16p [141] were produced by Dr. Márcia Oliveira (Universidade do Porto, Porto, Portugal), the anti-Pex13p antibody [145] was produced by Dr. Alexandra Gouveia (Universidade do Porto, Porto, Portugal) and the antibody directed to rat PMP24 [138] was produced by Dr. Carlos Reguenga (Universidade do Porto, Porto, Portugal). The rabbit antibodies directed to GFP [108] and rat Pex14p [146] were provided by Dr. Marc Fransen (Katholieke Universiteit Leuven, Leuven, Belgium). The anti-PMP70 antibody [147] was a kind gift of Dr. Wilhelm W. Just (Universität Heidelberg, Heidelberg, Germany). The antibodies directed to catalase (catalog no. RDI-CATALASEabr; Research Diagnostics, Inc.), KDEL (catalog no. ab12223; Abcam), cytochrome c (catalog no. 556433; BD Pharmingen) and c-Myc (catalog no. 11667149001; Roche Applied Science) were purchased. On Western blotting analysis, rabbit and mouse antibodies were detected with alkaline phosphatase-conjugated anti-rabbit and anti-mouse antibodies (Sigma), respectively. Anti-Pex3p and anti-Pex16p IgGs were purified by affinity chromatography by Dr. Márcia Oliveira (Universidade do Porto, Porto, Portugal). Total IgGs were purified from rabbit anti-PMP70 and nonimmune sera using Protein A-Sepharose beads according to the manufacturer's instructions (Sigma). IgG preparations were diluted and concentrated for five times with SEM, using the Vivaspin columns as described above (section 3.3.).

3.12. Miscellaneous

Concentrations of recombinant proteins were determined from the absorbance at 280 nm in a buffer containing 6 M guanidine, 20 mM sodium phosphate, pH 6.5, by employing the respective extinction coefficients calculated by the ProtParam tool available at the ExPASy website (http://www.expasy.org).

Protein samples subjected to SDS-PAGE were heated in Laemmli sample buffer [143], centrifuged for 2 min at 15,000 *g* to remove insoluble material and loaded onto the gel. SDS-PAGE separation was performed in 0.75- or 1-mm-thick 12-16% polyacrylamide gels using the Laemmli discontinuous buffer system [143]. At the end of the electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 or blotted onto nitrocellulose membranes (Schleicher & Schuell) using a semi-dry system (The W.E.P. Company) according to the manufacturer's instructions.

Densitometric analysis of X-ray films was performed using the UN-SCAN-IT automated system. Apparent K_D and IC_{50} values were determined from the results of, respectively, three and five independent experiments, using the GraphPad Prism 4 software.

4. RESULTS

4. RESULTS

4.1. Construction and *in vivo* sorting of the reporter PMP, GFP-P24

The first step in the development of an *in vitro* import system involves obtaining a suitable reporter protein. Such reporter protein has to be efficiently targeted and inserted into the peroxisomal membrane. Importantly, it is essential that the correctly imported protein can be distinguished from the nonimported fraction. However, none of the mammalian PMPs are known to be processed or undergo any covalent modification (*e.g*. glycosylation or signal sequence processing) throughout their assembly pathway into the organelle membrane. Protease protection assays are often used to assess the insertion of a protein into a given membrane system. Ideally, a defined portion of the correctly inserted protein should be accessible and sensitive to the protease to produce an insertion "signature". Such pattern is related to the membrane topology of the protein. Without this criterion, the observed protected protein fraction may rather reflect the use of a low protease concentration or nonspecific adsorption to organelle membranes. Thus, the required reporter protein should be a PMP that provides a protease "signature" upon import into the peroxisome membrane.

In previous works with mouse and rat liver peroxisomes, several PMPs were subjected to protease protection assays (C. P. Guimarães, A. M. Gouveia and J. E. Azevedo, unpublished results). However, no protected fragments detected by immunoblotting were found for most of these PMPs. In contrast, rat liver PMP24 fully resisted to both trypsin and proteinase K digestion (C. Reguenga and J. E. Azevedo, unpublished results). This 24-kDa PMP is a highly basic protein that comprises 212 amino acid residues [138]. Moreover, PMP24 appears to have several transmembrane segments (Figure 5), probably with small cytosolic loops inaccessible to the protease. Thus, the fusion of a soluble and protease-sensitive domain to a PMP24 moiety might generate a reporter PMP with the required proteolytic "signature". Several PMP24-derived fusion proteins holding soluble tag domains were constructed. As discussed previously (see "Introduction", section 1.3.3.2.), truncations or addition of tags to a PMP can interfere with its targeting. Therefore, the *in vivo* subcellular localization of these modified PMP24 versions was verified upon transfection of CHO cells. This intracellular sorting was assessed by co-localization with the peroxisomal membrane marker Pex14p in immunofluorescence studies. One of the constructs displaying an exclusive peroxisomal localization (Figure 6) was selected and used in the subsequent experiments. This fusion protein was designated GFP-P24 and harbours the first 175 amino acid residues of

human PMP24, which includes all the predicted membrane-spanning domains (Figure 5). The reporter protein further comprises an N-terminal GFP domain, followed by a 12-amino acid linker, the PMP24 region and three C-terminal c-Myc epitopes (Figure 6B).

The membrane topology of GFP-P24 was analyzed by differential permeabilization of CHO cells. Under conditions of selective permeabilization of the plasma membrane, GFP was recognized by the anti-GFP antibody indicating that this N-terminal domain is exposed to the cytosol. Conversely, the c-Myc epitopes were detected only after permeabilization of all membranes, which suggests that these C-terminal tags are facing the peroxisomal matrix. On the whole, GFP-P24 fulfils the requisites needed for a valid reporter PMP to be tested in *in vitro* import experiments.

Figure 5. Sequence alignment of PMP24 proteins and their putative transmembrane domains.
Human (Hs) PMP24 is aligned with protein homologs from *Gallus gallus* (Gg), *Tetraodon nigroviridis* (Tn), *Caenorhabditis elegans* (Ce), *Ustilago maydis* (Um), *Cryptococcus neoformans* (Cn), *Gibberella zeae* (Gz), *Aspergillus niger* (An), *Yarrowia lipolytica* (Yl), *Candida albicans* (Ca), *Debaryomyces hansenii* (Dh), *Dictyostelium discoideum* (Dd). Transmembrane domains predicted using HMMTOP (http://www.enzim.hu/hmmtop) are shaded in yellow. The asterisk indicates arginine 175 of human PMP24.

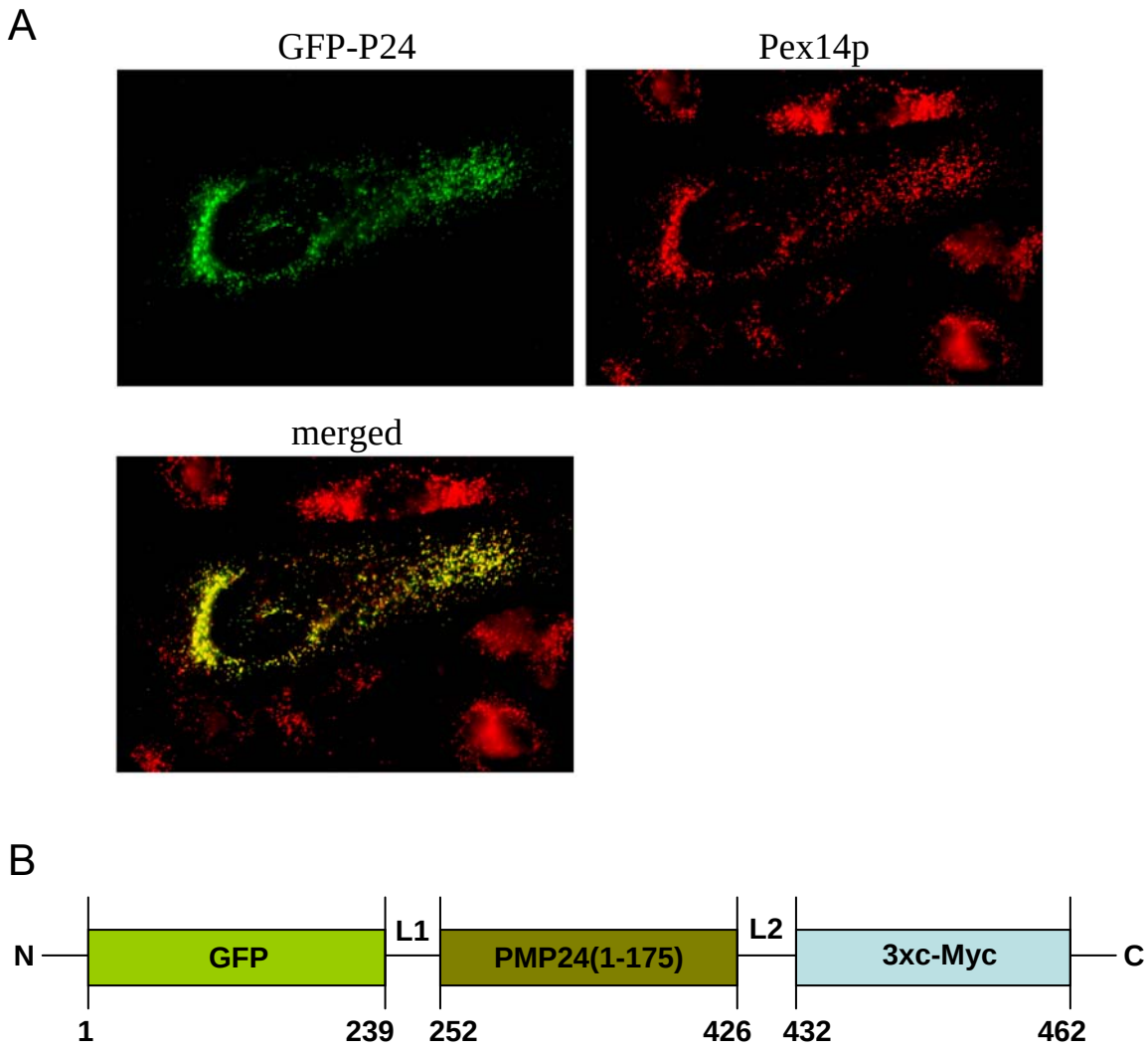

Figure 6. The reporter fusion protein GFP-P24 localizes to peroxisomes *in vivo*.
A. CHO cells were transiently transfected with a plasmid expressing the fusion protein GFP-P24 and examined by direct and indirect fluorescence microscopy to determine the intracellular localization. The punctuate structures observed with GFP-P24 are peroxisomes, as shown by their co-localization with endogenous Pex14p, a peroxisome membrane marker. B. Domain organization of the GFP-P24 fusion protein. From the N to the C terminus, GFP-P24 comprises GFP, a linker of 12 amino acids (L1, SGLRSRAQASNS), the first 175 amino acids of human PMP24, a short second linker (L2, GRKGI) and three copies of the c-Myc epitope.

4.2. A domain of GFP-P24 becomes protease-resistant upon *in vitro* membrane insertion

The *in vivo* results confirm that GFP-P24 is correctly targeted to peroxisomes, rendering it a suitable reporter PMP candidate for the *in vitro* import experiments. In the next step, GFP-P24 was synthesized *in vitro* in the presence of [^{35}S]methionine using a rabbit reticulocyte lysate. In this cell-free system, the fusion protein was properly

expressed and thus could be used in the *in vitro* import assays. To assemble the import reaction, the radiolabeled reporter protein was incubated with a PNS from rat liver as a source of organelles, in import buffer containing ATP (see “Experimental Procedures”, section 3.6.), for 20 min at 37 ºC (see the scheme in Figure 7). As a negative control, a protein consisting of amino acid residues 1-251 of GFP-P24 (thus comprising the soluble GFP and the linker of 12 amino acids but not the PMP24 moiety, see Figure 6B) was also used in these experiments. At the end of the incubation, the samples were centrifuged and the organelle pellets were gently resuspended in SEM buffer. The organelle suspensions were then divided into four identical aliquots. The first one was kept untreated and the second, the third and the fourth aliquot received detergents, proteinase K, and detergents plus proteinase K, respectively, as indicated in Figure 7. The protease treatment was performed on ice for 30 min, as described in “Experimental Procedures". When present, proteinase K was inhibited by the addition of PMSF to all aliquots. The samples that received no detergents were further separated by centrifugation into organelle pellet and supernatant. The proteins from all aliquots were precipitated with trichloroacetic acid and analyzed by SDS-PAGE followed by autoradiography.

As shown in Figure 7, a portion of the control GFP protein still sedimented with the organelles after the first and the second centrifugations (lanes 3 and 1, respectively), despite being soluble and not a membrane protein. This result illustrates the need of using a protease “signature” and shows that sedimentation with organelles alone is an insufficient criterion to assess specificity in this type of assays. Proteinase K digestion of samples containing this protein generated a smaller fragment of 28 kDa. The presence of this fragment is consistent with a tight GFP structure largely resistant to proteolysis [148]. This 28-kDa protease-resistant domain is present in the detergent-treated aliquot (lane 6) and in the supernatant fraction (lane 5) but is absent from the organelle pellet (lane 4). Therefore, the protease treatment eliminates the nonspecific adsorption to membranes of the control protein. Regarding GFP-P24, a large amount of this protein sedimented with the organelles after both the first and second centrifugations (lanes 9 and 7, respectively). Most important, treatment of import reactions containing GFP-P24 with proteinase K yields two protease-resistant fragments. One of the fragments displays the same gel migration as the one of the control GFP protein, and is also observed in the supernatant fraction (lane 11) and in the detergent-solubilized sample (lane 12) but not in the organelle pellet (lane 10). Indeed, this fragment of 28 kDa corresponds to GFP as it could be immunoprecipitated with the anti-GFP antibody (Figure 8, lane 3). The other fragment has a smaller mass (14 kDa) and presents distinct features. This fragment sediments with the organelles (Figure 7, lane 10) and can be immunoprecipitated using the anti-PMP24 antibody (Figure 8, lane 2). Thus, it contains the PMP24 moiety.

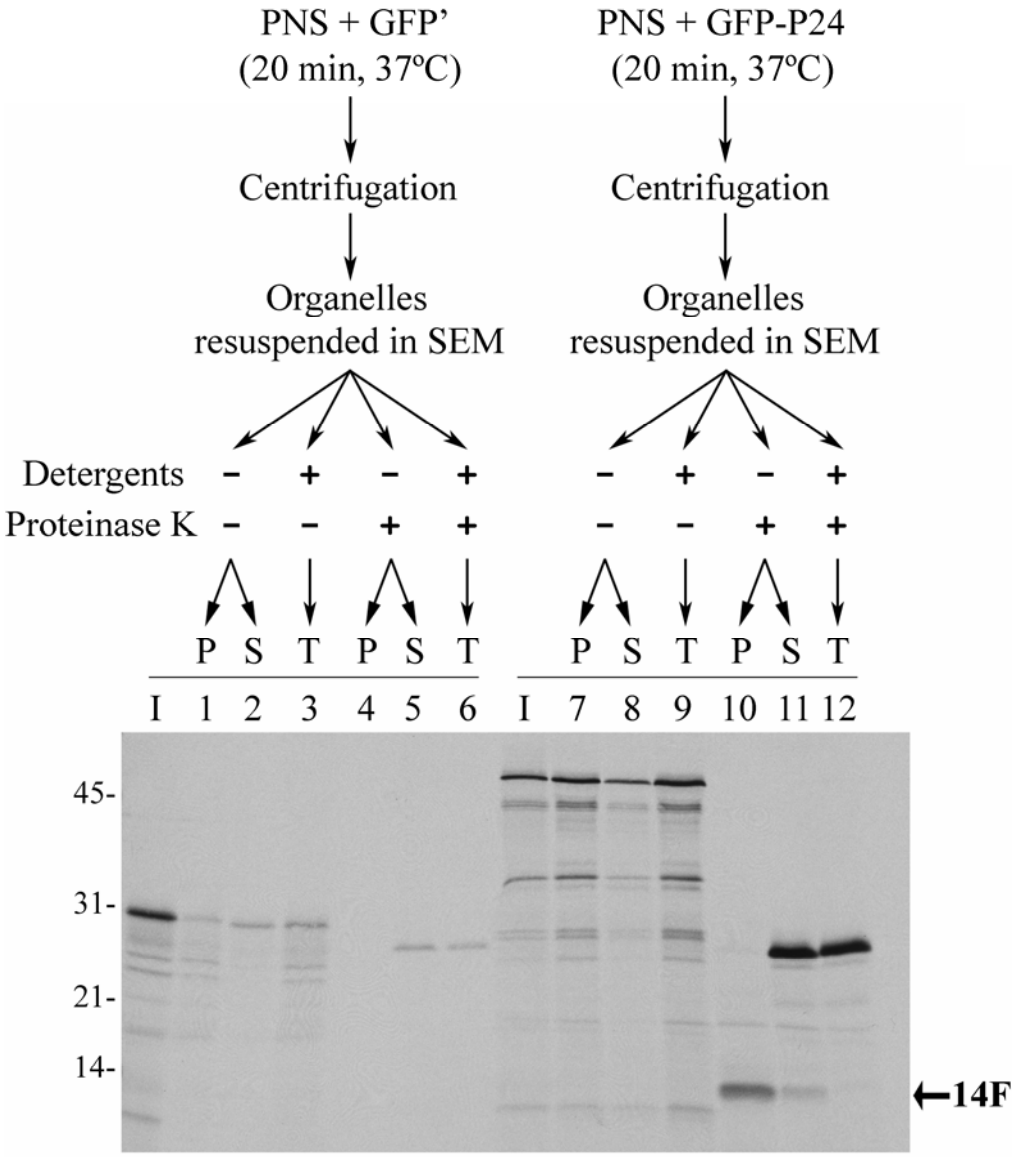

Figure 7. A fragment of ^{35}S-labeled GFP-P24 becomes protease-resistant and is found in the organelle fraction upon incubation with a rat liver PNS.

An *in vitro* synthesized ^{35}S-labeled GFP control protein (GFP', lanes 1-6) or ^{35}S-labeled GFP-P24 (lanes 7-12) was incubated with PNS fractions in import buffer supplemented with ATP for 20 min at 37ºC. After the import reaction, the organelles were sedimented, resuspended in SEM buffer and equally divided into four aliquots. The first aliquot was kept untreated and afterwards separated into organelle pellet (P, lanes 1 and 7) and supernatant (S, lanes 2 and 8). The second aliquot was treated with detergents as described in "Experimental Procedures" (T, lanes 3 and 9). The third aliquot was subjected to proteinase K digestion and later separated into an organelle pellet (P, lanes 4 and 10) and a soluble fraction (S, lanes 5 and 11). The fourth aliquot was treated both with detergents and proteinase K (T, lanes 6 and 12). At the end of the experiment, the total proteins in all samples were recovered by trichloroacetic acid precipitation. After SDS-PAGE separation, the gels were blotted onto nitrocellulose membranes followed by autoradiography. Lanes I: 2.5% of the reticulocyte lysates containing the ^{35}S-labeled proteins added to the initial import reactions. Note that lanes T and lanes P plus S represent one-fourth of the initial import reactions. Numbers to the left indicate the molecular masses of protein standards in kDa. 14F: 14-kDa protease-resistant fragment of GFP-P24.

Furthermore, the 14-kDa domain is completely degraded by proteinase K in the presence of detergents (Figure 7, lane 12). This result shows that the acquired protease-protected status derives from membrane insertion rather than from intrinsic resistance of the domain. Finally, the yield of radiolabeled GFP-P24 conversion into the 14-kDa fragment during the import assays was estimated to be 15-20% from the densitometric analysis of dried gels. Taken together, these data indicate that ^{35}S-labeled GFP-P24 was successfully inserted into organelle membranes through the PMP24 region with the GFP domain facing the cytosol and accessible to proteases, as expected from the *in vivo* observations. Moreover, the membrane insertion shields a 14-kDa domain from proteinase K degradation providing the proteolytic "signature" to monitor the import process.

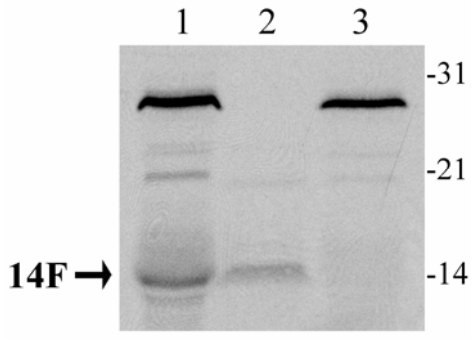

Figure 8. Identification of the protease-resistant fragments of ^{35}S-labeled GFP-P24.
A PNS fraction was incubated with ^{35}S-labeled GFP-P24 in import buffer for 30 min at 37 ºC, using a five-fold increase of the standard import reaction. After proteinase K treatment, the organelle suspensions were diluted 10 times with immunoprecipitation buffer containing protease inhibitors (see "Experimental Procedures"). The solubilized samples were centrifuged and the clarified supernatant was divided into three aliquots. The first aliquot (1/5 of total) was kept on ice throughout the experiment and then subjected to trichloroacetic acid precipitation (lane 1). The second and the third aliquots (2/5 of total each) were immunoprecipitated by using the anti-PMP24 (lane 2) or the anti-GFP (lane 3) antibody, respectively. An autoradiograph of an SDS-PAGE gel blotted onto a nitrocellulose membrane is shown. Numbers to the right indicate the molecular masses of protein standards in kDa. 14F: 14-kDa protease-resistant fragment of GFP-P24.

4.3. Characterization of GFP-P24 membrane interaction

The properties of the 14-kDa protease-resistant fragment were further characterized. Despite several attempts, this 14-kDa domain could not be immunoprecipitated by the anti-c-Myc antibody. Strikingly, full-length GFP-P24 from reticulocyte lysates also did not immunoprecipitate with this antibody. The reasons behind this behaviour are presently unknown. Thus, no additional information regarding the membrane topology of this domain of the reporter PMP could be obtained.

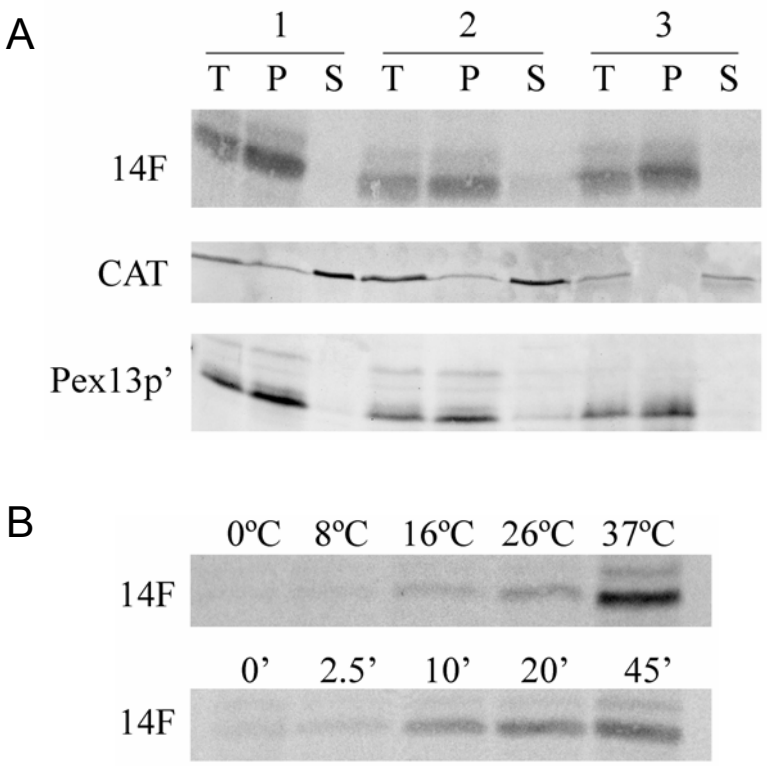

Figure 9. ^{35}S-Labeled GFP-P24 is inserted as an intrinsic membrane protein in a temperature- and time-dependent manner.

A. The 14-kDa fragment of GFP-P24 behaves as a transmembrane protein. After proteinase K treatment of standard import reactions, the organelles were sedimented and extracted either by sonication in SEM buffer (condition 1; see "Experimental Procedures") or 0.5 M NaCl in SEM buffer (condition 2), or by resuspension in 0.1 M Na_2CO_3, pH 11.5 (condition 3). The samples were halved and one half was kept on ice as a total control (lanes T). The other half was separated into membrane (lanes P) and soluble fraction (lanes S) by centrifugation at 135,000 *g* for 1 h. Proteins in the samples were precipitated with trichloroacetic acid, and subjected to SDS-PAGE and Western blotting. After autoradiography to detect the radiolabeled 14-kDa fragment (14F), the nitrocellulose membrane was probed with antibodies directed to catalase (CAT) and Pex13p (Pex13p'). The latter serum recognizes a 28-kDa fragment of Pex13p on proteinase K-treated peroxisomes [53]. B. ^{35}S-Labeled GFP-P24 was incubated with PNS fractions for 20 min at the indicated temperatures (upper panel) or at 37 ºC for the indicated minutes (lower panel). The samples were treated with proteinase K, subjected to trichloroacetic acid precipitation, and analyzed by SDS-PAGE followed by nitrocellulose membrane transfer and autoradiography, as usual. 14F: 14-kDa protease-resistant fragment of GFP-P24.

Treatment of organelle membranes with aqueous solutions exhibiting extremes of ionic strength or pH values has been used to distinguish intrinsic membrane proteins from the peripheral membrane-associated ones [53, 142, 145]. As shown in Figure 9A, the protease-protected 14-kDa fragment was not extracted from the membrane fraction either by sonication in low or high ionic strength buffers or by incubation at alkaline pH (Figure 9A). Likewise, the intrinsic membrane protein Pex13p was also not extracted from the peroxisome membrane under the same conditions. In contrast, the peroxisomal matrix

protein catalase was found in the soluble phase under all conditions employed. Hence, the 14-kDa domain behaves as an intrinsic membrane protein, additionally confirming that GFP-P24 is in fact inserted into organelle membranes.

The acquisition of resistance to proteinase K by the 14-kDa domain reflects the membrane insertion of GFP-P24. A kinetic analysis of this process was performed (Figure 9B). These experiments show that membrane insertion of GFP-P24 is both temperature- and time-dependent. After 20 min of incubation at 37 ºC, high amounts of the reporter PMP acquire a protease-resistant status whereas no detectable import occurs at 0 or 8 ºC. At 37 ºC, the import of GFP-P24 converges rapidly to steady-state values, with a large fraction inserted after only 10 min of incubation.

In any biological process, an important aspect that must be studied to understand its mechanism regards the energetics of that process. To characterize the energetics of the membrane insertion of GFP-P24, import reactions were carried out in the presence of ATP, GTP or nonhydrolyzable analogs (ATPγS or GTPγS). In other import reactions, both the PNS fractions and the GFP-P24-containing reticulocyte lysates were treated with apyrase, an NTP-degrading enzyme. Strikingly, no significant differences were observed among these distinct conditions (Figure 10). Therefore, energy in the form of ATP or GTP is not required to drive the reporter PMP import process.

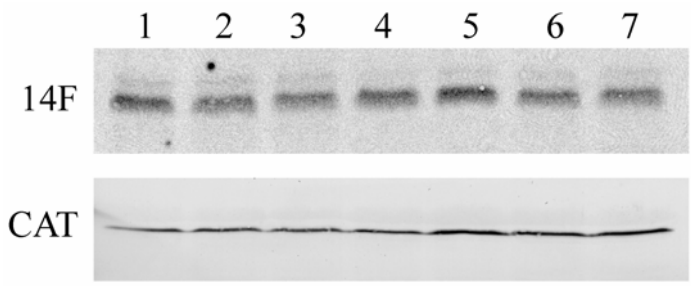

Figure 10. *In vitro* import of ^{35}S-labeled GFP-P24 is independent of ATP and GTP hydrolysis.
In vitro import reactions were performed at 37 ºC without exogenously added nucleotides (lane 1), with both PNS fractions and reticulocyte lysates (containing ^{35}S-labeled GFP-P24) treated with apyrase (lane 2) or with heat-inactivated apyrase (lane 3), or in the presence of 5 mM ATP (lane 4), 5 mM ATPγS (lane 5), 0.5 mM GTP (lane 6) or 0.5 mM GTPγS (lane 7). After proteinase K treatment, the samples were precipitated with trichloroacetic acid and processed for SDS-PAGE separation followed by blotting onto a nitrocellulose membrane. Upon autoradiography, the membrane was probed with an antibody directed to the peroxisomal enzyme catalase (CAT) to control loadings on gel. 14F: 14-kDa protease-resistant fragment of GFP-P24.

4.4. *In vitro* specific import of GFP-P24 into peroxisomes

The above described experiments strongly suggest that the reporter protein, GFP-P24, is inserted into some membrane system in a process that is time- and temperature-

dependent but does not require ATP or GTP hydrolysis. However, those experiments do not reveal the identity of the membrane system(s) in which insertion of GFP-P24 is occurring. As discussed in “Introduction” (section 1.3.3.2.), much controversy subsists on whether PMPs are imported directly to peroxisomes or sorted first to the ER. Thus, considering the small time windows used in the developed assay and the fast GFP-P24 membrane insertion (see Figure 9B), the identification of the membrane system targeted by the reporter PMP gains further interest. In order to clarify this issue, PNS fractions were subjected to standard import reactions with radiolabeled GFP-P24 for 7.5 min at 37 ºC. After this short incubation, the organelle suspension was treated with proteinase K and loaded onto the top of a discontinuous Nycodenz gradient (see “Experimental Procedures”, section 3.8.). At the end of the centrifugation, the gradient was fractionated and analyzed by SDS-PAGE and immunoblotting. The gradient distribution of peroxisomes, ER and mitochondria was assessed with organelle-specific antibodies (Figure 11).

In these gradients, peroxisomes present a double distribution appearing in two different regions, as reported before in another study [52]. One population is found in fractions 8-10, which corresponds to the 25% (w/v) Nycodenz step, wherein large portions of both ER and mitochondria are also present. In contrast, the other peroxisome population is highly pure and is detected in fractions 2-3, corresponding to the 30%-45% (w/v) Nycodenz interface, where none of the two other organelles are found. As shown in Figure 11, the dual distribution of peroxisomes in these gradients is clearly illustrated by Pex13p, an intrinsic protein of the peroxisomal membrane [149-151]. Although catalase is also a peroxisomal protein, a slightly different distribution is observed, with an additional population remaining at the top of the gradient (fractions 11-14). Since catalase is a soluble matrix enzyme, this behaviour is explained by leakage of peroxisomes during the preparation of PNS fractions. The gradient distribution of the protease-resistant fragments of ^{35}S-labeled GFP-P24 yields a very clear result. The 28-kDa GFP fragment stays at the top of the gradient (fractions 11-14) along with other soluble proteins like free catalase. In sharp contrast, the 14-kDa domain distribution perfectly matches the one of Pex13p, showing that GFP-P24 is specifically inserted into the peroxisome membrane despite the presence of all the other organelles in these import reactions. In addition, the parallelism between GFP-P24 and Pex13p profiles, after only 7.5 min of import, strongly suggests that the reporter PMP is inserted into the membrane of mature peroxisomes from the very beginning with no passage through the ER.

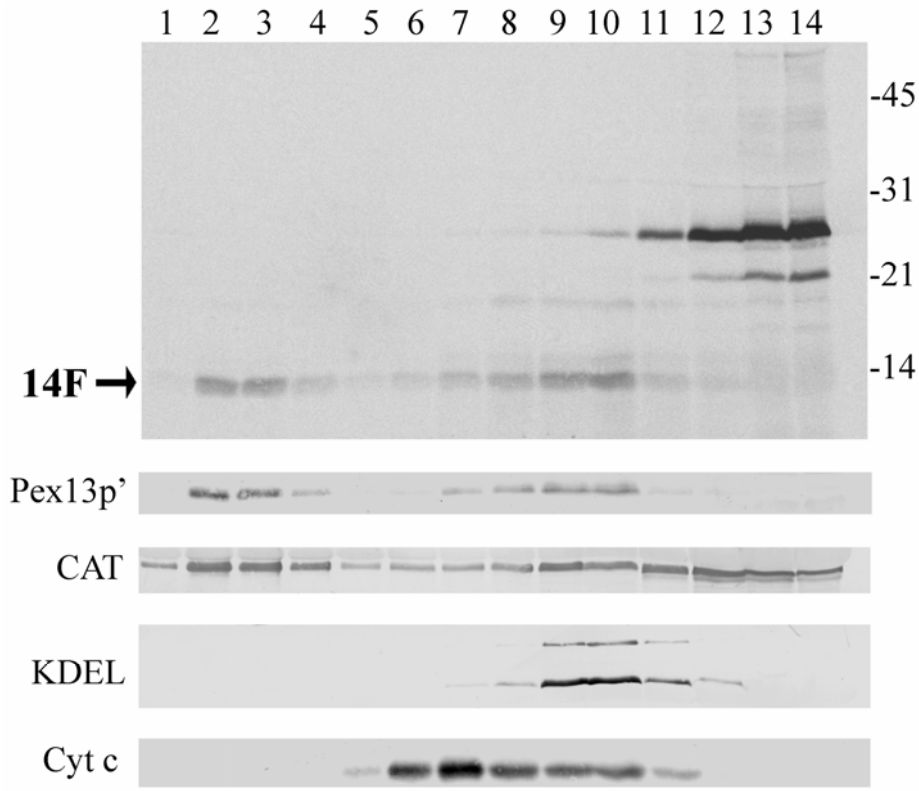

Figure 11. ^{35}S-Labeled GFP-P24 is specifically imported into the peroxisome membrane *in vitro*.
PNS fractions were incubated with ^{35}S-labeled GFP-P24 in import buffer for 7.5 min at 37 ºC. The organelle suspension was then treated with proteinase K, diluted with SEM buffer and applied onto the top of a discontinuous Nycodenz gradient (see "Experimental Procedures", section 3.8.). After centrifugation at 48,000 *g* for 2 h at 4 ºC, the gradient was fractionated from the bottom (lane 1) to the top (lane 14) into fourteen equal fractions. Aliquots of 1/4 of each fraction were precipitated with trichloroacetic acid and subjected to SDS-PAGE analysis, followed by blotting onto a nitrocellulose membrane. Upon autoradiography to detect the ^{35}S-labeled proteins (top panel), the membrane was probed with antibodies directed to markers of different organelles, as follows: anti-cytochrome c (Cyt c; mitochondrial marker), anti-KDEL (KDEL; recognizes the ER proteins GRP72 and GRP98), anti-catalase (CAT; peroxisomal matrix protein) and anti-Pex13p (Pex13p'; PMP, see legend to Figure 9). Numbers at the right indicate the molecular masses of protein standards in kDa. 14F: 14-kDa protease-resistant fragment of GFP-P24.

4.5. Involvement of Pex3p and Pex19p in the *in vitro* import of GFP-P24 into the peroxisomal membrane

The *in vitro* import system was next used to address the involvement of Pex3p and Pex19p in the targeting and insertion of GFP-P24 into the peroxisome membrane. As discussed in the "Introduction" section, these two peroxins are the central components responsible for the biogenesis of PMPs. This *in vitro* system offers the possibility to analyze their role within time windows of minutes rather than the steady-state scale used in most studies.

Pex3p has been proposed to act as a docking factor for Pex19p at the peroxisomal surface (see “Introduction”, section 1.3.3.6.). The involvement of this peroxin was first assessed by preincubating PNS fractions with IgGs directed to Pex3p and then subjecting the organelle suspensions to standard import reactions with radiolabeled GFP-P24. Similarly, import reactions using PNS fractions pretreated with anti-Pex16p, anti-PMP70 or preimmune IgGs were performed as well. While none of the latter IgGs affected the import of GFP-P24, the anti-Pex3p IgGs caused a strong inhibition in the insertion of the reporter PMP (Figure 12A). This result directly implies Pex3p on the mechanism of GFP-P24 import into the peroxisomal membrane. Furthermore, a recombinant version of Pex3p consisting of its cytosolic domain (thus lacking the N-terminal transmembrane domain, see “Introduction”, section 1.3.3.3.), Pex3p(34-373), was also included in these assays. Interestingly, the addition of this Pex3p fragment to the assays also resulted in the inhibition of the import of GFP-P24, displaying a 10 nM IC_{50} (Figure 12B, upper panel). This observation suggests that the recombinant Pex3p version interacts with some component of the PMP import pathway hampering its function, and further corroborates the participation of this peroxin in the GFP-P24 import process. A Pex19p complex is the most obvious candidate for a target of this recombinant Pex3p protein.

In order to verify the involvement of Pex19p in the GFP-P24 sorting pathway, standard import reactions were carried out in the presence of increasing amounts of recombinant Pex19p. Remarkably, rather than any stimulation in the import of GFP-P24, the opposite effect is observed at higher concentrations of the recombinant protein (Figure 12B, lower panel). Indeed, this recombinant Pex19p protein exhibits a 260 nM IC_{50} for the insertion of GFP-P24 into the peroxisomal membrane. Most likely, high amounts of recombinant Pex19p start interfering with the import mechanism of GFP-P24, possibly by hindering Pex3p at the peroxisomal membrane (see “Discussion”). Interestingly, when recombinant Pex19p is previously subjected to heat treatment, the inhibitory activity of the protein in the import reactions is preserved (Figure 12B, lower panel, lane Δ+). Such treatment is expected to irreversibly denature a globular protein and disrupt its function, as observed for Pex3p (Figure 12B, upper panel, lane Δ+). However, a major portion of Pex19p polypeptide chain is natively unfolded ([112], see section 1.3.3.4. of “Introduction”) and thus insensitive to this treatment. The observation that heat-treated Pex19p retains its properties on native PAGE analysis and its interaction with Pex3p (I. S. Alencastre, C. P. Grou and J. E. Azevedo, unpublished observations) corroborates this interpretation.

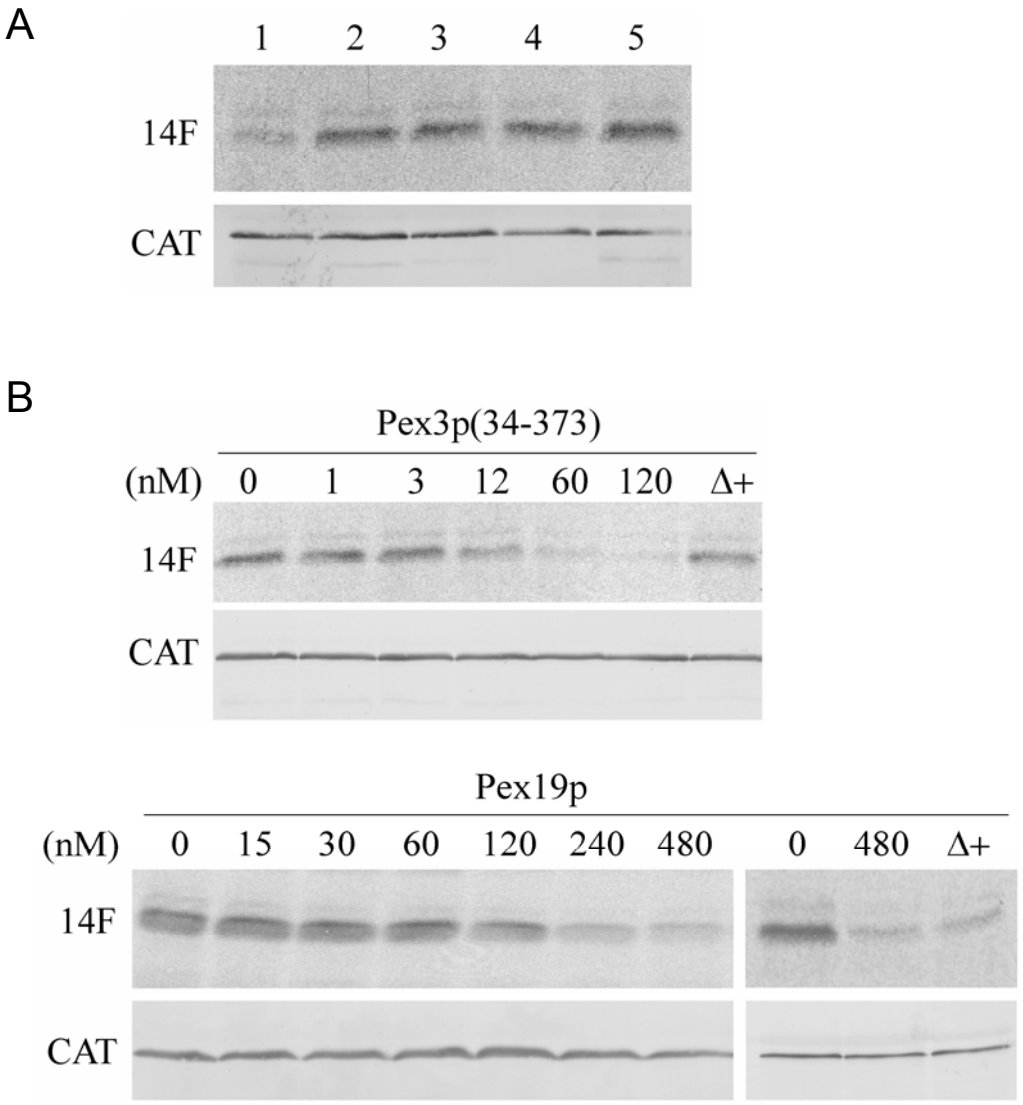

Figure 12. Pex3p and Pex19p participate in the *in vitro* import of ^{35}S-labeled GFP-P24.

A. PNS fractions were treated for 20 min on ice with 8 µg of immunopurified IgGs directed to Pex3p (lane 1) or Pex16p (lane 2), or total IgGs purified from an anti-PMP70 (lane 3) or nonimmune (lane 4) serum. As a control, a PNS fraction was preincubated under the same conditions with SEM buffer (lane 5). After the preincubation, the PNS fractions were subjected to standard import reactions with ^{35}S-labeled GFP-P24. The samples were processed as described in legend to Figure 10. B. ^{35}S-Labeled GFP-P24 was incubated with PNS fractions in import buffer containing increasing amounts of recombinant Pex3p(34-373) (upper panel) or recombinant Pex19p (lower panel) for 20 min at 37 ºC. The numbers refer to the concentration in nM of the recombinant protein used in each import reaction. In parallel, other import reactions were performed in the presence of the highest amount of the respective recombinant protein pretreated by heat at 95 ºC for 5 min (lane Δ+). After proteinase K treatment, the samples were processed as described in legend to Figure 10. CAT: catalase. 14F: 14-kDa protease-resistant fragment of GFP-P24.

4.6. Pex19p interacts with GFP-P24 in the cytosol and mediates its insertion into the peroxisome membrane

The observation that addition of recombinant Pex19p to the import reactions does not stimulate GFP-P24 insertion into the peroxisomal membrane may have several explanations (see previous section). For instance, this recombinant version of Pex19p could lack the capacity to bind GFP-P24. Alternatively, it is possible that the interaction of Pex19p with PMPs has to occur earlier, during or shortly after their translation. In order to address this possibility, different recombinant versions of Pex19p (full-length Pex19p, Pex19p(31-299) or Pex19p(1-124)) were added at different points in the life of the reporter PMP. Thus, in the experiment presented in Figure 13, the recombinant proteins were added either to the reticulocyte lysates during the *in vitro* synthesis of GFP-P24 (lanes 2, 4 and 6), or to the PNS fractions at the *in vitro* import assays (lanes 1, 3 and 5). In each sample, the final concentration of the recombinant protein in the import reaction was the same, 6 nM. As shown in lanes 1, 3 and 5, inclusion of any of these recombinant Pex19p proteins in import mixtures comprising standard GFP-P24-containing lysates (*i.e.* GFP-P24 synthesized in the absence of recombinant proteins) has no effect in the efficiency of the import process. Strikingly, dramatic changes in the import efficiencies are observed depending on whether GFP-P24 was synthesized in the presence or absence of the recombinant proteins. In fact, inclusion of full-length Pex19p in the *in vitro* translation of GFP-P24 leads to a 2.5-fold increase in the peroxisomal membrane insertion of the reporter PMP (compare lanes 1 and 2). In sharp contrast, addition of recombinant Pex19p(31-299) to the translation reaction results in a strong decrease in the amount of imported GFP-P24 (lane 4). This truncated version of Pex19p lacks the strongest Pex3p-binding site presumably necessary for docking at peroxisomal membrane, but preserves the integrity of the cargo-binding region (see “Introduction”, sections 1.3.3.4.-1.3.3.6.). Furthermore, when recombinant Pex19p(1-124) is added to the *in vitro* translation of GFP-P24 (lane 6), no significant differences in the amount of protease-protected 14-kDa fragment are obtained. In the case of Pex19p(1-124), the Pex3p-docking domain is present whereas the cargo-binding region is absent (see “Introduction”, sections 1.3.3.4.-1.3.3.6.). Altogether, these results indicate that the import competence of GFP-P24 is ensured by Pex19p molecules present at the translation level. Therefore, the presented data suggest that Pex19p forms a complex with newly synthesized GFP-P24 in the cytosol, and that this complex is the substrate for the import machinery at the peroxisomal membrane.

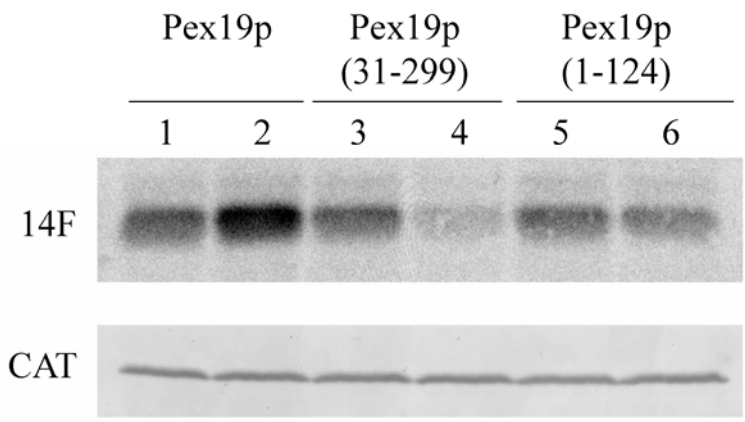

Figure 13. Inclusion of recombinant versions of Pex19p in the *in vitro* translation of ^{35}S-labeled GFP-P24 strongly influences its import efficiency.

^{35}S-Labeled GFP-P24 was synthesized *in vitro* using a reticulocyte lysate supplemented with recombinant Pex19p (lane 2), Pex19p(31-299) (lane 4) or Pex19p(1-124) (lane 6), and afterwards subjected to standard *in vitro* import reactions with PNS fractions. Alternatively, ^{35}S-labeled GFP-P24 synthesized in the absence of recombinant proteins was incubated with PNS fractions containing recombinant Pex19p (lane 1), Pex19p(31-299) (lane 3) or Pex19p(1-124) (lane 5) and subjected to standard import reactions as well. In all samples, the final concentration of the recombinant proteins in the import assay was 6 nM. The yield of the different reticulocyte lysates in ^{35}S-labeled GFP-P24 was previously quantified by SDS-PAGE and autoradiography, so that the amounts of the radiolabeled reporter used in each import reaction did not vary more than 20%. Proteinase K-treated samples were processed as described in legend to Figure 10. CAT: catalase. 14F: 14-kDa protease-resistant fragment of GFP-P24.

4.7. Native gel analysis of Pex19p interactions with Pex3p

The experiments involving radiolabeled GFP-P24 translated in the presence of recombinant versions of Pex19p strongly suggest that protein complexes comprising the reporter PMP and these recombinant proteins are formed within a cytosolic fraction, the reticulocyte lysate. Moreover, the nature of the recombinant proteins used had major effects in the import competence of GFP-P24. Indeed, full-length Pex19p yields a strong stimulation in the import process while the truncated version Pex19p(31-299), lacking the putative Pex3p-docking domain, has a large inhibitory outcome. Thus, these protein-protein interactions were further analyzed by employing a different method, the native PAGE. The behaviours of recombinant Pex19p, Pex19p(31-299), Pex19p(1-124) and Pex3p(34-373) under this electrophoretic technique were first characterized. Each of the recombinant Pex19p versions was preincubated with increasing amounts of Pex3p(34-373) and subjected to native PAGE analysis (Figure 14). As expected, both full-length Pex19p and Pex19p(1-124) interacted with Pex3p(34-373) (Figure 14, panels A and B, respectively) since these versions include the strongest N-terminal Pex3p-binding domain.

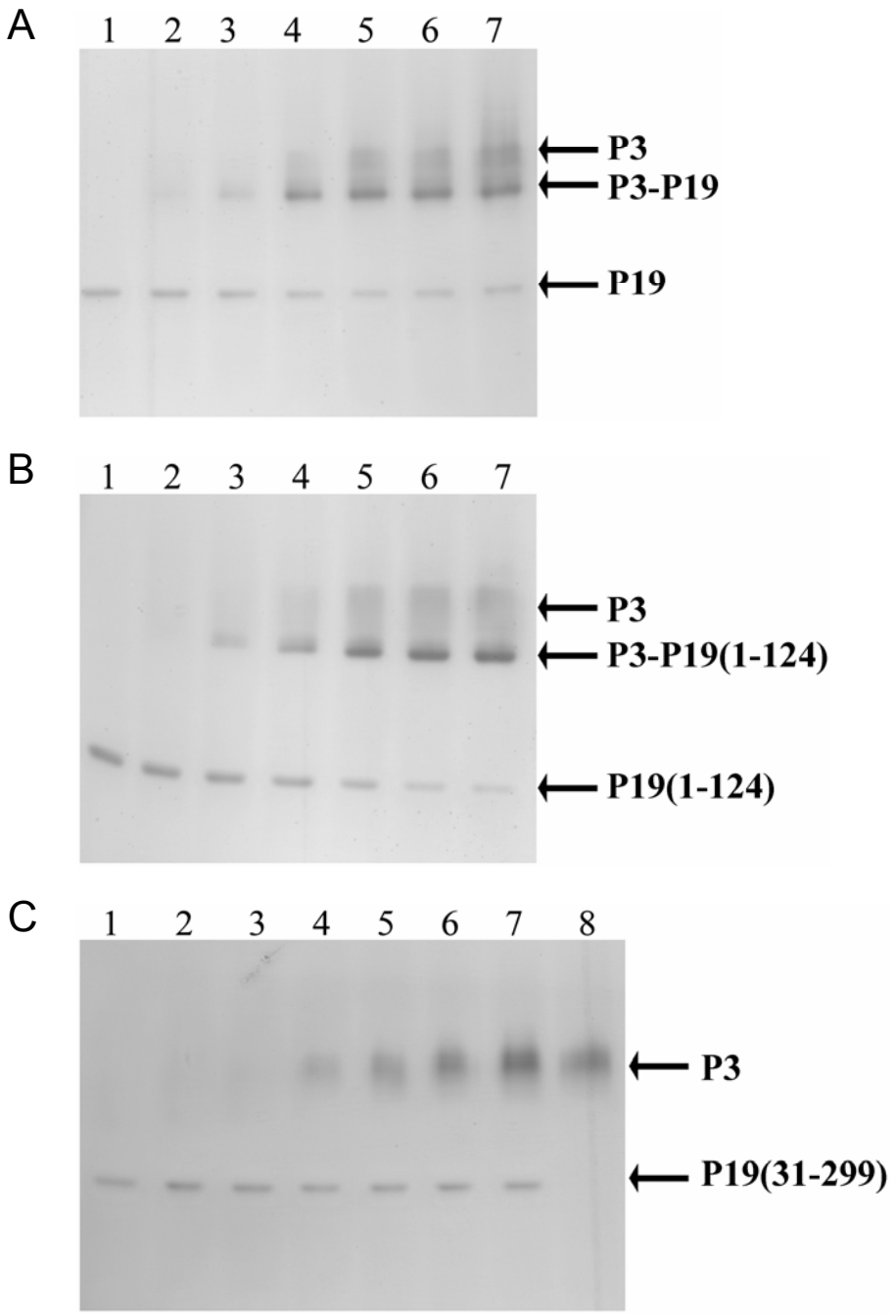

Figure 14. Interactions of Pex19p, Pex19p(1-124) and Pex19p(31-299) with Pex3p(34-373) by native PAGE analysis.

A constant amount (1 µg) of recombinant Pex19p (P19; panel A), Pex19p(1-124) (P19(1-124); panel B) or Pex19p(31-299) (P19(31-299); panel C) was incubated with increasing amounts of recombinant Pex3p(34-373) (P3; 0, 1, 2, 4, 6, 8 and 10 µg in lanes 1-7, respectively) for 30 min at room temperature. After this incubation, the samples were subjected to native PAGE as described in "Experimental Procedures" (section 3.10.). Coomassie-blue stained gels are shown. In panel C, 10 µg of recombinant Pex3p(34-373) alone were applied to lane 8. The arrows refer to the migration position of the recombinant proteins and formed complexes.

Interestingly, Pex19p seems to display a higher affinity towards Pex3p(34-373) when compared to its N-terminal fragment Pex19p(1-124), as suggested by their estimated K_D values of ~3.4 μM and ~26 μM, respectively. This affinity difference probably derives from the second, though weaker, Pex3p-binding site which is absent in Pex19p(1-124) (see "Introduction", section 1.3.3.4.). Although Pex19p(31-299) harbours this second domain, no interaction with Pex3p(34-373) was detected under these electrophoretic conditions (Figure 14C). Therefore, this Pex3p-binding site alone is insufficient to promote complex formation, at least under the used conditions. Taken together, these results corroborate the Pex3p-binding properties previously reported for these recombinant versions of Pex19p [116] and render native PAGE a useful approach to study such protein-protein interactions.

4.8. GFP-P24 forms trimeric complexes involving Pex19p and Pex3p

As mentioned in the previous section, the experiments which included GFP-P24 synthesized in the presence of recombinant Pex19p versions suggest that protein complexes between the reporter PMP and the recombinant proteins were formed *in vitro*. Therefore, protein complexes involving radiolabeled GFP-P24 were also analyzed using the native PAGE technique. When a standard GFP-P24-containing reticulocyte lysate (*i.e.* no recombinant proteins were added during the translation) is subjected to this electrophoretic analysis, a slow-migrating cluster of radioactive sharp bands is observed (Figure 15, arrow a). In addition, a faint and diffuse smear with a faster migration is also detected (lane 1). These two radioactive populations were confirmed as GFP-P24 after subjecting a full lane from a native gel to a second dimension by SDS-PAGE separation. The sharp bands observed at the top of the gel may result from precipitation of the partially hydrophobic GFP-P24 protein. Alternatively, they can represent a high molecular mass GFP-P24-containing complex with rabbit proteins from the lysate, possibly the chaperonin TCP1 ring complex (TRiC). In fact, such complex was previously identified for PMP22 using a rabbit reticulocyte lysate as well [137]. In the same study, PMP22 was also detected in another complex comprising an unidentified 40-kDa protein. Of these two PMP22-containing complexes, the latter showed much higher import efficiency [137]. Therefore, this 40-kDa protein seems to correspond to rabbit Pex19p. It should be noted that the rabbit peroxin is present during the *in vitro* translation of GFP-P24 despite being apparently in limiting amounts, as judged from the effect of the recombinant Pex19p proteins.

In these native gels, GFP-P24 synthesized in the presence of recombinant Pex19p displays a distinct pattern (lane 2). The group of slow-migrating sharp bands is now considerably weaker, whereas most of the reporter protein concentrates in a more intense smear migrating behind a sharp band (arrow c). Remarkably, the intensity of this sharp band is greatly increased when a GFP-P24-containing reticulocyte lysate is supplemented with recombinant Pex19p prior to electrophoresis (lanes 3 and 4). These results show that Pex19p abundance both during the *in vitro* synthesis and the gel run strongly increases the fraction of GFP-P24 that can migrate in this sharp band, probably by maintaining the reporter PMP in a soluble state and preventing its aggregation. Moreover, these observations also suggest that such complexes are somewhat labile under the electrophoretic conditions used. Therefore, high amounts of recombinant Pex19p in the gels prevent complex dissociation to a larger extent.

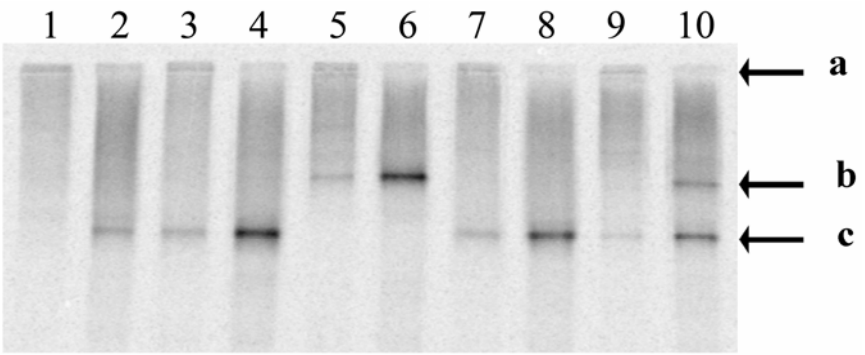

Figure 15. ^{35}S-Labeled GFP-P24 forms dimeric complexes with Pex19p and trimeric complexes involving Pex19p and Pex3p(34-373) in native PAGE analysis.

^{35}S-Labeled GFP-P24 was synthesized *in vitro* using a rabbit reticulocyte lysate under standard conditions (lanes 1, 3, 5, 7 and 9) or supplemented with recombinant Pex19p (lanes 2, 4 and 6) or Pex19p(31-299) (lanes 8 and 10). Before electrophoresis, most samples received 1 µg of recombinant Pex19p (lanes 3-6) or Pex19p(31-299) (lanes 7-10). Some samples also received 1.5 µg of recombinant Pex3p(34-373) (lanes 5, 6, 9 and 10). After the addition of recombinant proteins, the samples were subjected to native PAGE as described in "Experimental Procedures" (section 3.10.). A fluorograph of a dried gel is shown. The arrows indicate a cluster of sharp bands at the top of the gel containing GFP-P24 (arrow a), a complex comprising GFP-P24, Pex19p proteins and Pex3p(34-373) (arrow b) and a complex involving GFP-P24 and Pex19p proteins (arrow c).

Interestingly, inclusion of recombinant Pex3p(34-373) in these samples leads to a shift of the sharp band to an upper region of gel (lanes 5 and 6, arrow b). This result corroborates the former band as the dimeric complex GFP-P24-Pex19p (arrow c), and indicates that the new band (arrow b) corresponds to a trimeric complex comprising GFP-P24, Pex19p and Pex3p(34-373). The truncated Pex19p(31-299) version was also

included in these experiments both during the translation of the reporter PMP and prior to electrophoresis (lanes 7 and 8). In these samples, the observed pattern is similar to the one obtained with full-length Pex19p, although the sharp band displays a slightly lower mobility. However, when the same samples receive recombinant Pex3p(34-373), only a small amount of the GFP-P24 complex is shifted to an upper region of the gel (lane 10). Most likely, this different behaviour reflects the absence or disruption of the strongest Pex3p-binding domain in Pex19p(31-299). Nevertheless, a small portion of trimeric complex is indeed observed, which suggests that Pex19p(31-299) is still able to bind Pex3p(34-373) although with lower affinity. As shown above, however, these two recombinant proteins alone did not form a complex under the same electrophoretic conditions (Figure 14C). Therefore, the interaction between Pex19p(31-299) and Pex3p(34-373) in these native gels seems to be promoted by GFP-P24.

4.9. Cargo PMP dependence of Pex19p binding affinity for Pex3p

Native PAGE analysis allowed the detection of dimeric complexes between GFP-P24 and Pex19p, and trimeric complexes comprising the reporter PMP, Pex19p and Pex3p(34-373). Thus, this technique can be used to analyze how the presence of the cargo PMP, GFP-P24, affects the binding properties of Pex19p for Pex3p. To address this issue, steady amounts of recombinant Pex19p and of a reticulocyte lysate containing radiolabeled GFP-P24 were mixed and incubated with increasing quantities of recombinant Pex3p(34-373). The samples were then subjected to native PAGE analysis followed by blotting onto a nitrocellulose membrane and exposure to an X-ray film. In Figure 16, a Ponceau S-stained membrane (lower panel) and an autoradiograph (upper panel) of the same experiment are shown. At lower substoichiometric concentrations of Pex3p(34-373), unloaded recombinant Pex19p is essentially observed in a free state (lower panel). Conversely, Pex19p molecules loaded with GFP-P24 readily bind Pex3p(34-373) under exactly the same conditions (upper panel). Therefore, these observations indicate that Pex19p interactions with Pex3p are modulated by the cargo PMP whose binding greatly increases the affinity of Pex19p for Pex3p.

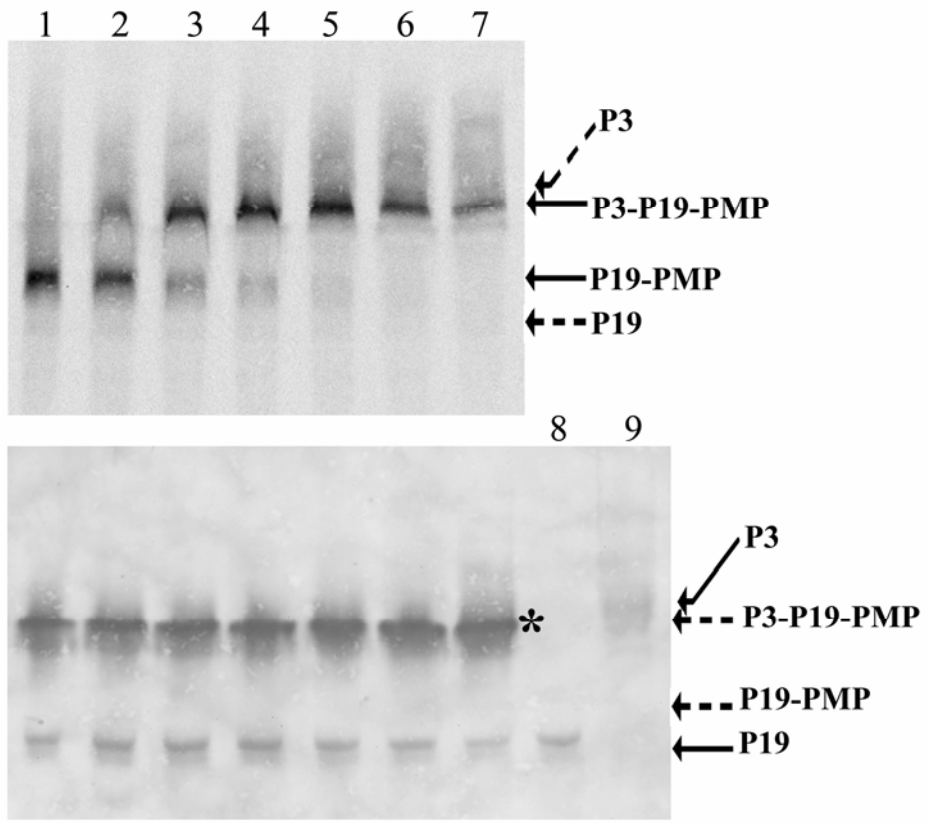

Figure 16. Binding of GFP-P24 increases the affinity of Pex19p for Pex3p.

Increasing amounts of recombinant Pex3p(34-373) (0, 93.5, 187, 325, 750, 1500 and 3000 ng in lanes 1-7, respectively) were incubated with recombinant Pex19p (2 µg) and ^{35}S-labeled GFP-P24 synthesized in the presence of recombinant Pex19p. After incubation for 30 min at room temperature, the samples were subjected to native PAGE followed by blotting onto a nitrocellulose membrane and autoradiography (see "Experimental Procedures", section 3.10.). An autoradiograph (upper panel) and a Ponceau S-stained membrane (lower panel) of the same experiment are shown. Lane 8 contains 2 µg of recombinant Pex19p whereas lane 9 holds 3 µg of Pex3p(34-373). The arrows refer to the gel migration of recombinant Pex19p (P19), recombinant Pex3p(34-373) (P3) and the complexes formed with ^{35}S-labeled GFP-P24 (PMP). The asterisk in the lower panel indicates the hemoglobin band derived from the rabbit reticulocyte lysate.

5. DISCUSSION

5. DISCUSSION

In this work, an *in vitro* import system was developed to characterize the specific targeting and insertion of a PMP into the peroxisome membrane. As previously discussed, this kind of systems has the advantage of being experimentally open and providing a kinetic perspective. Conversely, most of the other studies have used steady-state strategies to address this subject. The first step in the development of the *in vitro* import system was the construction of a reporter PMP that could provide a protease "signature" to assess the correctly inserted protein fraction. For this purpose, an engineered version of PMP24 was constructed and validated *in vivo* to be used as reporter in the *in vitro* assay. This fusion PMP, GFP-P24, fulfils the protease "signature" criterion and was shown to be specifically imported into the peroxisomal membrane. In addition to protease resistance, the membrane insertion of the reporter PMP was also corroborated in experiments in which organelle membranes were extracted by sonication in low or high ionic strength solutions or incubated with alkali. A kinetic characterization of the *in vitro* GFP-P24 import was performed showing a time- and temperature-dependent membrane insertion. These experiments revealed a strong dependence on temperature with high levels of insertion at 37 ºC, but largely decreasing at 26 ºC or 16 ºC and essentially undetected at lower temperatures. Furthermore, this import occurs very rapidly at 37 ºC, with a substantial amount of inserted GFP-P24 being detected after only 10 min of incubation. Hence, the PMP import pathway appears to be completed within a very short time window.

Several years ago, a similar strategy was used to follow the insertion of *in vitro* translated PMP22 and PMP70 into the membrane of peroxisomes [84, 85]. To assess membrane integration of PMP22, the authors digested the samples with subtilisin. Indeed, imported PMP22 becomes entirely resistant to this protease [84]. In the case of PMP70, the authors relied solely on carbonate extraction [85]. A major difference between the previous systems and the one developed in this work concerns the fact that purified peroxisomes were used in those studies, whereas the present one used PNS fractions from rat liver. The reason behind this choice derives from the fact that PMP import into purified peroxisomes does not rule out the hypothesis that such pathway represents a minor contribution to mature peroxisomes, whereas in the presence of all of the other organelles the primary sorting pathway may be distinct, for instance, via the ER (see "Introduction", section 1.3.3.2.). Thus, the finding that a reporter PMP is imported into peroxisomes, even though all the other abundant subcellular organelles are present in the PNS fraction used, demonstrates the specificity of the system.

This observation gains special relevance considering the current dispute regarding the ER involvement in peroxisome membrane biogenesis. As discussed in “Introduction" (section 1.3.3.2.), the longstanding model states that PMPs are synthesized on free cytosolic ribosomes and post-translationally inserted into the organelle membrane [37, 82], whereas some other authors have proposed that peroxisomes bud from the ER with at least some PMPs travelling first through this compartment [83, 87]. This last concept is supported by some recent data suggesting that two PMPs, particularly yeast Pex3p and mammalian Pex16p, reach the peroxisomal membrane via the ER [81, 88-90]. Although these studies raise many reservations (see "Introduction”, section 1.3.3.2.), it should be emphasized that Pex3p and Pex16p are not ordinary PMPs since they participate in the biogenesis of the remaining PMPs. Therefore, it is possible that these two peroxins are sorted through a pathway distinct from all the other PMPs. Indeed, it was suggested that Pex3p is imported independently of Pex19p [109]. Nevertheless, such traffic of Pex3p or Pex16p through the ER could also imply that newly synthesized PMPs are primarily targeted and inserted in this compartment, reaching the peroxisomes at a later stage. However, the observation that, after only 7.5 min of import reaction, GFP-P24 localizes to mature peroxisomes and not to the ER strongly suggests that it is inserted directly from the cytosol into the peroxisomal membrane. In fact, this very short time period renders a putative passage through the ER highly unlikely.

At the time the studies of PMP22 and PMP70 import were performed, the data regarding peroxisome membrane biogenesis were still very limited and the protein components involved were unknown. Presently, our understanding of this process has improved and the peroxins mediating the PMP sorting pathway are identified. Thus, these mechanisms could be more thoroughly analyzed. As shown in Figure 12B (lower panel), addition of increasing amounts of recombinant Pex19p to the *in vitro* import reactions has no stimulatory effect but rather inhibits GFP-P24 insertion into the peroxisomal membrane at higher concentrations. Although Pex19p is directly involved in the PMP sorting mechanism, this result is not surprising. The absence of stimulation of the import process is consistent with the properties of the Pex19p-PMP interaction. Pex19p was reported to have a chaperone-like activity in binding newly synthesized PMPs and preventing their aggregation by shielding hydrophobic domains [112]. Moreover, the results from Figure 13 indicate that these complexes must be formed during or immediately after PMP translation (see below). Therefore, additional Pex19p molecules available at the import level do not contribute to the membrane insertion of the reporter PMP. The inhibition observed when using large concentrations of recombinant Pex19p is probably related to the fact that recombinant Pex19p still interacts with Pex3p(34-373) in its unloaded state. This recombinant version of Pex3p contains the cytosolic exposed domain involved in Pex19p

binding (see “Introduction”, section 1.3.3.3.). Thus, recombinant Pex19p is also likely to interact with peroxisomal Pex3p in the *in vitro* import assays, hampering its function when present at high concentrations and preventing the docking of Pex19p-cargo complexes.

This ability of Pex19p to bind Pex3p even when no PMP is being carried does not appear to be restricted to this recombinant version. Indeed, overexpressed Pex19p versions show a Pex3p-dependent peroxisomal localization although no peroxisomal proliferation is observed in these cells [74, 95, 96]. Furthermore, a truncated version comprising the Pex3p docking domain but lacking the PMP-binding region displayed exactly the same behaviour [95]. Altogether, these observations suggest that free Pex19p is in fact capable of interacting with Pex3p when the intracellular levels of the former are artificially raised. However, under normal physiological concentrations, the affinity of unloaded Pex19p towards peroxisomal Pex3p is probably insignificant. In the *in vitro* import assays, recombinant Pex19p inhibited GFP-P24 import with an IC_{50} value of 260 nM, whereas the native PAGE K_D value estimated for the Pex19p-Pex3p(34-373) interaction was much higher, ~3.4 μM. Considering that in this electrophoretic technique the samples are subjected to nonequilibrium and nonphysiological conditions, this apparent K_D value is likely to be overestimated. Indeed, in a recent study this interaction was reported to have a K_D value in the nanomolar range (3.4 nM), determined by surface plasmon resonance [111]. Nevertheless, the apparent K_D figures estimated by native PAGE analysis are definitely valuable when comparing the binding affinities of the different recombinant versions of Pex19p towards Pex3p(34-373).

The inclusion of recombinant Pex19p versions in the *in vitro* translation of GFP-P24 subsequently subjected to *in vitro* import reactions yielded results of most important consequence. The addition of full-length Pex19p to the *in vitro* synthesis of the reporter PMP leads to a marked stimulation in its peroxisomal import, whereas the presence of Pex19p(31-299) during translation produces a strong inhibition of the import process. In stark contrast, no differences in the normal import levels are obtained with exactly the same final concentrations of the recombinant proteins in import reactions using GFP-P24 translated in the absence of exogenous Pex19p. Therefore, these data indicate that Pex19p proteins form a complex with GFP-P24 at the translation level that determines the PMP import competence. Additional free Pex19p molecules (recombinant or the rat liver ones) available at later stages do not contribute to this import competence. These observations imply that those complexes formed during GFP-P24 synthesis correspond to the substrates recognized by the import machinery at the peroxisome membrane. The properties of the Pex19p proteins included in these complexes then establish the final outcome. When full-length Pex19p is present, the complexes interact with Pex3p, and GFP-P24 is eventually inserted into the peroxisomal membrane. In contrast, Pex19p(31-

299) lacks the strongest Pex3p-binding domain apparently required for docking at the peroxisomal surface, and thus is incapable of delivering the reporter PMP and mediating its insertion. Taken together, these results are the first mechanistic evidence that Pex19p functions primarily in binding newly synthesized PMPs in the cytosol and transporting them to the peroxisome membrane. In line with these conclusions, both IgGs directed to Pex3p and recombinant Pex3p(34-373) resulted in a decrease of imported GFP-P24. The anti-Pex3p IgGs hinder the peroxin at the peroxisome membrane and therefore the docking sites for the Pex19p-cargo complexes. On the other hand, native PAGE analyses confirmed that recombinant Pex3p(34-373) is capable of interacting both with free and cargo-loaded Pex19p. Hence, this Pex3p version, comprising the cytosolic-exposed domain, probably sequesters the Pex19p-cargo complexes preventing their docking at the peroxisomal import machinery.

A relevant aspect regarding the experiments involving recombinant Pex19p lies in the concentrations used when compared to the endogenous values. At present, the physiological concentration of Pex19p remains unknown for all organisms. Nevertheless, a few considerations should be made on this subject. In this regard, morphometric data available for the rat hepatocyte may provide some helpful hints [152]. From this data, one Pex19p protein per peroxisome corresponds to a cytosolic concentration of 0.23 nM. Therefore, 6 nM (the amount used in the import assays from Figure 13) of cytosolic Pex19p would represent 26 molecules per peroxisome in these cells. Moreover, rat liver Pex3p seems to have the same abundance of Pex14p [153] which was estimated in a previous study to represent 0.25% of the total rat liver peroxisomal protein [50]. In agreement, a band of 38 kDa from highly purified rat liver peroxisome membranes, consisting of Pex3p and PMP52 [154], represented 0.37% of total peroxisomal protein [145]. The *in vitro* import assays described in this work are performed in 100 µl final volume containing 450 µg of PNS protein, wherein peroxisomes correspond to 2.5% of total rat liver protein. According to these data, the endogenous 42-kDa Pex3p from rat peroxisomes is estimated to be at 6.6 nM in the import reaction. This concentration value is roughly the same of recombinant Pex19p in the experiments from Figure 13. Thus, the concentrations of recombinant Pex19p used in those assays are apparently not in a molar excess towards peroxisomal Pex3p.

The native PAGE method employed in this study emerged as a useful technique when investigating the interactions involving Pex19p, Pex3p and a cargo PMP (GFP-P24). As a first approach, the binding properties of Pex19p for Pex3p were analyzed using different recombinant versions of Pex19p and Pex3p(34-373). In these experiments, full-length Pex19p binds Pex3p(34-373) with a higher affinity than does Pex19p(1-124). On the other hand, Pex19p(31-299) interacts quite weakly with Pex3p(34-373) since, under

the electrophoretic conditions, a complex was only detected in the presence of the cargo GFP-P24. These results are compatible with the proposed existence of two Pex3p-binding domains on Pex19p, a strong one at the N terminus and a weaker one partially overlapping with the cargo-interacting region (see “Introduction”, section 1.3.3.4.). Thus, the observed binding properties for these recombinant proteins are in agreement with the ones previously obtained in the two-hybrid system [116]. Furthermore, dimeric complexes between Pex19p and GFP-P24 and trimeric complexes comprising Pex3p, Pex19p and GFP-P24 were also detected in these native gels. Hence, the dimeric complexes correspond to cargo-loaded Pex19p whereas the trimeric complexes may represent the docking of cargo-loaded Pex19p to peroxisomal Pex3p. Similar trimeric complexes involving other cargo PMPs were also described in other studies [112, 133]. Considering that all these protein complexes could be observed under native PAGE, the effect of the cargo PMP on Pex19p affinity for Pex3p was analyzed as well. As shown in Figure 16, the binding of the cargo GFP-P24 to Pex19p increases its affinity for Pex3p. This property is expected so that only cargo-loaded Pex19p is targeted to the docking and insertion machinery, and free Pex19p is not occupying Pex3p docking sites. Thus, the binding of the cargo may induce some conformational changes in Pex19p enhancing the affinity of its N-terminal domain for Pex3p. Alternatively, it is possible that Pex3p directly interacts with the cargo protein within the Pex19p-PMP complex, mediating the subsequent insertion steps. Elucidation of this issue will surely help understanding the mechanisms of docking and insertion into the peroxisomal membrane. Importantly, this difference in affinity may have major implications on the energetics of Pex19p export back to the cytosol. Indeed, the strong decrease in the affinity of Pex19p for Pex3p after the cargo PMP is delivered probably drives this export step. Therefore, it is likely that no ATP or GTP hydrolysis is required for recycling Pex19p.

The *in vitro* import system developed in this work was also used to assess the energetics of the docking and insertion mechanisms of the reporter PMP into the peroxisomal membrane. Remarkably, the import process was insensitive to high amounts of ATP, GTP or their nonhydrolyzable analogs, and also to apyrase which catalyzes the hydrolysis of ATP and other nucleotides. Hence, these results indicate that none of the steps from the peroxisomal docking of Pex19p-cargo complexes until the ultimate PMP membrane insertion involve energy in the form of ATP or GTP. Furthermore, once the Pex19p-cargo complex is formed during translation or shortly after it, the whole PMP import pathway may be energy independent. Indeed, all the subsequent steps are likely to be energetically favourable. Thus, considering that PMPs are highly hydrophobic, their membrane integration may drive the docking and insertion steps. The export of Pex19p back into the cytosol is probably ensured by the decrease in its affinity for Pex3p upon

cargo release (see above). These findings are consistent with the energetics reported for the *in vitro* import of rat PMP22 and PMP70 [84, 85] and *A. thaliana* PMP22 [155], although the latter authors suggest that ATP may stimulate the assembly of the PMP at the peroxisomal membrane. In sharp contrast, Matsuzono and co-workers recently suggested that cargo-loaded Pex19p is targeted to peroxisomes in an ATP-dependent manner [133]. This apparent discrepancy may be related with the features of the *in vitro* system used in that study. In fact, the authors did not use a reporter PMP but rather followed the traffic of radiolabeled Pex19p between the organelle pellet and the supernatant of import reactions. Therefore, the cargo PMPs transported by radioactive Pex19p are presumably supplied by the PNS fraction used in those assays which, in fact, was never demonstrated. Here, it was shown that import-competent Pex19p-cargo complexes have to be formed during or immediately after the translation step of the PMP. Thus, an ATP-requiring step may be related to the PMP synthesis itself or to a putative transfer from a chaperone to Pex19p. Nevertheless, the experimental criteria used by Matsuzono and colleagues raise many reservations regarding the relevance of those observations. As discussed before, the *in vitro* import system developed here relies on a protease protection assay in which a protease-resistant fragment of the reporter PMP represents the protein fraction that is correctly inserted into the peroxisome membrane. However, Matsuzono and co-workers defined the import event solely by sedimentation of the radiolabeled protein with the organelle fraction, which is a clearly insufficient criterion. Moreover, such organelle pellets were obtained upon ultracentrifuging 250-µl samples at 100,000 *g* for 30 min [133]. In contrast, the organelles pellets from the *in vitro* import assays performed in the present work were obtained by centrifuging the 1-ml samples at 15,000 *g* for 15 min in a typical tabletop microcentrifuge. The problem of relying on such criterion is well illustrated by the results with a control GFP protein shown in Figure 7. Although this protein is highly soluble, it still sediments with organelles even after two centrifugation steps in standard conditions (*i.e.* at 15,000 *g* for 15 min).

The results described in the present work provide a new insight into PMP biogenesis and clarify some essential aspects of the Pex19p-mediated import pathway. Indeed, it was shown that import-competent Pex19p-cargo complexes are formed in the cytosol during or shortly after PMP translation and represent the substrates recognized by the peroxisome docking and insertion machinery. Altogether, the presented data constitute compelling evidence supporting a Pex19p role as PMP cycling receptor. In this model, Pex19p interacts with the nascent PMP either during or immediately after its translation on free cytosolic ribosomes (see Figure 17). The second possibility may require a chaperone such as TRiC to assist the protein folding and transfer to Pex19p. This peroxin loaded with the PMP then acts as a cycling receptor and transports it to

peroxisomes. At the organelle membrane, cargo-loaded Pex19p (but not the free peroxin) docks on Pex3p and delivers the PMP which ultimately becomes inserted into the lipid bilayer, via ATP- and GTP-independent mechanisms. It remains to be determined whether Pex19p is also involved in subsequent steps such as membrane integration or assembly/disassembly of protein complexes. After releasing the PMP, free Pex19p is exported back to the cytosol because its affinity for Pex3p is now much smaller. Therefore, this step is probably energetically favourable as well. In the cytosol, exported Pex19p binds once more to newly synthesized PMPs and catalyzes further cycles of transportation. Since Pex19p displayed all the properties of a mobile receptor for this particular PMP, it is likely that the same biogenesis pathway also applies to the other PMPs, perhaps with the exception of Pex3p and Pex16p as these peroxins appear to be directly involved in this process. However, it is still possible that a different protein mediates the sorting of other PMPs to the peroxisomal membrane, specially the ones which appear to have separate mPTSs and Pex19p-binding sites. Bearing in mind the concerns regarding these observations (see “Introduction”, section 1.3.3.7.), such hypothesis seems unlikely. Taken as a whole, the developed *in vitro* import system and the native PAGE analysis presented in this work have proven to be extremely helpful strategies to study the PMP import pathway and their protein components.

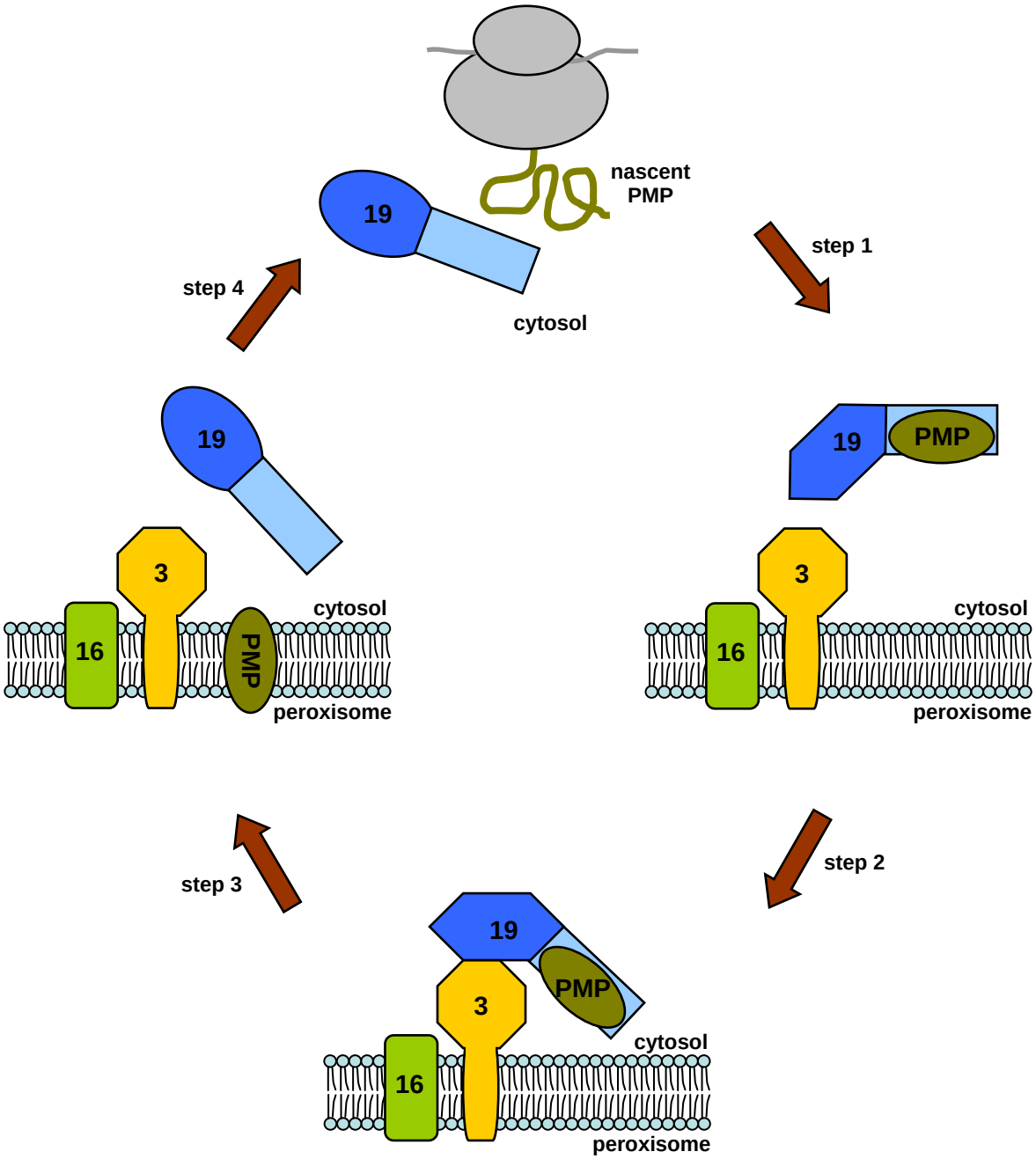

Figure 17. Model for the Pex19p-mediated import of PMPs.

Step 1: Pex19p (19) interacts with a nascent PMP through its C-terminal domain (light blue). This cargo binding occurs either during or immediately after PMP synthesis on cytosolic ribosomes and may be assisted by chaperones. Step 2: Pex19p then functions as an import receptor and transports the PMP to the peroxisomal membrane. Cargo-loaded Pex19p displays a higher affinity for peroxisomal Pex3p (3), where it docks via its N terminus (dark blue). Step 3: After the docking, Pex19p delivers the PMP which eventually becomes inserted into the lipid bilayer. Both the docking and insertion steps are ATP and GTP independent. Pex16p (16) may also participate in these steps. Free Pex19p has much lower affinity for Pex3p and is released back to the cytosol, apparently without the need of an energy input. Step 4: In the cytosol, free Pex19p is now again available to interact with newly synthesized PMPs and mediate further cycles of PMP import.

6. FUTURE PERSPECTIVES

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Despite the progress in recent years together with the significant contribution from this work, many important questions concerning peroxisome membrane biogenesis remain to be answered. The Pex19p-mediated PMP import pathway was characterized here using a modified version of PMP24 as a reporter cargo protein. Considering that PMP24 is an ordinary PMP, the attained conclusions probably also apply to most or even all of the other PMPs, except Pex3p and Pex16p which are directly implicated in the biogenesis of this class of proteins. Nevertheless, a yet unknown different sorting pathway may exist for some other PMPs. Therefore, other reporter PMPs should be tested in the *in vitro* import system developed in this work. In this regard, Pex14p, which also yields a protease-resistant fragment upon proteinase K digestion of the organelles [156], was subjected to import reactions. This PMP exhibited the same behaviour of GFP-P24 in these *in vitro* import assays (C. P. Grou, M. P. Pinto and J. E. Azevedo, unpublished results). Additional PMPs will also be tested with this *in vitro* system. Another important aspect regards whether a chaperone and/or other factors mediate the folding and transfer of the nascent PMP from the ribosome to Pex19p. Furthermore, the production of a soluble Pex19p-binding peptide, capable of maintaining the peroxin in a loaded state, would surely be a useful tool for various purposes such as competition studies and analysis of binding properties. These issues are currently being addressed.

As previously discussed, the biogenesis of PMPs apparently constitutes a remarkably simplified system comprising only three protein components, Pex3p, Pex16p and Pex19p. In yeast species, Pex16p is either absent or serves a different purpose (*Y. lipolytica*), rendering Pex3p and Pex19p as the only proteins of such machinery [32, 79]. Moreover, mammalian Pex16p is thought to regulate early events in peroxisomal membrane biogenesis, especially in *de novo* formation [80, 81], and has not been linked to import of PMPs into mature peroxisomes. In addition, considering that the docking and insertion steps are independent of ATP and GTP hydrolysis, no other protein factors seem to be required. Therefore, a minimum *in vitro* import system was envisaged consisting of Pex3p reconstituted into liposomes, Pex19p and a reporter PMP. Thus, reconstitution of full-length Pex3p into liposomes resembling the phospholipid content of the peroxisomal membrane [157, 158] was attempted. For this purpose, full-length Pex3p was first expressed in bacterial cells. However, in contrast to its cytosolic domain, Pex3p(34-373), the expression levels of the full-length peroxin were extremely low. Subsequently, several strategies were followed in an attempt to produce recombinant Pex3p. Recently, these expression problems were overcome and reasonable amounts of the protein were obtained in a mostly insoluble state. Following solubilization with detergents, Pex3p was

then subjected to reconstitution in the presence of different ratios of phospholipids. Unfortunately, so far Pex3p failed to reconstitute in these experiments. Presently, reconstitution assays are being performed using different solubilization conditions. A successful reconstitution of Pex3p into liposomes would allow carrying out *in vitro* import reactions using Pex19p loaded with a reporter PMP. Then, it could be assessed whether such minimum *in vitro* reconstituted system is sufficient to promote PMP membrane insertion. Specific import of a reporter PMP in this *in vitro* reconstituted system would have major implications. In fact, such result would imply that Pex16p is not essential in this process and rather plays a distinct role in peroxisomal membrane biogenesis. Additionally, this observation would indicate that no further proteins are required. In the case of a negative outcome, coupled reconstitution of Pex3p and Pex16p should be attempted. In summary, reconstitution of a minimum PMP *in vitro* import system would certainly be enlightening. Indeed, reconstitution of a biological system is the ultimate goal in biochemistry.

7. REFERENCES

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