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Functional Characterization of the Human Peroxins PEX3 and PEX19, Proteins Essential for Early Peroxisomal Membrane Biogenesis

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Abstract
Abstract

Peroxisomes are single-membrane-bound organelles present in virtually all eukaryotic cells. Their significance in human metabolism is illustrated by the existence of severe inherited diseases caused by the failure of peroxisomal biogenesis (peroxisomal biogenesis disorders; PBDs). Proteins required for peroxisomal assembly are termed “peroxins” and are encoded by at least 23 PEX-genes. In most cases, defects in PEX genes lead to a disruption of peroxisomal matrix protein import, whereas various peroxisomal membrane components are synthesized and accumulate in peroxisomal membrane remnants (“peroxisomal ghosts”). Since yeast mutants in PEX3 and PEX19 have been shown to lack peroxisomal ghosts, these peroxins were expected to be involved in the early stages of peroxisomal membrane synthesis. This work focused on the functional characterization of human PEX3 and PEX19 to gain insights into their role in human biology and disease. PEX19 was shown to interact with three peroxisomal ABC half-transporters (Adrenoleukodystrophy protein ALDP, Adrenoleukodystrophy-related protein ALDRP, and the 70 kDa peroxisomal membrane protein PMP70), confirming its role as a broad specific peroxisomal membrane-protein binding-protein. As a prerequisite for these protein-protein interaction assays, the human ALDR gene was characterized. Proteins encoded by two PEX19 splice variants showed a considerable functional diversity as to peroxisomal membrane protein binding and with respect to induction of peroxisomal formation in PEX19-deficient human fibroblasts. Farnesylation of PEX19 was shown not to be essential for these functions. These data provide the first experimental evidence for specific biological functions of the different predicted domains of the PEX19 protein. In order to investigate the molecular details of peroxisomal assembly and to evaluate experimental treatment strategies for the peroxisomal biogenesis disorders, data useful for the future generation of a mouse model with targeted disruption of the PEX3 gene were presented. The human PEX3 gene was characterized with the goal to identify a yet unknown human PEX3-deficient phenotype. Two inactivating PEX3 mutations were detected in two PBDs-patients exhibiting a severe and lethal Zellweger syndrome phenotype. They showed a total lack of morphologically recognizable peroxisomal membrane remnants in their fibroblasts. Expression of the wild type PEX3 cDNA in the mutant cell lines restored peroxisomal biogenesis, establishing PEX3 as a key factor in early human peroxisome synthesis. Taken together, the combined data presented here provide evidence for the essential role of PEX3 and PEX19 in the initial steps of an alternative human peroxisome-formation pathway. In contrast to the theory that peroxisomes can arise exclusively by growth and division of preexisting peroxisomes, this alternative pathway does not require morphologically recognizable peroxisomes.
Zusammenfassung
Zusammenfassung

CHAPTER I:

Introduction
1 Peroxisomes: Morphology and Metabolic Functions

Membrane biogenesis and its regulation is one of the mayor foci in modern molecular cell biology (Schatz & Dobberstein, 1996). Despite their simple architecture, peroxisomes represent a diverse group of organelles, with varied metabolic activities (van den Bosch et al., 1992). Like the Golgi apparatus, they actually constitute a dynamic organelle population consisting of many structurally and functionally distinct compartments that differ in their import competency for various proteins (Titorenko & Rachubinski, 2001b). Therefore, the processes of peroxisomal membrane assembly and maintenance have begun to attract the interest of a broad range of scientists. In addition to the protein import into organelles such as the ER, Golgi apparatus, mitochondria, nucleus and chloroplasts, this would complete the knowledge about highly specific intracellular trafficking systems in eukaryotes that mediate the biosynthesis of the different intracellular compartments.

Peroxisomes, the last of the major subcellular organelles to be discovered, are present in virtually all eukaryotic cells. In a morphological study, they were introduced by Rhodin (1954) as small spherical oval bodies in the proximal convoluted tubular epithelium of the mouse kidney (Rhodin, 1954). Because of the lack of functional identity, Rhodin named them microbodies. The biochemical characterization of peroxisomes and their recognition as distinct organelle was established in the 1960’s: De Duve and Baudhuin observed that catalase, urate oxidase and d-amino acid oxidase were associated with particles different from lysosomes, microsomes, and mitochondria (De Duve & Baudhuin, 1966). The term ‘peroxisome’ was introduced by de Duve and Baudhuin because these organelles contain at least one hydrogen-peroxide-producing oxidase and a catalase to decompose the hydrogen peroxide (De Duve & Baudhuin, 1966). Breidenbach and Beevers named the corresponding organelle in plants glyoxysomes because they contained five enzymes of the glyoxylate cycle (Breidenbach & Beevers, 1967).

Peroxisomes have a relatively simple morphology (Fig. I-1): They are small spherical organelles, ranging in diameter from 0.3 to 1.5 µm, delimited by a single unit membrane, containing a electron-dense matrix and occasionally a granular or crystalline core. Their abundance ranges from less than a hundred to more than a thousand peroxisomes per cell.

Peroxisomes show remarkable metabolic diversity (Tab. I-1). Their size, number, protein composition and biochemical function vary depending on the organism, cell type, and/or environmental condition (Geraghty et al., 1999; Subramani, 1993; Wanders & Tager, 1998). Most of the enzymes that catalyze these reactions are found within the organelle matrix (lumen). In human, the granular matrix contains more than 50 matrix enzymes that participate in a wide variety of metabolic pathways including the β-oxidation of certain fatty acids and biosynthesis of ether phospholipids, cholesterol, bile acids, and polyunsaturated fatty acids.
Table I-1: Metabolic functions of peroxisomes

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Fungi</th>
<th>Plants</th>
<th>Mammals</th>
<th>Humans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio-synthesis</td>
<td>Degradation</td>
<td>Bio-synthesis</td>
<td>Degradation</td>
<td>Degradation</td>
</tr>
<tr>
<td>• lysine</td>
<td>• penicillin</td>
<td>• ether phospholipids</td>
<td>• ether phospholipids</td>
<td>• ether phospholipids</td>
</tr>
<tr>
<td>• decomposition of hydrogen peroxide</td>
<td>• decomposition of hydrogen peroxide</td>
<td>• cholesterol</td>
<td>• cholesterol</td>
<td>• cholesterol</td>
</tr>
<tr>
<td>• glyoxylate cycle</td>
<td>• glyoxylate cycle</td>
<td>• bile acids</td>
<td>• bile acids</td>
<td>• bile acids</td>
</tr>
<tr>
<td>• amino acids</td>
<td>• β-oxidation of fatty acids</td>
<td>• polyunsaturated fatty acids</td>
<td>• polyunsaturated fatty acids</td>
<td>• polyunsaturated fatty acids</td>
</tr>
<tr>
<td>• methanol</td>
<td>• α-oxidation of fatty acids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• β-oxidation of fatty acids</td>
<td>• ß-oxidation of fatty acids</td>
<td>• recomposition of hydrogen peroxide</td>
<td>• recomposition of hydrogen peroxide</td>
<td>• recomposition of hydrogen peroxide</td>
</tr>
<tr>
<td>• amino acids</td>
<td>• glyoxylate cycle</td>
<td>• amino acids</td>
<td>• amino acids</td>
<td>• amino acids</td>
</tr>
<tr>
<td>• purines</td>
<td>• β-oxidation of fatty acids</td>
<td>• purines</td>
<td>• purines</td>
<td>• purines</td>
</tr>
<tr>
<td>• some reactions of photorespiration</td>
<td>• β-oxidation of fatty acids</td>
<td>• prostaglandin</td>
<td>• α-oxidation of fatty acids</td>
<td>• α-oxidation of fatty acids</td>
</tr>
<tr>
<td>• β-oxidation of fatty acids</td>
<td>• β-oxidation of very long-chain fatty acids</td>
<td>• polyamines</td>
<td>• β-oxidation of very long-chain fatty acids</td>
<td>• β-oxidation of very long-chain fatty acids</td>
</tr>
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<td></td>
<td></td>
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</tbody>
</table>
2 Peroxisomal Diseases

The importance of peroxisomes in mammalian metabolism is illustrated by the existence of severe inherited metabolic diseases caused by peroxisomal malfunction. Single peroxisomal enzyme disorders result from a deficiency in a single peroxisomal enzyme that is not involved in the biogenesis of the organelle. Therefore, one single peroxisomal metabolic pathway is affected whereas the peroxisomes of the patients are of normal number and morphology. Table I-2 lists the single peroxisomal enzyme deficiencies identified so far. On the other hand, disorders associated with peroxisomal assembly are termed “Peroxisomal Biogenesis Disorders” (PBDs). These disorders affect several metabolic pathways of the peroxisome, as they result from a deficiency in the biogenesis of the whole organelle.

Table I-2: Single peroxisomal enzyme deficiencies

<table>
<thead>
<tr>
<th>Peroxisomal function involved</th>
<th>Disorder</th>
<th>Phenotype</th>
<th>Deficient enzyme/protein identified</th>
<th>Genetic basis resolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acid j-oxidation</td>
<td>X-ALD</td>
<td>X-ALD</td>
<td>ALDP</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Acyl-CoA oxidase deficiency</td>
<td>NALD-like</td>
<td>Acyl-CoA oxidase</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>D-Bifunctional protein deficiency</td>
<td>ZS/NALD-like</td>
<td>D-Bifunctional protein</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Thiolase deficiency</td>
<td>ZS-like</td>
<td>Peroxisomal thiolase 1</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Branched-chain acyl-CoA oxidase deficiency</td>
<td>No patients identified</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Branched-chain peroxisomal thiolase deficiency</td>
<td>No patients identified</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eterphospholipid biosynthesis</td>
<td>Racemase-deficiency</td>
<td>Late-onset neuropathy (Rhizomelic) chondrodysplasia punctata</td>
<td>Racemase</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>DHAPAT-deficiency</td>
<td>(Rhizomelic) chondrodysplasia punctata</td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Alkyl-DHAP synthase deficiency</td>
<td>(Rhizomelic) chondrodysplasia punctata</td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>Fatty acid 3-oxidation</td>
<td>Phytanoyl-CoA hydroxylase deficiency</td>
<td>Refsum disease</td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>Isoprenoid biosynthesis</td>
<td>Mevalonate kinase (MK) deficiency</td>
<td>1. Classical MK-deficiency 2. Hyper IgD/periodic fever syndrome</td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>Pipeolic acid degradation</td>
<td>Isolated hyperpipeolic acidemias</td>
<td>Variable (see text)</td>
<td></td>
<td>No</td>
</tr>
<tr>
<td>Glutaryl-CoA metabolism</td>
<td>Glutaryl-CoA oxidase deficiency</td>
<td>Glutaric aciduria type 3</td>
<td></td>
<td>No</td>
</tr>
<tr>
<td>Hydrogen peroxide metabolism</td>
<td>Catalase deficiency</td>
<td>Acatalasaemia</td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>Glyoxylate detoxification</td>
<td>Alanine:glyoxylate aminotransferase deficiency</td>
<td>Hyperoxaluria type 1</td>
<td></td>
<td>Yes</td>
</tr>
</tbody>
</table>

*a All patients described with presumed L-bifunctional protein deficiency have now been found to be deficient in D-bifunctional protein due to mutations in the D-bifunctional protein gene (see text).

* from (Wanders et al., 2001)
2.1 X-Linked Adrenoleukodystrophy: A Peroxisomal Single Enzyme deficiency

X-Linked Adrenoleukodystrophy (X-ALD; OMIM No. 300100) is the most common inherited peroxisomal disorder characterized by the abnormal accumulation of saturated very long chain fatty acids (VLCFAs; ≥C22:0) predominantly in myelin, adrenal cortex, and testis (Moser et al., 1995b). The biochemical defect is localized to the level of lignoceroyl-CoA synthesis, a step in the peroxisomal β–oxidation of very long chain fatty acids. The clinical manifestations of X-ALD are highly variable. In the case of the most frequent and severe childhood cerebral form, patients seem unaffected until the age of five to ten years, when there is onset of adrenal insufficiency and progressive neurological dysfunction (Moser et al., 1995b). After onset of symptoms the course is rapidly and progressive, leading to an apparently vegetative state within two to four years and to death at varying intervals thereafter (Gärtner et al., 1998c). Further phenotypes are ranging from the adolescent and adult onset cerebral form, the Adrenomyeloneuropathy affecting primarily the spinal cord, the Addison only form with isolated adrenal insufficiency, as well as asymptomatic forms. Because the various phenotypes occur within the same kindred, modifying genes and/or environmental factors might contribute to this phenomenon.

The Adrenoleukodystrophy gene (ALD), mutated in X-ALD, was identified by positional cloning (Mosser et al., 1993) and belongs to the family of ATP-binding cassette (ABC) transporters (Contreras et al., 1994; Kobayashi et al., 1994; Mosser et al., 1994) capable of transporting a wide variety of ligands across biological membranes. The ALD protein (ALDP, synonym: ABCD1), located within the peroxisomal membrane, is an ABC half-transporter composed of one hydrophobic transmembrane domain (TMD) and one hydrophilic nucleotide binding fold (Fig. I-2A). Three other ABC half-transporters have been located within the peroxisomal membrane: The Adrenoleukodystrophy-related protein (ALDRP, synonym: ABCD2) (Holzinger et al., 1997b; Lombard-Platet et al., 1996), the 70 kDa peroxisomal membrane protein (PMP70, synonym: ABCD3) (Kamijo et al., 1990) and the 69 kDa peroxisomal membrane protein (PMP69, synonym: ABCD4) (Holzinger et al., 1997a; Shani et al., 1997). ABC half-transporters are expected to act as dimers to form functional units. Recently, homo- as well as heterodimerization has been reported to occur between the carboxy-terminal halves of ALDP, ALDRP and PMP70 (Liu et al., 1999a). ALDRP or PMP70-cDNA, if transfected in fibroblast of a patient suffering from X-ALD, have been shown to correct the defect of β–oxidation indicating a functional redundancy of peroxisomal
ABC half-transporters (Braiterman et al., 1998; Kemp et al., 1998). Because ALDRP is the closest homologue of ALDP (Fig. I-2B), it might be a candidate for being a modifier gene in X-ALD, accounting for the heterogeneity of clinical phenotypes.

Figure I-2: Peroxisomal ABC half-transporters. A, Scheme of an ABC half-transporter. Transmembrane segments are shown as black boxes, the nucleotide binding fold as gray oval. B, Evolutionary relationship among human Adrenoleukodystrophy protein and its homologues. The picture was adapted from (Smith et al., 1999). ALDP: Adrenoleukodystrophy protein; ALDRP: Adrenoleukodystrophy-related protein; PMP70: 70 kDa peroxisomal membrane protein; PMP69: 69 kDa peroxisomal membrane protein; PXA1/2: Saccharomyces cerevisiae peroxisomal ABC-transporter 1/2; Hs: Homo sapiens; Rn: Rattus norvegicus; Mm: Mus musculus; Ce: Caenorhabditis elegans; Sc: Saccharomyces cerevisiae.
2.2 Peroxisomal Biogenesis Disorders

The most dramatic loss of peroxisome function is observed in the peroxisomal biogenesis disorders (PBDs). Defective biogenesis of the peroxisome leads to complex developmental and metabolic phenotypes that can be organized into two clinical spectra: (i) The Cerebro-hepato-renal syndrome spectrum with Zellweger syndrome (ZS) as the most severe example and neonatal adrenoleukodystrophy (NALD) and infantile Refsum disease (IRD) as milder variants. (ii) The second spectrum is distinctive with classical rhizomelic chondrodysplasia punctata (RCDP) as its exemplar (Gould et al., 2001). The PBDs are inherited in an autosomal recessive manner and, in aggregate, occur in approximately 1/50000 live births (Gould et al., 2001).

Zellweger syndrome is characterized by an array of neural, hepatic and renal defects, hence its original designation as cerebro-hepto-renal syndrome. Individuals with ZS exhibit severe neurological dysfunction and rarely survive their first year (Gould & Valle, 2000). NALD patients display similar but less severe phenotypes then those of ZS patients and can survive up to a decade. IRD patients are even more mildly affected, with some surviving up to their third decade (Moser et al., 1995a). Virtually all Zellweger spectrum patients are defective in both the PTS1 and PTS2 matrix protein import (Chapter I-3.1). Therefore, the Zellweger spectrum is generally associated with severe, moderate, or mild defects in virtually all peroxisomal functions (Gould et al., 2001). RCDP, by contrast, is characterized by a more specific clinical and metabolic phenotype. The typical clinical features include proximal shortening of the limbs (rhizomelia), punctate epiphyseal calcifications, cataracts, and severe developmental delay. At the metabolic level, RCDP patients have deficiencies in just two metabolic pathways: Plasmalogen biosynthesis and branched fatty acid oxidation, due to an isolated PTS2-specific protein import defect (Chapter I-3.1) (Gould et al., 2001).

In somatic cell fusion experiments, fibroblasts from patients with peroxisomal biogenesis disorders have been shown to segregate into at least 12 complementation groups (CGs) (Moser, 1999; Shimozawa et al., 1998). These groups are listed in Table I-3, but investigators in the United States, Japan, and the Netherlands have utilized different numbering systems. The existence of 12 CGs suggests strongly that PBDs phenotypes can be caused by at least 12 distinct genetic defects (Moser, 1999). Mutations in PEX-genes, encoding peroxisomal assembly proteins (“peroxins”), are known to be the molecular cause of the phenotypes
represented by different CGs. At the beginning of this thesis, the molecular defects in 7 of the 12 CGs had been identified. Now, gene identification strategies for the PBDs have determined the molecular basis of disease in all known PBD-CGs other than CG-A (Gould & Valle, 2000). The molecular and biochemical evidence that CG-G is associated with mutations in human \( PEX3 \) will be shown in appendix 6 of this thesis.

Table I-3: Peroxisomal Biogenesis Disorders Complementation Groups

<table>
<thead>
<tr>
<th>Complementation group(^a)</th>
<th>Gene(^b)</th>
<th>Phenotypes(^c)</th>
<th>%(^d)</th>
<th>References</th>
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<tbody>
<tr>
<td>KKI</td>
<td>Jap</td>
<td>Ams</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>E</td>
<td>2</td>
<td>( PEX1 )</td>
<td>ZS, NALD, IRD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td></td>
<td>( PEX5 )</td>
<td>ZS, NALD, IRD</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>( PEX12 )</td>
<td>ZS, NALD, IRD</td>
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<td>4, 6</td>
<td>C</td>
<td>3</td>
<td>( PEX6 )</td>
<td>ZS, NALD</td>
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<td>7, 5</td>
<td>B</td>
<td></td>
<td>( PEX10 )</td>
<td>ZS, NALD</td>
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<td></td>
</tr>
<tr>
<td>8</td>
<td>A</td>
<td>7</td>
<td>?</td>
<td>ZS, NALD, IRD</td>
</tr>
<tr>
<td>9</td>
<td>D</td>
<td></td>
<td>( PEX16 )</td>
<td>ZS</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>5</td>
<td>( PEX2 )</td>
<td>ZS, IRD</td>
</tr>
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<td>11</td>
<td>R</td>
<td>1</td>
<td>( PEX7 )</td>
<td>RCDP</td>
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<tr>
<td>12</td>
<td>G</td>
<td></td>
<td>( PEX3^c )</td>
<td>ZS</td>
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<tr>
<td>13</td>
<td>H</td>
<td></td>
<td>( PEX13 )</td>
<td>ZS, NALD</td>
</tr>
<tr>
<td>14</td>
<td>J</td>
<td></td>
<td>( PEX19 )</td>
<td>ZS</td>
</tr>
</tbody>
</table>

\(^a\)Nomenclature: KKI: Kennedy Krieger Institute, USA; Jap: Gifu University, Japan; Ams: Amsterdam
\(^b\)During preparation of this thesis, the molecular defects in CG-D (PEX16), CG-G (PEX3), CG-H (PEX13), and CG-J (PEX19) were discovered
\(^c\)The molecular and biochemical evidence that CG-G is associated with mutations in human \( PEX3 \) is shown in appendix 6 of this thesis
\(^d\)Phenotypes in each CG are from (Gould et al., 2001)

Most of the Zellweger spectrum patients do synthesize peroxisomal membranes and import peroxisomal membrane proteins normally. By contrast, a few ZS patients lack detectable peroxisomal membranes. This might due to defects in the synthesis of peroxisomal membranes, or in the targeting of peroxisomal membrane proteins to the peroxisome, or in the integration of the membrane proteins into the bilayer of the organelle, or all with a secondary deficiency of peroxisomal matrix enzymes. The molecular cause of defective peroxisomal membrane biogenesis in two patients displaying a severe ZS-phenotype will be described in appendix 6.
3 The Peroxisomal Biogenesis

Peroxisomes do not contain DNA or an independent protein synthesis machinery. Accordingly, peroxisomal proteins are encoded by nuclear genes, synthesized on free cytosolic polyribosomes and imported post-translationally (Lazarow & Fujiki, 1985). The assembly and maintenance of peroxisomes is complex and involves several interrelated processes (Fig. I-3). These include the generation of the peroxisomal membrane, the recognition of proteins destined for this particular organelle, the targeting and insertion of peroxisomal membrane proteins into this membrane, and the transport of peroxisomal matrix proteins to and across this same bilayer. There is a remarkable degree of homology between different species in respect to the factors involved in peroxisome biogenesis, and analysis of various model organisms has revealed general rules governing peroxisomal assembly: First, all known membrane and matrix proteins are targeted to the peroxisome post-translationally. Second, the peroxisomal import machineries and targeting signals for membrane and matrix proteins are distinct. Third, the peroxisomal import of membrane and matrix proteins is mediated by their receptors, either cytosolic or membrane-associated, that interact with the peroxisomal targeting signals of their cargo proteins. Last, both folded, oligomeric and
unfolded, monomeric polypeptides are imported into the peroxisomal matrix (Titorenko & Rachubinski, 2001a). On the other hand, in addition to these general rules, there are some organism-specific differences in the peroxisomal protein import (Tab. I-4).

**Table I-4: Common features and differences in peroxisomal protein import**

<table>
<thead>
<tr>
<th>Feature</th>
<th>S.c</th>
<th>Pp</th>
<th>H.p</th>
<th>Y.l</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Membrane proteins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Are targeted to peroxosomes post-translationally.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Many are sorted to peroxosomes directly from the cytosol by the peroxisomal membrane targeting signal 1 (mPTS1).</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>Some are sorted to peroxisomes indirectly through the endoplasmic reticulum, by the peroxisomal membrane targeting signal 2 (mPTS2).</td>
<td>+</td>
<td>?</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>Many are delivered to peroxisomes by a cytosolic receptor that interacts with the mPTS1.</td>
<td>+</td>
<td>-</td>
<td>?</td>
<td>-</td>
</tr>
<tr>
<td>Many are assembled in the peroxisomal membrane by a chaperone that does not interact with the mPTS1.</td>
<td>?</td>
<td>+</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td><strong>Matrix proteins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Are targeted to peroxosomes post-translationally.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Many are sorted to peroxosomes by the carboxy-terminal peroxisomal targeting signal 1 (PTS1).</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Some are sorted to peroxisomes by the amino-terminal peroxisomal targeting signal 2 (PTS2).</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PTS1- and PTS2-containing proteins are delivered to peroxisomes by cytosolic receptors.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PTS1 and PTS2 receptors are peroxisome-associated proteins that interact with the PTSs.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PTS2-containing proteins are delivered to peroxisomes by a cytosolic chaperone that does not interact with the PTS2.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Some are imported as folded, oligomeric proteins.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Some are imported as unfolded polypeptides.</td>
<td>?</td>
<td>+</td>
<td>+</td>
<td>?</td>
</tr>
</tbody>
</table>

*Note: Table I-4 continues.*

The proteins that are required for peroxisomal biogenesis are termed ‘peroxins’ and are encoded by *PEX* genes. Yeasts have emerged as convenient and powerful model organisms for the study of peroxisome biogenesis, therefore *PEX* genes have first been identified by genetic analysis of peroxisome-deficient mutants of yeast (Erdmann et al., 1989). While at least 23 peroxins (Tab. I-5) have been reported to be essential for peroxisomal biogenesis in yeast, the exact functions and interactions of only a limited number of these proteins have been determined so far. Human *PEX* genes have been identified by genetic phenotypicomplementation assays of Chinese hamster ovary cell mutants and by searching human expressed sequence tag databases using yeast *PEX* gene sequences (Tateishi et al., 1997). To date, 13 human orthologs of the 23 yeast *PEX* genes have been identified, and 11 human peroxins have been shown to complement the deficiencies of peroxisomal assembly in cells of PBD patients (Chapter I.2.2).
Table I-5: Features and functions of peroxins

<table>
<thead>
<tr>
<th>Peroxin</th>
<th>Subcell. location</th>
<th>Human ortholog</th>
<th>Peroxin Interactions</th>
<th>Features</th>
<th>Proposed function</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEX1</td>
<td>C/M/V</td>
<td>+</td>
<td>6</td>
<td>AAA ATPase</td>
<td>Matrix protein import, vesicle fusion</td>
</tr>
<tr>
<td>PEX2</td>
<td>M</td>
<td>+</td>
<td></td>
<td>Zn RING</td>
<td>Matrix protein import</td>
</tr>
<tr>
<td>PEX3</td>
<td>M</td>
<td>+</td>
<td>19</td>
<td>Membrane biogenesis; PMP import</td>
<td></td>
</tr>
<tr>
<td>PEX4</td>
<td>M</td>
<td>22</td>
<td>Ubc2p, binds Ub</td>
<td></td>
<td>PEX18 / PEX21 turnover</td>
</tr>
<tr>
<td>PEX5</td>
<td>C/P</td>
<td>+</td>
<td>7, 8, 12-14</td>
<td>TPR domains</td>
<td>PTS1 receptor</td>
</tr>
<tr>
<td>PEX6</td>
<td>C/M/V</td>
<td>+</td>
<td>1</td>
<td>AAA ATPase</td>
<td>Matrix import, vesicle fusion</td>
</tr>
<tr>
<td>PEX7</td>
<td>C/P</td>
<td>+</td>
<td>5, 13, 14, 18, 21</td>
<td>WD repeats</td>
<td>PTS2 receptor</td>
</tr>
<tr>
<td>PEX8</td>
<td>M</td>
<td>5, 20</td>
<td></td>
<td>Matrix protein import</td>
<td></td>
</tr>
<tr>
<td>PEX9</td>
<td>M</td>
<td></td>
<td></td>
<td>Matrix protein import</td>
<td></td>
</tr>
<tr>
<td>PEX10</td>
<td>M</td>
<td>+</td>
<td>12</td>
<td>Zn RING</td>
<td>Translocation machinery</td>
</tr>
<tr>
<td>PEX11</td>
<td>M</td>
<td>+</td>
<td>Two human isoforms</td>
<td></td>
<td>Proliferation, MCFA oxidation</td>
</tr>
<tr>
<td>PEX12</td>
<td>M</td>
<td>+</td>
<td>5, 10</td>
<td>Zn RING</td>
<td>Translocation machinery</td>
</tr>
<tr>
<td>PEX13</td>
<td>M</td>
<td>+</td>
<td>5, 7, 14</td>
<td>SH3 domain</td>
<td>Docking of receptors</td>
</tr>
<tr>
<td>PEX14</td>
<td>M</td>
<td>+</td>
<td>5, 7, 13, 14, 17</td>
<td>Phosphorylated</td>
<td>Docking of receptors</td>
</tr>
<tr>
<td>PEX15</td>
<td>M</td>
<td></td>
<td></td>
<td>Phosphorylated</td>
<td>Matrix protein import</td>
</tr>
<tr>
<td>PEX16</td>
<td>M</td>
<td>+</td>
<td></td>
<td>Membrane biogenesis, PMP import,</td>
<td></td>
</tr>
<tr>
<td>PEX17</td>
<td>M</td>
<td>14</td>
<td></td>
<td>Docking complex, PMP import</td>
<td></td>
</tr>
<tr>
<td>PEX18</td>
<td>C/P</td>
<td>7</td>
<td></td>
<td>PEX7 targeting / cycling</td>
<td></td>
</tr>
<tr>
<td>PEX19</td>
<td>C/P</td>
<td>+</td>
<td>Many PMPs</td>
<td>Can be farnesylated</td>
<td>PMP receptor or chaperone</td>
</tr>
<tr>
<td>PEX20</td>
<td>C/P</td>
<td>8</td>
<td></td>
<td>Thiolase dimerization / import</td>
<td></td>
</tr>
<tr>
<td>PEX21</td>
<td>C/P</td>
<td>7</td>
<td></td>
<td>PEX7 targeting / cycling</td>
<td></td>
</tr>
<tr>
<td>PEX22</td>
<td>M</td>
<td></td>
<td>4</td>
<td>PEX4 membrane anchoring</td>
<td></td>
</tr>
<tr>
<td>PEX23</td>
<td>M</td>
<td></td>
<td></td>
<td>Matrix protein import</td>
<td></td>
</tr>
</tbody>
</table>

*Table was adapted from (Purdue & Lazarow, 2001) and (Titorenko & Rachubinski, 2001a).  
*a: C: cytosol; M: peroxisomal membrane; P: peroxisome; V: vesicle. Multiple localization indicates either a bimodal distribution (PEX5, PEX7, PEX18, PEX19, PEX20, PEX21) or species differences (PEX1 and PEX6). Peripheral PMPs: PEX8 is on the inner face of the membrane, whereas PEX1, PEX6 (when found in peroxisomes), and PEX4 are found on the cystolic face. Most of the other membrane proteins are integral PMPs with domains exposed on the cystolic face.  
*b: Peroxin interaction may be stable (e.g. the docking complex) or transient (e.g. receptors). PMPs: peroxisomal membrane proteins  
*c: SH3: src homology domain 3; TPR: tetratricopeptide repeat; Ub: ubiquitin.  
*d: MCFA: medium chain fatty acids; PMPs: peroxisomal membrane proteins; PTS: peroxisomal targeting sequence.
3.1 Targeting and Import of Matrix Enzymes

Most of the identified peroxins are involved in the import of the soluble peroxisomal matrix enzymes from the cytosol into the peroxisomal lumen. The number of proteins involved in this process is far exceeding the number of proteins required by other translocation systems with the exception of nucleocytoplasmic transport (Smith & Schnell, 2001). Peroxisomal matrix proteins are synthesized with one of two types of intrinsic peroxisomal targeting signals (PTSs), that direct import into the organelle. Following synthesis in the cytoplasm, the PTS-containing proteins (ligands) are recognized and bound by specific soluble receptors. The receptor-ligand complex is then transported to the peroxisomal surface, where it docks via membrane associated docking factors. After translocation of the ligand into the peroxisomal matrix and dissociation of the receptor-ligand complex, the PTS receptor must be recycled back to the cytoplasm, where it can initiate another round of matrix protein import. All these steps are summarized in Fig. I-4.

**Figure I-4: Model for peroxisomal matrix protein import.** PEX5 and PEX7 act as receptors for newly synthesized PTS1- and PTS2-containing peroxisomal matrix proteins (ligands), respectively. The receptor-ligand complex is then transported to the peroxisomal surface, where it interacts with docking factors. PEX2, PEX10, and PEX12 act in matrix protein import downstream of docking, possibly in translocation. In the “extended shuttle model”, the receptors enter the peroxisomes as part of their cycles. PEX1 and PEX6 interact with one another upstream of PEX4 and PEX22 in the terminal steps (receptor recycling) of matrix protein import.
Peroxisomal matrix protein targeting signals and their receptors

There are two well-characterized classes of PTSs, known as PTS1 and PTS2. The majority of matrix enzymes are targeted via the PTS1 pathway. Previous studies on this targeting sequence characterized it as a C-terminal tripeptide with the consensus (S/A/C)-(K/R/H)-L (Gould *et al.*, 1989). PTS1 signals interact directly with their receptor PEX5, which is absolutely required for their targeting to peroxisomes. PEX5 is one to the more intensively investigated and better understood of the peroxins. Originally identified as the PTS1 receptor in the yeast *Pichia pastoris*, (McCollum *et al.*, 1993), it has been cloned from numerous species including mammals, yeast, invertebrates, plants, and protozoan parasites (Purdue & Lazarow, 2001). All these PEX5 orthologs share a region comprised of six to seven tetratricopeptide repeats (TPRs) within the C-terminal part of the protein, which is essential for PTS1 import and which interacts with the PTS1 signal (Brocard *et al.*, 1994; Dodt *et al.*, 1995; Fransen *et al.*, 1995; Gatto *et al.*, 2000; Terlecky *et al.*, 1995). PTS2 consists of a nine residue signal with a broad consensus sequence of (R/K)-(L/V/I)-x5-(H/Q)-(L/A/F), located internally or near the N-terminus. It directs the import of a small number of proteins via the soluble PEX7 receptor (Marzioch *et al.*, 1994). In addition to the well characterized PTS1 and PTS2 pathways, there might exist a third, as yet uncharacterized and possibly heterogeneous class of PTS. For example the peroxisomal *Saccharomyces cerevisiae* acyl-coenzyme A-oxidase, which lacks both PTS1 and PTS2, might be imported by a distinct machinery that is independent of both the PTS1 and PTS2 receptors (Zhang *et al.*, 1993).

In mammalians, two isoforms of the PTS1 receptor, the short isoform (PEX5S) and the long isoform (PEX5L) have been reported (Braverman *et al.*, 1998; Otera *et al.*, 1998). PEX5S interacts in the cytosol with PTS1 and is required for peroxisomal import of PTS1-targeted proteins only. The second isoform, PEX5L, forms a cytosolic complex with the PTS2-receptor PEX7 and is essential for peroxisomal import of both PTS1 and PTS2 proteins (Braverman *et al.*, 1998; Matsumura *et al.*, 2000; Otera *et al.*, 2000; Otera *et al.*, 1998). In contrast in yeast, only one form of the PTS1 receptor PEX5 exists. Unlike PEX7-mediated import of PTS2-containing peroxisomal matrix proteins in mammalian cells, this process in yeast does not require PEX5 (Hettema *et al.*, 1999; McCollum *et al.*, 1993).
Peroxisomal docking

After ligand-binding, the ligand-receptor complexes dock to the cytosolic surface of the peroxisomal membrane to initiate the cascade of peroxins-mediated events that leads to the entrance of the ligand into the peroxisomal matrix and recycling of the receptors to the cytosol. Despite the overall similarity of this process in different organism, there are some mechanistic differences between higher and lower eukaryotes.

In mammalians, the initial docking site for both the PTS1 and the PTS2 complexes on the cytosolic surface of the peroxisomal membrane is PEX14 (Fransen et al., 1998; Otera et al., 2000). After their interaction with PEX14, the PTS1 and PTS2 receptors, together with their ligands, are transferred to other components of the import machinery, including PEX10, PEX12, and PEX13, which directly interact with PEX5L and PEX5S (Chang et al., 1999a; Otera et al., 2000). The peroxins PEX2, PEX10 and PEX12, all known to be members of the RING finger superfamily, might be involved in the translocation of the ligands, either alone or together with their receptors, across the membrane into the peroxisomal matrix (Chang et al., 1999a; Otera et al., 2000).

In yeast, a putative docking complex for both PTS1 and PTS2 pathways consists of three membrane-associated peroxins: PEX13, PEX14 and PEX17 (Girzalsky et al., 1999), (Elgersma et al., 1996; Huhse et al., 1998; Urquhart et al., 2000). These peroxins might also be part of, or functionally cooperate with, a translocation machinery for PTS1-containing proteins. Further steps in the import of peroxisomal matrix proteins involve the transfer of PEX5 and PEX7, either alone or together with their ligands, from the docking complex to PEX2, PEX10 and PEX12 (Collins et al., 2000).

Translocation across the peroxisomal membrane

It is well established that only unfolded, monomeric proteins can cross the ER and mitochondrial membranes, and folding and oligomerization of these proteins occur only within these organelles. By contrast, completely folded and oligomeric proteins can be imported into the matrix of the peroxisome (Glover et al., 1994; Walton et al., 1995; Walton et al., 1992). Furthermore, a key discussion in the recent literature concerns whether the PTS-receptors enter the peroxisomal lumen as part of their cycles. This “extended shuttle model”, as proposed for the PTS1-receptor PEX5, predicts that after docking at the peroxisomal membrane, PEX5 remains bound to its substrate and is translocated into the matrix along with its ligand (Dammair & Subramani, 2001; Smith & Schnell, 2001). A challenge for the future
will be to define the mechanism by which this folded and oligomeric proteins translocate across the peroxisomal membrane. The role of the putative translocases PEX2, PEX10 and PEX12 and of chaperones like Hsp40 and Hsp70 in this process needs to be clarified. Different models for the transport of oligomeric proteins across the peroxisomal membrane have been proposed. One possibility is that the translocon assembles at the site of translocation in response to the docking of the receptor-ligand-complex. This would allow the formation of protein-conducting channels of a variety of sizes in response to the size of the transport substrate. Disassembly of the translocon immediately following membrane translocation would minimize the free diffusion of molecules across the channel, thereby maintaining the critical permeability of the organelle (Smith & Schnell, 2001). Other models involve vesicle transport via a system resembling modified endocytosis of the peroxisomal membrane (McNew & Goodman, 1994). A third model proposes that, similar to the cytoplasm-to-vacuole targeting of aminopeptidase (Teter & Klionsky, 1999), oligomeric complexes of matrix proteins, which are preassembled in the cytosol, can be sequestered in vesicles that subsequently fuse with the peroxisomes (Titorenko & Rachubinski, 2001a).

**Receptor recycling**

The recycling of the PTS receptors back to the cytosol might require several peroxins: PEX8, PEX4, PEX1, and PEX6. PEX8 interacts with the PTS1 receptor (Rehling et al., 2000). The peripheral membrane protein PEX4 is a member of the E2 family of ubiquitin-conjugating enzymes (Wiebel & Kunau, 1992) and is anchored at the cytosolic face of the peroxisomal membrane through its interaction with the integral membrane protein PEX22 (Koller et al., 1999). It has been proposed that PEX4 may be involved in PEX5 recycling from the matrix to the cytosol (van der Klei et al., 1998) or in “quality control” at the peroxisomal membrane (Subramani et al., 2000). PEX1 and PEX6 are ATPases belonging to the family of “ATPases associated with various cellular activities” (AAA). They are the only known peroxins with ATPase activity, however, reports of their subcellular localization and proposed function show striking differences among species (Purdue & Lazarow, 2001).


3.2 Peroxisomal Membrane Biogenesis

Organelle membranes are composed of proteins, phospholipids and a variety of other lipids, such as sterols and fatty acids, but it is unlikely that organelle membranes could form in the absence of integral membrane proteins. Therefore, the import of peroxisomal membrane proteins may be an essential aspect of peroxisome membrane biogenesis. The first hypothesis for peroxisomal membrane biogenesis suggested that peroxisomes bud from the ER (Novikoff & Shin, 1964). The “growth and division model” proposed by Lazarow and colleagues replaced this theory, reporting that peroxisomes are formed by growth and division via the posttranslational import of newly synthesized proteins into preexisting peroxisomes (Lazarow & Fujiki, 1985).

Like peroxisomal matrix enzymes, the integral peroxisomal membrane proteins are synthesized on free polyribosomes in the cytosol and posttranslational targeted to, and inserted into, the peroxisomal membrane (Lazarow & Fujiki, 1985). This process involves the protection of the hydrophobic transmembrane segments of the membrane proteins from aggregation and maintenance of their import-competent conformations during and after synthesis in the cytosol (Just & Diestelkotter, 1996; Pause et al., 1997), the targeting of proteins to the peroxisomal membrane via membrane targeting signals, docking to the membrane, and ATP hydrolysis-independent insertion of the proteins into the peroxisomal membrane (Diestelkotter & Just, 1993; Imanaka et al., 1996; Just & Diestelkotter, 1996).

Targeting of membrane proteins to the peroxisome

Integral peroxisomal membrane proteins lack functional PTS1 and PTS2 signals, and their import is independent of the PTS1- and PTS2-receptors PEX5 and PEX7 (Chang et al., 1999b; Hettema et al., 2000). Therefore, integral peroxisomal membrane proteins are thought to be imported into peroxisomes by a distinct targeting mechanism from that used by peroxisomal matrix enzymes. Multiple studies have attempted to characterize the targeting information in integral peroxisomal membrane proteins, but the knowledge of well-defined peroxisomal membrane protein targeting signals (mPTSs) is limited. Dyer and colleagues first described an internal targeting signal for the 47-kDa peroxisomal membrane protein (PMP47) of Candida boidinii (Dyer et al., 1996). This signal is characterized by a basic amino acid cluster directed to the peroxisomal membrane in addition to a cytosolic domain located in front of a transmembrane segment. We demonstrated that the N-terminal 40 amino acids of
human PEX3 are sufficient to target a reporter protein (GFP) to the peroxisome (Kammerer et al., 1998). These 40 amino acids include a highly conserved positive charged cluster (RNKKK, amino acids 11-15) as well as the first transmembrane segment (amino acids 16-33) (Kammerer et al., 1998; Soukupova et al., 1999). Furthermore, we were able to define a targeting region of peroxisomal ABC half-transporters containing a highly conserved 14 amino acid motif positioned immediately prior to the first transmembrane helix (Landgraf et al., 2003). In general, transmembrane domains within or close to peroxisomal targeting signals are crucial for targeting function: In PEX3, PEX11β, PEX14, and PEX22 one single transmembrane domain is directly integrated in the targeting signal (Koller et al., 1999; Sacksteder et al., 2000; Soukupova et al., 1999). Two transmembrane domains are required for the targeting of PMP22 (Brosius et al., 2001), and three for PMP34 (Wang et al., 2001). Besides the transmembrane domains, basic amino acid clusters also influence the peroxisomal membrane protein targeting. These clusters are stretches of at least five amino acids consisting of at least two basic amino acids and mainly aliphatic residues (Biermanns & Gartner, 2001; Brosius et al., 2001). Interestingly, some peroxisomal integral membrane proteins (yeast PMP47, human PMP34, human PEX13, human and rat PMP22) seem to be targeted via two distinct non-overlapping internal targeting signals, one at the N-terminus and one at the C-terminus of these proteins (Brosius et al., 2001; Jones et al., 2001; Wang et al., 2001).

Two models for targeting of peroxisomal membrane proteins have been proposed (Brosius et al., 2001): First, after synthesis in the cytosol, the peroxisomal membrane proteins are recognized and bound at their positively charged basic cluster to a reporter protein located in the peroxisomal membrane. The protein is then integrated into the peroxisomal membrane. Another model involves cytosolic docking proteins. The docking protein picks up the peroxisomal membrane protein in the cytosol and the regions containing the positively charged clusters might mediate this binding. The membrane proteins complexed to their cytosolic acceptors are then transported to the peroxisome. There the complex associates with the peroxisomal membrane either directly or by binding to a component of the peroxisomal membrane insertion machinery. Thereafter, the peroxisomal membrane protein is then integrated into the bilayer of the organelle. Then the docking protein shuttles back to the cytosol where it can receive new substrates.
The control of peroxisome proliferation

In both yeast and mammalian cells, the abundance of peroxisomes can vary significantly depending upon the environmental conditions. A well-characterized example of peroxisomal proliferation is the response of rodent liver cells to peroxisome proliferating agents, like fenofibrate. These agents act by stimulating the nuclear hormone receptor PPARα and to a lesser extent PPARγ, which, in turn, alter the transcription of numerous target genes (Wahli et al., 1999). Members of the PEX11 family function as positive regulators of peroxisome division, but are not required for peroxisomal protein import (Erdmann & Blobel, 1995; Marshall et al., 1995; Schrader et al., 1998). In human, two isoforms, PEX11α and PEX11β, have been reported (Passreiter et al., 1998; Schrader et al., 1998). PEX11β is expressed at robust levels in virtually all tissues, whereas PEX11α is expressed in a restricted set of tissues, primarily those that response to peroxisome proliferating agents (Passreiter et al., 1998; Schrader et al., 1998). Therefore, it has been proposed that PEX11β may mediate constitutive peroxisome proliferation, whereas PEX11α may mediate hormone- and diet-induced changes in peroxisome abundance (Schrader et al., 1998).
3.3 PEX3 and PEX19, Peroxins Expected to be Involved in the Early Steps of Peroxisomal Biogenesis

Most of the peroxins known today are involved in the peroxisomal matrix protein import. Therefore, defects in these genes lead to a disruption of peroxisomal matrix protein import, whereas various peroxisomal membrane components are synthesized and accumulate in peroxisomal membrane remnants, described as “peroxisomal ghosts”. Nevertheless, yeast cells defective in the farnesylated protein PEX19 (Götte et al., 1998) and in the integral membrane protein PEX3 (Baerends et al., 1996; Höhfeld et al., 1991; Wiemer et al., 1996) seem to have not even such recognizable peroxisomal ghosts. This has led to the suggestion that these two peroxins might be implicated in the early steps of peroxisomal biogenesis. We have previously cloned and characterized the human ortholog of yeast PEX3, former termed PAS3 in Saccharomyces cerevisiae (Höhfeld et al., 1991), PER9 in Hansenula polymorpha (Baerends et al., 1996), and PAS2 in Pichia pastoris (Wiemer et al., 1996). Human PEX3 encodes for an integral membrane protein of 373 amino acids with a molecular weight of 42 kDa and is localized at the peroxisome. As can be expect for a peroxisomal assembly protein, PEX3 is expressed in all human cells and tissues analyzed (Kammerer et al., 1998). In yeast, PEX3 has been shown to interact with PEX19 in vivo (Götte et al., 1998). PEX19 was originally isolated from a Chinese hamster cell line. It was localized to the outer surface of the peroxisome, exhibited a C-terminal farnesyl group and was therefore designated Pf (peroxisomal farnesylated protein) (James et al., 1994). The human ortholog of the hamster protein was firstly described as HK33 (housekeeping gene encoding a 33 kDa protein) due to its ubiquitous transcription pattern (Braun et al., 1994). For human PEX19, the intrinsic property for C-terminal farnesylation was demonstrated in vitro (Kammerer et al., 1997). The covalent attachment of prenyl lipids, for example farnesyl groups or geranylgeranyl groups, by specific transferases is indispensable for the cellular sorting of some proteins (Clarke, 1992). These proteins exhibit the C-terminal sequence CaaX (“CaaX-box”), wherein ‘C’ is cysteine, ‘a’ is an aliphatic amino acid, and ‘X’ may represent one of several amino acids, predicting whether farnesyl groups or geranylgeranyl groups are attached (Marshall, 1993; Moomaw & Casey, 1992; Moores et al., 1991; Reiss et al., 1991) (Fig. 1-5). Posttranslational prenylation seems to play a major role in cellular processes, such as targeting, membrane association, and vesicle fusion events (Gorvel et al., 1991; Hancock et al., 1989; Kato et al., 1992b). PEX19 is the only peroxin known to own a C-terminal CaaX-box. In Saccharomyces cerevisiae, PEX19 is partly farnesylated in vivo and it was shown that the interaction of
PEX19 and PEX3 strongly depends on the presence of the farnesyl group (Götte et al., 1998). The question whether farnesylation of human PEX19 is essential for its function in peroxisomal biogenesis will be further examined in this thesis.

Figure I-5: Scheme for protein prenylation. The C-terminal CaaX-box predicts whether farnesyl or geranylgeranyl groups are attached.
CHAPTER II

Aims of the Thesis
Aims of the Thesis

The genes coding for human PEX3 and PEX19, two peroxisomal assembly proteins (“peroxins”), were recently cloned and characterized by our research group. (Kammerer et al., 1998; Kammerer et al., 1997). Studies in yeast supported the hypothesis that PEX19 and its interacting protein PEX3 are involved in the early steps of peroxisomal assembly (Baerends et al., 1996; Götte et al., 1998; Höhfeld et al., 1991). The main objective of this thesis was to gain insights into the functional role of human PEX3 and PEX19 in human biology and disease.

One task was to gain further knowledge on the protein-protein interaction network, regarding both interactions between peroxins and interactions between peroxins and other proteins. For this purpose, we aimed to extend the list of proteins harboring the ability to bind to PEX19.

In addition, we planned to perform experiments to investigate functional key properties of the human PEX19 protein, in particular the role of C-terminal farnesylation. The definition of functional regions of human PEX19 by the characterization of the encoded proteins of truncated PEX19 splice variants (Kammerer et al., 1997) was expected to contribute to the knowledge on specific protein domains of PEX19.

PEX19 as well as PEX3 were candidate genes for human peroxisomal biogenesis disorders. Identification of yet unknown mutations in these two PEX-genes in candidate patients was expected to provide a natural human model for the elucidation of the very early steps of human peroxisome formation. Alternatively, the generation of animal (mouse) models, defective in PEX3 and PEX19 respectively, could address these issues.
CHAPTER III

Results and Discussion
1 PEX19 Interacts With a Variety of Peroxisomal Membrane Proteins

PEX19 has previously been shown to interact with PEX3 in yeast (Götte et al., 1998). The characterization of this and other protein-protein interactions in human might contribute to a more detailed model of early human peroxisomal biogenesis. In order to investigate protein-protein interactions expected to occur between PEX19 and other proteins, we performed cDNA library screens using the LexA yeast two-hybrid system. Different LexA-PEX19 constructs (amino acids 1-299, 7-295, 7-166 and 139-299 of PEX19), if used as a bait, had been shown to be self-activating in the yeast two hybrid system (C. J. Glöckner, personal communication). Therefore, we tested directly the interaction between PEX19 and the Adrenoleukodystrophy Related Protein (ALDRP), a PMP we previously cloned and characterized in our laboratory (Holzinger et al., 1997b). ALDRP belongs to the peroxisomal subgroup of ABC half-transporters (Chapter I.2.1 and Appendix 1). A contribution of ABC transporters to peroxisomal biogenesis cannot be excluded since it has been suggested that PMP70, another peroxisomal ABC half-transporter, might play an important role in peroxisomal assembly (Gärtner & Valle, 1993; Gärtner et al., 1992). Furthermore, ALDRP is of special interest because it is the closest homologue of the Adrenoleukodystrophy Protein (ALDP), known to be the causative gene in X-Linked Adrenoleukodystrophy (X-ALD). Therefore, ALDRP is expected to act as a modifier gene in X-ALD, being responsible for the heterogeneity of clinical phenotypes. Ectopic ALDR expression or overexpression could possibly have a beneficial effect on the disease. First, the genomic organization, the putative promoter region and the chromosomal localization of the ALDR gene were characterized (Appendix 1). The human ALDR gene extents over 33 kb on chromosome 12q12 and consists of 10 exons. The genomic organization of ALD and ALDR is highly similar, confirming the expected close homology between both genes and a recent divergence from a common ancestor (Appendix 1). Furthermore, the ALDR full-length cDNA was completed and the ALDR protein was shown to be exclusively localized at the peroxisome (Appendix 1).

Using a yeast two-hybrid screen with parts of ALDR as a bait, we initially identified an interaction between ALDRP and PEX19 (Appendix 2). Further yeast two-hybrid and in vitro interaction assays confirmed this protein-protein interaction and, moreover, identified two other peroxisomal ABC half-transporters (ALDP and PMP70) being able to bind to PEX19 (Appendix 2). With regard to ALDP, the PEX19-interaction occurs in an internal region of ALDP that was shown to be important for the targeting of the protein to the peroxisome. This
provides evidence for one proposed biological function of PEX19 as PMP-receptor (further discussed in Chapter III-3). In vitro binding assays revealed that the farnesylated wild type PEX19 and a farnesylation-deficient PEX19 variant did not differ in their abilities to bind to ALDP (Appendix 2). Therefore, we further characterized the influence of PEX19-farnesylation as to PMP-interaction and induction of peroxisomal biogenesis using the encoded proteins of PEX19 splice variants (Chapter III-2 and Appendix 3).

At the beginning of this thesis, PEX3 was the only PMP known to interact with PEX19 (Götte et al., 1998). We extended the list of proteins known to interact with PEX19 including not only peroxins, like PEX3, but also other PMPs, like the peroxisomal ABC half-transporters ALDP, ALDRP, and PMP70. Today, PEX19 protein has been shown to bind a variety of peroxisomal membrane proteins (Fig. III-1) including peroxins, ABC transporters, and other PMPs (Fransen et al., 2001; Gloeckner et al., 2000; Sacksteder et al., 2000; Snyder et al., 2000; Snyder et al., 1999a; Snyder et al., 1999b). Therefore, PEX19 is known to act as a broad specific PMP-binding protein.

![Figure III-1: PEX19 binds multiple peroxisomal membrane proteins.](image-url)

Figure III-1: PEX19 binds multiple peroxisomal membrane proteins. PEX19 has been shown to interact with a set of peroxisomal membrane proteins (PMPs), including peroxins (PEX2, PEX3, PEX10, PEX11, PEX12, PEX13, PEX14, PEX16, PEX17, and PEX22), peroxisomal ABC half-transporters (ALDP, ALDRP, and PMP70), and other PMPs (PMP22, PMP24, and PMP34). PEX19 is farnesylated at the C-terminal farnesylation consensus sequence (CaaX-box). Protein-protein interactions that are characterized in this thesis are highlighted in dark.
2 Characterization of PEX19 Splice Variants: Functional Diversity and New Insights in the Role of Posttranslational Farnesylation

PEX19 does not equally bind to all of its known PMP-interactors. Full-length PEX19 is required for efficient interaction with PEX10, PEX11αβ, PEX12, and PEX13. On the other hand, PEX19 has distinct binding sites for PEX3 and PEX16 (Fransen et al., 2001), suggesting that PEX19 may bind to these PMPs simultaneously. Nevertheless, distinct PMP-binding sites of PEX19 had not been linked to a specific domain organization of the protein. We therefore characterized the domain organization of PEX19 by database analysis (Appendix 3). The protein is composed of three domains in direct succession (Fig. III-2), which are exclusively shared by ortholog PEX19 proteins of other species. However, these domains have not yet been linked to specific biological functions. Interestingly, the encoded proteins of two PEX19 splice variants, each truncated in exactly one domain, resemble this modular domain organization (Fig. III-2): The variant PEX19ΔE2 lacks the N-terminal domain D1, whereas PEX19ΔpE8 lacks the C-terminal domain D3 including the farnesylation consensus sequence. We took advantage of these splice variants in order to assess their functional ability as to PMP binding, induction of peroxisomal membrane synthesis and the dependence of posttranslational farnesylation of PEX19.

<table>
<thead>
<tr>
<th>domain structure</th>
<th>PMP interaction a</th>
<th>functional complementation b</th>
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<tr>
<td>full-length PEX19</td>
<td>E1 E2 E3 E4 E5 E6 E7 E8</td>
<td>+</td>
</tr>
<tr>
<td>PEX19ΔE2</td>
<td>E3 E4 E5 E6 E7 E8</td>
<td>+/-</td>
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<tr>
<td>PEX19pΔE8</td>
<td>E1 E2 E3 E4 E5 E6 E7 E8</td>
<td>+</td>
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Figure III-2: Functional diversity of different PEX19 splice variants. Scheme of the domain structure of PEX19 (gray) and of the encoding proteins of the PEX19 splice variants (white). The dashed box at the C-terminus of PEX19pΔE8 is indicating amino acids that differ from the wild type PEX19 sequence because of frame shifts. aProtein-protein interactions were analyzed between PEX19 variant proteins and the peroxisomal ABC transporters (ALDP, ALDRP, and PMP70) and two PEX3 variants (full-length PEX3 and Δ66aaPEX3): (+) the variant binds to all PMPs examined; (+/-) the variant binds to all PMPs examined except Δ66aaPEX3. bFunctional complementation assays were performed in PEX19-deficient fibroblast: (+) restoration of functional peroxisomes; (-) no restoration of functional peroxisomes. The corresponding experiments are described in appendix 3. D1: domain 1 (ProDom-ID PD339396); D2: domain 2 (ProDom-ID PD024170); D3: domain 3 (ProDom-ID PD329985); E1-E8: exons 1-8; PMP: peroxisomal membrane protein; jagged line: farnesyl group.
Functional diversity of \textit{PEX19} splice variants

With regard to PMP-interaction, the encoded proteins of both \textit{PEX19} splice variants (PEX19\textDelta E2 and PEX19\textDelta pE8) interact with peroxisomal ABC transporters (ALDP, ALDRP and PMP70) and with full-length PEX3. In contrast, PEX19\textDelta pE8 interacts with a truncated PEX3 protein lacking its N-terminal peroxisomal targeting sequence, whereas PEX19\textDelta E2 does not (Appendix 3). Functional complementation studies in \textit{PEX19}-deficient human fibroblasts revealed that transfection of \textit{PEX19}\textDelta pE8-cDNA leads to restoration of both, peroxisomal membranes and functional peroxisomes, whereas transfection of \textit{PEX19}\textDelta E2-cDNA does not restore peroxisomal biogenesis. Taken together, these data provide the first experimental evidence for the functional diversity of \textit{PEX19} splice variants as to PMP-interaction and induction of peroxisomal assembly (Fig. III-2). Functional diversities of splice variants as to protein-protein-interactions has been reported for many proteins. For example, PYK2 (proline-rich tyrosine kinase 2), but not its isoform PRNK (PYK2-related non-kinase), interacts with p130\textsuperscript{cas} (Crk-associated substrates) and Graf (GTPase regulator associated with focal adhesion kinase), indicating a PRNK-mediated regulation mechanism in certain cells (Xiong \textit{et al.}, 1998). Among the mammalian peroxins, the appearance of different splice variants has been reported for the PTS1-receptor PEX5 (Braverman \textit{et al.}, 1998; Otera \textit{et al.}, 1998). Two variants (PEX5S and PEX5L) are involved in the import of peroxisomal matrix enzymes in a different manner (Chapter I.3.1). In addition, humans, like other mammals, contain two forms of PEX11, namely PEX11\textalpha{} and PEX11\textbeta{}. Based on the differences in their tissue-specific patterns of expression and their differential regulation by peroxisome proliferation agents, it has been proposed that PEX11\textbeta{} may mediate constitutive peroxisome proliferation, whereas PEX11\textalpha{} may mediate hormone- and diet-induced changes in peroxisome abundance (Schrader \textit{et al.}, 1998). In addition to distinct functions, tissue- or cell specific expression of different splice variants has been reported for many genes (Atamas, 1997; Strehler & Zacharias, 2001). In the case of \textit{PEX19}, the full-length variant has been described to be the predominant transcript in mRNA of several cells and tissues in human. However, the variant \textit{PEX19}\textDelta E2 has also been reported to account for a significant level of total human \textit{PEX19} mRNA (Kammerer \textit{et al.}, 1997). Interestingly, the ratio between both variants was completely reversed in uterine tissue (Kammerer \textit{et al.}, 1997). The question whether this reversed expressions level in uterine tissue is linked to the functional diversity of \textit{PEX19} splice variants has to be subjected to further examinations.
The role of farnesylation of PEX19

Among the peroxins known today, only PEX19 owns a C-terminal consensus sequence (CaaX-box; Chapter I.3.3) with the intrinsic property of being modified by posttranslational farnesylation. Farnesylation of *Saccharomyces cerevisiae* PEX19 was shown to be essential for the proper function of the protein in peroxisome biogenesis (Götte *et al.*, 1998). In contrast, this modification seems to be absent and dispensable for the function PEX19 in *Pichia pastoris* (Snyder *et al.*, 1999a). The human PEX19 protein has been reported to be farnesylated *in vitro* (Kammerer *et al.*, 1997) and *in vivo* (Matsuzono *et al.*, 1999). There was a controversial discussion at the beginning of this thesis whether farnesylation is essential for the function of PEX19 in human peroxisomal biogenesis (Matsuzono *et al.*, 1999) or has only an ancillary effect (Sacksteder *et al.*, 2000). By performing functional complementation studies, we clearly demonstrated that expression of *PEX19ΔpE8*-cDNA, encoding for a C-terminally truncated PEX19 protein that cannot be modified by a farnesyl group (Kammerer *et al.*, 1997), could restore functional peroxisomes in *PEX19*-deficient human fibroblasts (Appendix 3). We therefore concluded that posttranslational farnesylation is not essential for PEX19 to induce peroxisomal membrane synthesis in human. *In vivo*, however, we cannot rule out the possibility that farnesylation may enhance PEX19 function, perhaps in a regulating manner. Moreover, farnesylation might influence or regulate other functions of the PEX19 protein. It has been reported that farnesylation motif of PEX19 affects the binding properties for PEX10, PEX11αβ, PEX12, and PEX13, but not those for PEX3 and PEX16 (Fransen *et al.*, 2001). For PEX3, we were able to confirm these data. Furthermore, we demonstrated that farnesylation of PEX19 is not necessary for its interaction with peroxisomal ABC half-transporters (Appendix 3). Altogether, farnesylation might be able to regulate the interaction of PEX19 with different PMPs, perhaps in an intracellular location-specific manner, ensuring proper sorting and trafficking of the distinct PMPs.
3 Proposed Biological Functions of PEX19

In yeast, PEX19 and PEX3 are required for the proper localization of PMPs, and cells deficient in these peroxins mislocalize their PMPs to the cytosol (Hettema et al., 1999). Human PEX19 is a predominantly cytoplasmic, partly peroxisomal protein, and loss of PEX19 results in the absence of detectable peroxisomal structures, the destabilization of many integral PMPs, and the mislocalization of other PMPs to the mitochondrion (Sacksteder et al., 2000). Due to these observations, several models of PEX19 function are possible: First, PEX19 might act as a soluble receptor that binds newly synthesized PMPs in the cytosol and subsequently directs the PMPs to the peroxisomal membrane. Second, PEX19 might be a PMP-specific chaperone that keeps PMPs in an import-competent conformation. Third, PEX19 might facilitate the insertion of PMPs into the peroxisomal membrane. Last, PEX19 might play a role in peroxisomal membrane stabilization or maturation.

Interestingly, our in vitro targeting studies suggested that the PEX19-interaction domain of the peroxisomal ABC-transporter ALDP coincides with the region of ALDP that is required for its targeting to the peroxisome (Appendix 2). Similar results have been reported for other PMPs (PMP70, PEX11β, and PEX14), indicating that the targeting elements of some PMPs retain the ability to bind to PEX19 (Sacksteder et al., 2000). Furthermore, mislocalization of PEX19 has been shown to result in mislocalization of newly synthesized PMPs. These data, combined with the observation that a small but significant amount of PEX19 is associated with the outer surfaces of peroxisomes, made PEX19 to be a prime candidate for a cycling PMP-receptor protein (Gould & Valle, 2000). However, an increasing number of PMPs have been reported that bind to PEX19 at regions distinct from their targeting regions (Fransen et al., 2001; Snyder et al., 2000). Random mutagenesis studies of PEX13 demonstrated that the PEX19-binding domain and the peroxisomal targeting signal could be functionally separated (Fransen et al., 2001). Furthermore, interactions between PEX19 and PMPs were not reduced upon inhibition of new protein synthesis, suggesting that they occur with preexisting, and not newly synthesized, pools of PMPs (Snyder et al., 2000). In Pichia pastoris, the organelle-associated pool of PEX19 interacts with the PMPs at the peroxisome, and this interaction may occur at the cytosolic site of the membrane (Snyder et al., 2000). Taken these data together, the role of PEX19 as cycling PMP-receptor has been challenged. It has been suggested that PEX19 may have a chaperone-like role at the peroxisomal membrane. Nevertheless, since PEX19 is predominantly present in the cytosol, this peroxin most likely has also other
biological functions (Fransen et al., 2001). Recently, $PEX19$ mutants in *Yarrowia lipolytica* have been shown capable of forming structures that are morphologically similar to wild-type peroxisomes and that are surrounded by a membrane whose protein composition is similar to wild-type peroxisomes (Lambkin & Rachubinski, 2001). A significant reduction in the levels of the integral membrane protein PEX2 in $PEX19$-mutants of *Yarrowia lipolytica* lends support to the proposal that PEX19 is important to the stability of PMPs, or of the peroxisomal membrane (Lambkin & Rachubinski, 2001). Although the overall mechanism of peroxisomal assembly has been conserved during evolution, the role played by individual peroxins, like PEX19, may not necessarily be exactly the same in different organism. In general, compared with the well-defined peroxisomal matrix protein receptors PEX5 and PEX7, it is not likely that PEX19 exhibits an analogous receptor function for all known PMPs. PEX19 may act in a more PMP-stabilizing manner, at or distinct-from the peroxisomal membrane. The question whether the cytosolic and peroxisomal intracellular localizations of PEX19 are linked to distinct biological functions of the PEX19 protein has to be subjected to further examinations.
4 PEX3, a Key Factor in Early Human Peroxisome Synthesis

PEX3 or PEX19 mutants in yeast have been shown to lack functional peroxisomes (Baerends et al., 1996; Götte et al., 1998; Höhfeld et al., 1991). Therefore their human orthologs had been expected to be candidate genes for peroxisomal biogenesis disorders (PBDs; Chapter I.2.2). However, human phenotypes defective in these genes had not been reported at the beginning of this thesis. Shortly thereafter, an inactivating mutation in human PEX19, causing Zellweger syndrome in CG-J of the PBDs, was reported (Matsuzono et al., 1999). Like their yeast counterparts, human cells defective in PEX19 were shown to lack even peroxisomal membrane remnants. It was suggested that PEX19 might be involved at the very initial stages of peroxisomal membrane assembly, even before the import of matrix proteins (Matsuzono et al., 1999). Based on the observation that the cellular phenotypes of yeast cells defective in PEX3 and PEX19 are resembling each other, it was tempting to speculate that besides PEX19-mutants, mammalian cells with an inactivating PEX3 mutation might also entirely lack peroxisomal membrane remnants.

The generation of a mouse model by targeted disruption of the PEX3 gene is one strategy to clarify the exact contribution of PEX3 to the formation of peroxisomes. Mouse models defective in PEX genes have already been described for PEX2, which encodes for a zinc-binding PMP, and for PEX5, the import receptor for the most peroxisomal matrix proteins (Baes et al., 1997; Faust & Hatten, 1997). Besides their ability to study the molecular details of peroxisomal assembly, these models could provide suitable in vivo systems to study disease mechanisms, and experimental treatment strategies for the peroxisomal biogenesis disorders. As a first step to generate a PEX3-deficient mouse model, the mouse PEX3 gene was characterized (Appendix 4): The gene spans a region of about 30 kb, contains 12 exons, and is located on band A of chromosome 10. The 2 kb cDNA encodes for a mouse PEX3 protein of 372 amino acids (42 kDa). Compared to human PEX3 (373 amino acids), both proteins reveal an overall amino acid identity of 93.8%. The putative mouse PEX3 promoter region exhibits characteristic housekeeping features, and PEX3 expression was identified in all tissues analyzed, but could not be induced by fenofibrate. Taken together, these data are experimental prerequisites for the future generation of a mouse model defective in PEX3.

To prove the hypothesis that PEX3 might be a candidate gene for peroxisomal biogenesis disorders, we searched for a PEX3-deficient human phenotype. As a prerequisite, we analyzed
CHAPTER III: RESULTS AND DISCUSSION

the genomic structure, the putative promoter region, the chromosomal localization and fine-mapping of the human PEX3 gene (Appendix 5). The gene spans a region of about 40 kb on chromosome 6q23-24. The human PEX3 gene is composed of 12 exons and 11 introns, and these data were useful to design a strategy for PEX3 mutation analysis based on PCR amplification and sequencing of amplicons from genomic DNA. This method was applied to screen the PBD complementation groups CG-E, CG-C, CG-B, CG-A, and CG-D for a PEX3-mutation, but no differences to the wild-type PEX3 sequence were detected (Appendix 5). In the meantime, CG-E, CG-C, CG-B, and CG-D had been clarified to be caused by defects of PEX1, PEX6, PEX10, and PEX16, respectively (Table I-3). Only the causative gene in CG-A of the PBDs has not been identified so far.

Later on, fibroblasts from two Zellweger patients (PBDG-01 and PBDG-02), assigned to an additional complementation group CG-G (Poulos et al., 1995), were available for further studies. Fibroblasts of this complementation group had been shown to lack peroxisomal membrane remnants (Shimozawa et al., 1998), and this was confirmed by performing own immunofluorescence microscopy analyses with fibroblasts of both patients (Appendix 6, Fig A6-2): In both mutant cell lines, PMPs are either degraded, as proposed for ALDP and PMP70, or mislocalized to the mitochondria, as shown for PEX14. Because of the lack of morphologically recognizable PMP-containing peroxisomal membranes, the cell lines of the two patients were excellent candidates for a PEX3 mutation. By performing PEX3 mutation analysis, we identified homozygous PEX3 mutation in both patients, each leading to a C-terminal truncation of PEX3 (Appendix 6, Fig. A6-3): One of the mutations involved a single-nucleotide insertion in exon 7, whereas the other was a single-nucleotide substitution eight nucleotides form the normal splice site in the 3’ acceptor site of intorn 10, which interferes with correct splicing. Expression of the wild-type PEX3 cDNA in the mutant cell lines restored peroxisomal biogenesis, whereas transfection of the mutated cDNA did not (Appendix 6, Fig. A6-4). This confirmed that the causative gene had been identified, being responsible for Zellweger syndrome in CG-G. Biochemically, we were able to demonstrate that truncated PEX3 proteins of patient PBDG-01, lacking approximately the C-terminal halve, could not bind to full length PEX19 in vitro (Appendix 6, Fig. A6-5). Although this does not necessarily reflect the situation in vivo, this lack of interaction between both peroxins could functionally be responsible for the impaired peroxisomal membrane assembly. In yeast, this protein-protein interplay between PEX3 and PEX19 appears to be a prerequisite for
generation of preperoxisomal structures that can acquire and stabilize newly synthesized PMPs (Hettema et al., 2000).

The molecular and biochemical evidence shown in appendix 6 leaves little doubt that PEX3 mutations are responsible for Zellweger syndrome in CG-G of the peroxisomal biogenesis disorders. So far, only mutations in human PEX16 and PEX19 have been reported to cause the unusual cellular phenotype characterized by the lack of functional peroxisomes and of detectable peroxisomal membranes (Matsuzono et al., 1999; South & Gould, 1999). Here, we complete the list of “early peroxins” including human PEX3. Expression of wild-type PEX3, PEX16, or PEX19 restored functional peroxisomes in the respective mutant cell line, providing evidence for the essential role of these early peroxins in a human peroxisome-formation pathway that does not require morphologically detectable preexisting peroxisomal structures. This challenges the theory that peroxisomes arise exclusively by growth and division of preexisting peroxisomes and establishes PEX3 as a key factor in early human peroxisome synthesis.
5 Two-Path Model for Peroxisome Membrane Synthesis in Mammalian Cells

Prior the discovery of peroxin-deficient cells that lack peroxisomal ghosts, it had been suggested that peroxisomes arise exclusively by growth and division from preexisting peroxisomes (Lazarow & Fujiki, 1985). In appendix 6, we describe that expression of wild-type PEX3 restored the peroxisomal biogenesis in PEX3-deficient human fibroblasts that have been shown to lack even morphologically detectable peroxisomal membranes. Corresponding results have been obtained for PEX16- and PEX19-deficient cells, respectively (Honsho et al., 1998; Matsuzono et al., 1999; South & Gould, 1999). Taken together, these observations of peroxisomal formation in the absence of morphologically recognizable peroxisomal membranes challenge the theory that peroxisomes arise exclusively from preexisting peroxisomes and suggest a model that incorporates two distinct paths for peroxisomal membrane synthesis (Fig. III-3) (Gould & Valle, 2000).

![Figure III-3: Speculative two-path model for peroxisomal membrane biogenesis.](image)

(1) Growth and division model of peroxisome biogenesis. Growth results from the PEX5- and PEX7-mediated uptake of matrix proteins and involves the matrix-protein import machinery composed of several peroxins (Chapter I.3.1). Division is mediated by PEX11. PEX3, PEX16, and PEX19 would function primarily in PMP-import. (2) In the second path, PEX3 and/or PEX16 and/or PEX19 mediate the formation of a nascent peroxisome from a pre-peroxisomal vesicle. Nascent peroxisomes import further PMPs, leading to the assembly of the peroxisomal matrix protein import machinery. Thereafter, matrix proteins can be imported, leading to mature peroxisomes.
The primary path would involve the growth and division of preexisting peroxisomes. Growth of the organelle would result from the uptake of newly synthesized matrix proteins, a process that involves all peroxins of the matrix protein import machinery including the receptors PEX5 and PEX7 (Chapter I.3.1). The peroxins PEX3, PEX16, and PEX19 would function primarily in PMP-import. Peroxisome division would be mediated by PEX11.

The two-path model also proposes a second route for peroxisomal formation in which PEX3 and/or PEX16 and/or PEX19 mediate the formation of a nascent peroxisome from a pre-peroxisomal vesicle, probably some other endomembranes of the cell. Such vesicle would be competent for the import of further PMPs, leading to the assembly of the peroxisomal matrix protein import machinery. Thereafter, matrix proteins could be imported, leading to mature, metabolic active peroxisomes, which could be further divided by ‘growth and division’.

Such a two-path model is attractive because it could explain: (a) the absence of morphologically recognizable peroxisomal membranes in PEX3-, PEX16-, and PEX19-deficient mammalian cells and the ability to synthesize peroxisomes in these cells when the correct gene is expressed (Honsho et al., 1998; Matsuzono et al., 1999; Muntau et al., 2000a; South & Gould, 1999; South et al., 2000); (b) the extremely slow, stepwise nature of peroxisome formation after expression of the correct gene in PEX3-PEX16-, or PEX19-deficient cells lacking peroxisomal membranes (Matsuzono et al., 1999; Muntau et al., 2000a; South & Gould, 1999); (c) the PEX11-mediated hyperproliferation of peroxisomes and the presence of only a few large peroxisomes in PEX11-mutants (Erdmann & Blobel, 1995; Marshall et al., 1995; Schrader et al., 1998); (d) the ability of the N-terminal 50 amino acids of PEX3 to induce the formation of vesicles in the vicinity of the nuclear membrane in PEX3-deficient Hensenula polymorpha cells and the fact that these vesicles are the specific target for peroxisome development after subsequent synthesis of full-length PEX3 (Faber et al., 2002). Nevertheless, the major weakness of this model is that it does not explain the vesicle-fusion-events observed in the yeast Yarrowia lipolytica (Titorenko et al., 2000). But apart from the degree of homology between different species in respect to general rules governing peroxisomal assembly, several well-established differences in the peroxisomal protein import have been reported to occur between different organisms (Tab. I-4). Therefore, it is tempting to speculate that there might be some organism-specific differences in the general model of peroxisomal membrane synthesis.
Further Perspectives: Identification of the Origin of the Endomembrane Template

The peroxisomal membrane is a typical endomembrane, approximately 6.5–7 nm thick, with a phospholipid/protein ratio of 140-200 nmol/mg (Lazarow, 1984). The phospholipids are principally phosphatidyl choline and phosphatidyl ethanolamine, with a tendency toward somewhat longer-chain fatty acids than other membranes (Schneiter et al., 1999). The ER is site of biosynthesis of these phospholipids, just as it is the site of synthesis of most mitochondrial phospholipids (Lazarow & Fujiki, 1985). To day, it is still unclear how these phospholipids are transported from the ER to the peroxisome. Furthermore, apart from the biosynthesis of these lipids, it has been proposed that some integral peroxisomal membrane proteins shuttle through the ER on their route to the peroxisome (Baerends et al., 1996; Kammerer et al., 1998; Kunau & Erdmann, 1998). Taken together, these data made the ER as prime candidate for being the origin of the pre-peroxisomal vesicles, formed in the PEX3/PEX16/PEX19-mediated second route of peroxisomal membrane synthesis. However, PEX3-mediated peroxisome membrane synthesis has been reported to occur independently of COPI- and COPII-dependent membrane trafficking through the ER (South et al., 2000). Furthermore, the targeting of PEX2, PEX3, and PEX16 was not affected by inhibitors of COPI and COPII that block vesicle transport in the early secretory pathway of the ER (Voorn-Brouwer et al., 2001). Together, these data suggest that the ER is not involved in PMP targeting and peroxisome formation. On the other hand, these data do not definitively rule-out a possible role for the ER in peroxisomal biogenesis because not all vesicle-budding and –fusion processes depend on COPI and COPII (Latterich et al., 1995). For example in plants, it has been reported that vesicles can be formed at the ER that are morphologically distinct from COP-coated vesicles (Toyooka et al., 2000). Recently, it has been demonstrated that the synthesis of the N-terminal 50 amino acids of PEX3 in PEX3-deficient Hensenula polymorpha cells is associated with the formation of vesicular membrane structures, and that the nuclear membrane is the donor membrane compartment of these vesicles (Faber et al., 2002). Therefore, a challenge for the future will be to clarify the origin for the endomembranes that will be further converted to peroxisomal structures.
References


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Appendix 1

Full Length cDNA Cloning, Promoter Sequence and Genomic Organization of the Human Adrenoleukodystrophy Related (ALDR) Gene Functionally Redundant to the Gene Responsible for X-Linked Adrenoleukodystrophy

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Abstract

X-linked adrenoleukodystrophy (X-ALD) is a functional defect of the ALD Protein (ALDP), an ABC half-transporter localized in the peroxisomal membrane. It is characterized by defective very long chain fatty acid (VLCFA) β-oxidation resulting in progressive cerebral demyelination. Since individual mutations in the ALD-gene may result in a variety of clinical phenotypes the existence of modifying genetic factors has been proposed. The adrenoleukodystrophy related protein (ALDRP) a close homolog of ALDP has been shown to complement the defect of VLCFA oxidation if transfected into X-ALD cells or chemically induced in ALDP-deficient mice. Chemical ALDRP induction holds a potential for a novel therapeutic strategy. We report here the exclusively peroxisomal localization of human ALDRP, the full length cDNA, the transcriptional start and 2.4 kb of the putative promoter region DNA sequence. The human ALDR gene extends over 33 kb on chromosome 12q12 and consists of 10 exons. The gene structure is highly similar to the ALD-gene indicating a recent divergence from a common ancestor. The putative human promoter sequence contains a novel motif conserved in peroxisomal ABC-transporters in the mouse. Our data will enable sequence analysis in X-ALD patients to determine a possible role of ALDRP as a modifier and provide tools for the study of therapeutic ALDRP induction.
Introduction

X-linked adrenoleukodystrophy (X-ALD; McKusick 300100) (Gärtner et al., 1998c; Moser et al., 1995b) is the most frequent peroxisomal with an incidence of 0.8 to 1.6 per 100,000 live births (Bezman & Moser, 1998; Heim et al., 1997). It is characterized by progressive demyelination of the nervous system and adrenocortical insufficiency with marked heterogeneity of clinical manifestations ranging from the childhood cerebral form leading to premature death within several years to asymptomatic forms. Other forms include the cerebral adult form, adrenomyeloneuropathy (AMN) mainly affecting the spinal cord of adults and isolated adrenal insufficiency. The identification of the ALD-gene by positional cloning (Mosser et al., 1993) demonstrated the corresponding gene product to be a member of the ABC-transporter protein family. These integral membrane proteins are involved in the transport of various substrates across biological membranes. Mutational analysis in X-ALD patients showed no predictable genotype-phenotype correlation (Gärtner et al., 1998c). Individuals from a family carrying the same and no other mutation in the ALD-gene may be affected by any of the clinical phenotypes (Berger et al., 1994). ALDP is likely to be involved in the peroxisomal transport or catabolism of very long chain fatty acids (VLCFA; >C22) since a defect of peroxisomal VLCFA β-oxidation resulting in elevated VLCFA in plasma and tissues is the biochemical correlate of all forms of X-ALD. It has been proposed that ALDP might be a transporter translocating Coenzyme A-activated VLCFA into the peroxisome, the exclusive site of β-oxidation of VLCFA (Verleur et al., 1997).

The cDNA of the mouse adrenoleukodystrophy related protein (ALDRP) was cloned by a PCR-based search for novel ABC-transporters in the mouse genome (Lombard-Platet et al., 1996). In the mouse ALDRP is mainly expressed in brain, adrenal glands and liver. ALDRP is the closest of three ALD homologs belonging to the peroxisomal subgroup of ABC half-transporters also including PMP70 (synonym: PXMP1) and PMP69 (synonyms: P70R, PXMP1-L). PMP70 has been proposed to be involved in peroxisome biogenesis, the function of PMP69 is unknown. ABC-half-transporters are expected to form dimers in order to constitute a functional transporter complex. Homodimerization or interaction with another ABC-half-transporter has been proposed. The only two yeast peroxisomal ABC-half-transporters Pat1 and Pat2 have been shown to associate with each other and to be involved in β-oxidation of fatty acids (Shani et al., 1996). We have recently cloned the complete coding region of the human ALDR-cDNA (although lacking the full 5´ and 3´ ends) and demonstrated an expression pattern similar to mouse ALDRP mRNA different from other
peroxisomal ABC-transporters (Holzinger et al., 1997b). Analysis of human cell lines and mouse tissues showed mirror expression of ALD and ALDR genes suggesting a similar function in different tissues (Troffer-Charlier et al., 1998).

ALDR or PMP70 cDNA - if transfected into X-ALD-fibroblasts - have been shown to correct the defect of β-oxidation indicating a functional redundancy of peroxisomal ABC-transporters (Braiterman et al., 1998; Kemp et al., 1998). ALDR mRNA has previously been demonstrated to be inducible by the peroxisome proliferator fenofibrate in the rat (Albet et al., 1997). Kemp and colleagues showed inducibility by 4-phenylbutyrate of PMP70 and ALDRP in mouse and human normal and ALDP-deficient cultured fibroblasts. Moreover, 4-phenylbutyrate treatment of cultured human X-ALD fibroblasts as well as 4-phenylbutyrate feedings of ALDP-deficient mice was able to correct the biochemical defect of peroxisomal β-oxidation. Singh and colleagues (Pahan et al., 1998; Singh et al., 1998) have reported that Lovastatin (an inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase) and sodium phenylacetate as well as 8-Br-cAMP, Forskolin und Rolipram stimulated the beta-oxidation of lignoceric acid (C24:0) and normalized the elevated levels of VLCFA in X-ALD skin fibroblasts. This effect is independent of ALDP. Whether the effect was attributable to the induction of other peroxisomal ABC-transporters has not been determined yet. These findings have raised hopes for a new therapeutic strategy for X-ALD and have enhanced the medical interest in the ALDR gene and its regulation of expression. Ectopic ALDR expression or overexpression could possibly have a beneficial effect on the disease. The example of the induction of fetal hemoglobin in hemoglobinopathies such as sickle-cell disease and beta-thalassemia have demonstrated the feasibility of an approach employing the induction of a „surrogate gene“(Fibach et al., 1993).

We report here the sequence of the 5´ and 3´ untranslated regions of the human ALDR cDNA, the exclusively peroxisomal localization of human ALDRP, the transcriptional start, the putative promoter sequence including the description of a motif conserved in fenofibrate-inducible peroxisomal ABC-transporters and the detailed genomic structure of the gene including exon-flanking intronic sequence. These data will be useful tools to investigate a possible role of ALDRP in the determination of the clinical course of X-ALD and will enable further analysis of the mechanism of therapeutic induction of ALDRP. Knowledge of this mechanism might lead to the development of new therapeutic agents for the treatment of X-ALD.
Material and Methods

cDNA cloning and determination of the transcriptional start
A human 1365-bp ALDRP cDNA probe ranging from bp 1336 to 2700 in the updated NCBI Genbank entry No. AJ000327 was generated by polymerase chain reaction from randomly primed human liver cDNA. This probe was [α-32P]dATP labeled by hexanucleotide random priming and used for screening the human fetal brain library No. 507 from the Resource Center of the German Human Genome Project, Heidelberg/Berlin (Lehrach, 1990). A clone (ICRF P507D21235Q11) containing a 3547 bp insert including the full coding region was isolated. It contained 135 bp of DNA sequence at the 5’ untranslated region and 1190 bp at the 3’ untranslated region including the polyA-signal. Cloning of the full 5’ untranslated region to determine the transcriptional start was performed by RACE-PCR (Rapid Amplification of cDNA Ends) using the human brain adaptor-ligated cDNA kit (Marathon, Clontech). RACE products were subcloned into the pGemTeasy vector (Promega) and sequenced. The presence on mRNA level of the 5’ untranslated region identified was confirmed by RT-PCR from human brain mRNA. 3’- RACE-PCR was performed accordingly to determine a possible alternative mRNA 3’ end.

Genomic library screening and determination of the genomic structure
A human genomic DNA library generated with pCYPAC2 vector and genomic DNA derived from cultured skin fibroblasts with an average insert size of approximately 100 kb (Ioannou et al., 1994) was screened with the 3547 bp human ALDR cDNA probe mentioned above. The probe was [α-32P]dATP labeled by random priming and three positive clones were isolated (Screening Service of the Resource Center of the German Human Genome Project, Heidelberg/Berlin, (Lehrach, 1990)). Genomic fragments containing complete introns were PCR-amplified from pCYPAC2 DNA templates using cDNA-derived oligonucleotide primers. pCYPAC2 DNA preparation was performed by ion-exchange columns (Qiagen midiprep) according to the manufacturer’s protocol for P1-derived constructs. PCR amplification of intron-containing fragments was performed using the Expand Long Template system (Boehringer Mannheim). To estimate intron sizes the PCR products were separated on 0.7% agarose gels and compared to appropriate molecular size markers. The PCR-products were directly sequenced from either end after purification from oligonucleotides (Qiagquick, Quiagen) without further subcloning using the amplification oligonucleotide primers. Sequencing was also performed directly from pCYPAC DNA templates. All sequencing was performed by the rhodamine fluorescent dideoxy terminator method on an ABI 377 sequencer.

Southern blot analysis of pCYPAC clones
The three isolated pCYPAC ALDR clones were characterized by nonradioactive Southern blotting of EcoRI, HindIII and XbaI restriction digestions using the 3547-bp ALDR cDNA as a probe. The probe was digoxigenin-labeled using the PCR DIG Probe Synthesis Kit (Boehringer Mannheim). Transfer of DNA fragments onto nylon membranes was performed by an alkaline capillary method (Turboblotter, Schleicher and Schuell) according to the manufacturer. Hybridization and chemoluminescent detection by an alkaline phosphatase-labeled anti-digoxigenin antibody and the substrate CDP-Star (Boehringer Mannheim) followed the manufacturer’s protocol.

Cloning of the putative promoter region
Genomic DNA from the region upstream from the transcriptional start was cloned by identification by nonradioactive Southern blotting of the appropriate fragment size using an exon 1-specific probe in a HindIII digest of the pCYPAC clone 3 (LLNLP704H12113Q16) and ligation of a band of appropriate size into the pCDNA3 vector (Invitrogen). A 1700 bp HindIII fragment was amplified from this ligation reaction with an exon1-specific reverse oligonucleotide primer and a T7 vector-specific primer and sequenced. Additionally, a restriction-digested adaptor-ligated genomic DNA library (Genome Walker Kit, Clontech) was used to obtain sequence information from the putative promoter region.

Subcellular localization of human ALDR
In order to determine the subcellular localization of human ALDRP, epitope-tagged plasmid expression constructs containing a myc/hexahistidine tag (pcDNA3.1 Myc/His, Invitrogen) or a hemagglutinin tag at the COOH-terminus were generated by PCR and sequenced. Cos7 cells were transfected with these constructs using Lipofectamine (Life Technologies) according to the manufacturer and indirect immunofluorescence double staining with was performed after 72 hours using mouse monoclonal antibodies against the HA or Myc epitopes and rabbit anti-catalase antibodies to identify peroxisomes. Species-specific tetrarhodamidio-isothiocyanate-(TRITC)-labeled goat anti-mouse IgG antibodies and fluoresceine-isothiocyanate (FITC)-labeled goat anti-rabbit
IgG antibodies (Dianova, Hamburg) were used as secondary antibodies.

**Cytogenetic mapping using fluorescence in situ hybridization (FISH) analysis**

Human metaphase cells were prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes according to standard procedures. FISH was performed using pCYPAC2-DNA from clone 3 (LLNL-P704H12113Q16) which contained the complete *ALDR* gene. pCYPAC2-DNA was labeled with biotin-14-dUTP (Life Technologies) by nick-translation and preannealed with Cot-1 DNA (Life Technologies). Detection and visualization was achieved using the avidin-fluorescein isothiocyanate/anti-avidin antibody system described elsewhere (Lichter *et al.*, 1988; Lichter *et al.*, 1990) and chromosomes were identified by staining with 4,6-diamino-2-phenylindole dihydrochloride (DAPI).

![Figure A1-1: Immunofluorescence analysis of the subcellular localization of human ALDRP. Human hemagglutinin epitope-tagged ALDRP (A) colocalizes with catalase (B) a peroxisomal marker.](image-url)
Results

Full length cDNA cloning and determination of the transcriptional start

Human fetal brain cDNA library screening in combination with 5’ RACE PCR yielded a 3968 bp human ALDR cDNA. The longest 5’ RACE products defined the transcriptional start. Several clones containing identical RACE products were identified as the longest. PCR amplification of ALDR cDNA across exon-intron boundaries excluded an artifact of the 5’ RACE procedure resulting from genomic DNA amplification. A 1500-bp ALDR-specific product could be amplified from human randomly primed brain cDNA with an exon 2 specific reverse oligonucleotide primer and a forward primer from the start of the transcript.

3’ RACE procedures to determine a possible alternative 3’ mRNA end yielded the same poly-A signal as the clone identified in cDNA library screening. The total length of 3968 bp of the cloned cDNA is in good agreement with the size estimation of the human ALDR transcript in Northern blot analysis of 4.2 kb (Albet et al., 1997) if a variable length of the poly-A tail is considered. Our previous NCBI GenBank entry No. AJ000327 containing the human ALDR cDNA sequence was updated accordingly.

Human ALDRP is an exclusively peroxisomal protein

Indirect immunofluorescence double staining using human ALDR cDNA HA or Myc/hexahistidine epitope-tagged at the COOH-terminus showed that human ALDRP colocalized with catalase, a well-established peroxisomal marker enzyme (Fig. A1-1A and A1-1B). The exclusively peroxisomal localization of human ALDRP was expected in analogy to the mouse data available (Lombard-Platet et al., 1996) but had not been directly shown previously.

Characterization of a human pCYPAC-2 clone containing the complete ALDR gene

Three clones from a human genomic DNA pCYPAC-2 library were identified with an ALDR-specific cDNA probe (clone 1: LLNL704H03232Q13; clone 2: LLNL704K24206Q13; clone 3: LLNL704H12113Q16). Rescreening of these clones was performed by Southern blot analysis on EcoRI/HindIII/XbaI digests using the 3547 bp cDNA derived from the above-mentioned ALDR cDNA clone (ICRF P507D21235Q11) as probe (Fig. A1-2A). Clone No. 3 was found to contain additional ALDR-specific bands compared to the two others and was further characterized. Fluorescence in situ hybridization
analysis using this clone as probe showed a single signal on chromosome 12q12 (Fig. A1-2B). This locus has been previously reported for the human ALDR gene using a cDNA probe (Savary et al., 1997). Exon-exon PCR-analysis using oligonucleotide primers derived from the 5’ and 3’ cDNA ends revealed that pCYPAC-2 clone 3 (LLNL704H12113Q16) contained the entire ALDR gene including the 5’ flanking region containing the putative promoter.

**Figure A1-2: Characterization of three human ALDR pCYPAC clones.** A. Southern blot analysis of EcoRI, HindIII and XbaI digests using a human ALDR cDNA probe Clone No.3 contained more specific bands and was found to contain the entire ALDR gene. B. Fluorescence in situ hybridization analysis using clone No. 3 as probe. A single signal was obtained at chromosome 12q12.

**Striking similatrity of the genomic organization of the human ALDR gene with the ALD gene**

The complete exon-intron-structure could be determined by exon-exon PCR. All introns could be amplified using the pCYPAC-2 clone as template. All intron sizes were determined by agarose gel electrophoresis exon-exon PCR fragments in relation to standard size markers. The human ALDR gene contains 10 exons and covers approximately 33 kb of genomic DNA. Exon 1 contains the largest fragment of the protein coding sequence. The exon-intron structure of the human ALDR gene is presented in Fig. A1-3A. A comparison of the human ALD and ALDR genes is shown in Fig. A1-3B.
Computational analysis of the putative ALDR promoter region

A genomic DNA sequence of 2.4 kb of the putative promoter region upstream from the transcriptional start was analyzed. A TATA-Box (TATATTCT) was found 64 bp upstream of the transcriptional start defined by the longest RACE PCR product obtained. A repetitive element of the ALU-family is present from nt 1155-1401. Computer-aided search for potential binding sites to known regulatory factors using MatInspector (Quandt et al., 1995) revealed several Sp1 and AP1 sites. We searched for peroxisome proliferator responsive elements (PPRE), the cis-acting sequences thought to mediate a response to peroxisomal proliferators. The consensus PPRE sequence consists of two direct AGG(A/T)CA repeats separated by a single nucleotide (Dreyer et al., 1992; Green, 1993; Juge-Aubry et al., 1997). A survey of 2353 bp 5′ flanking DNA of the ALDR gene displayed no perfect matches for the consensus sequence or for the exact sequence of 16 known PPREs (Juge-Aubry et al., 1997). Several PPRE-like sequences, spaced by one nucleotide (DR+1), were observed. One PPRE-like sequence repeat with a four-nucleotide spacer (DR+4) was detected by the MatInspector program as an antisense-oriented thyroid hormone response element (TRE)-like binding site (Subauste & Koenig, 1995) combined with an orphan receptor binding site (Giguere et al., 1994) separated by four nucleotides (2529 - AGGTCG AATCAGGCCA - 2514). A similar sequence (AGGTCGAATGAGGTCA ; bp 1118 - 1133 in Genbank entry
No. AJ009991) is present 177 nucleotides upstream from the transcriptional start site of the mouse \textit{ALDR} gene. This element was not found in the known sequences of the mouse or human \textit{ALD}, mouse \textit{PMP70} or \textit{P70R} promoter regions. A detailed comparison of the mouse and human orthologs revealed a sequence (GTAGTAAACATGAACATT), which was also present in the upstream region of the mouse \textit{ALDR} gene (AAGAAAAACATGAAGAAA) and in antisense orientation in the 5' flanking DNA of the \textit{PMP70} gene (AAGAAAAACATGAAGAAA). This sequence shows no homology to any known PPRE or other described cis-acting elements but however is conserved between these three genes and is located at a comparable distance from the initiation codon: at 1162, 1122, and 1355 nucleotides in the human \textit{ALDR}, the mouse \textit{ALDR} and the mouse \textit{PMP70} gene respectively (Fig. A1-4B).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure_a1-4.png}
\caption{Computational analysis of the putative promoter region. A. Linear map demonstrating the transcriptional start (angled arrow), potential Sp1 binding sites (full circles), potential AP1 sites (open circles), the ORF (bold bar) and the motifs discussed. B. Identification of a conserved motif in the putative promoters of mouse \textit{ALDR} and \textit{PMP70} genes. Conservation of the central fraction (8 bp) of this motif at a similar distance from the start codon in the putative human \textit{ALDR} promoter.}
\end{figure}
**Discussion**

No cure is currently available for X-linked adrenoleukodystrophy. Dietary regimens have failed to revert the progressive nature of the disease (for review see Gärtn er et al., 1998c). Successful bone marrow transplants have been reported although the general risk is high and measures have to be started early in the course of the disease (Gärtn er et al., 1998c). Alternative therapeutic strategies are therefore under investigation. One such approach suggests the induction of ALDR gene since some functional redundancy to the ALD gene has been shown.

We have previously reported the sequence of a human ALDR cDNA fragment containing the complete coding region but lacking the full 5’ and 3’ ends. Here we report the full length cDNA including 556 bp of the 5’ untranslated and 1189 bp of the 3’ untranslated ends. The length of this cDNA sequence corresponds to the data from Northern blot analysis. Transcript variants (such as observed in the mouse) have not been detected. Although only 191 bp of the sequence corresponding to the 557 bp human 5’ UTR are transcribed in mouse (Fig. A1-4A) the entire 557 bp are highly conserved between human and mouse (72.8% bp identity under the permission of 27 gaps). There are eight start codons within the human ALDR 5’ untranslated region, however all of them are closely followed by a termination codon. Most of these ATG codons are in a very week initiation context with the exception of one ATG at the position 2809-2811. This start codon has a G residue following the ATG codon and a purine, A, three nucleotides upstream (Kozak, 1991). In theory, this upstream translation initiation site that runs into a terminator codon after six amino acids, thereby creating a small upstream ORF, should reduce but not abolish translation of the major downstream ORF (Abastado et al., 1991). Interestingly, an expression construct containing 135 bp upstream from the ORF (including the ATG codon at position 2809-2811) is effectively translated as determined by immunofluorescence and western blot analysis and is able to restore β-oxidation in X-ALD fibroblasts (Berger et al., unpublished data). A regulatory effect of this ATG codon on translation efficiency seems very likely.

The human ALDR protein is localized exclusively in the peroxisome as shown by immunocytological analysis. It belongs thus to the group of peroxisomal ABC-half-transporters as has been deduced from mouse data and sequence similarity.
The genomic organization of the human *ALDR* gene shows striking similarity to the structure of the *ALD* gene, its closest homolog (Fig. A1-3B). This is additional evidence for a recent divergence of these two genes from a common ancestor. No apparent gene structure similarity with the remaining peroxisomal ABC-transporters (PMP70, PMP69) has been observed. The mirror expression of ALDP and ALDRP (Troffer-Charlier *et al.*, 1998) i.e. tissues with high ALDP expression are low in ALDRP (and vice versa) has been interpreted as ALDP and ALDRP serving the same or very similar functions in different tissues. Kemp *et al.* (Kemp *et al.*, 1998) have recently demonstrated that the β-oxidation defect in X-ALD can be corrected by transfection of *ALDR* cDNA or induction of *ALDR* transcription. The authors concluded that there must be redundancy of the function of peroxisomal ABC-transporters. Since X-ALD has a wide spectrum of clinical manifestations - even within a family with identical ALD phenotype - the existence of modifying genetic factors independent of the *ALD* gene has long be postulated. The data on suspected functional redundancy render this hypothesis attractive and the *ALDR* gene is a plausible candidate for such a modifier determining (together with an obligate defect in the *ALD* gene) the clinical course of X-ALD. Since ALDRP is predominantly expressed in the brain sequence analysis in X-ALD patients without the knowledge of the gene structure is difficult and has not been undertaken to date. The sequence data presented here will allow the search for polymorphisms in the *ALDR* gene using genomic DNA as starting material within the complete coding region as well as the 5´and 3´ untranslated regions of the mRNA and intronic sequence.

The fact that *ALDR* transcription can be upregulated by 4-phenylbutyrate leading to a correction of β-oxidation in human X-ALD fibroblasts (Kemp *et al.*, 1998) has immediate therapeutic implications. The induction of a „surrogate gene“ of overalappping or identical function is a principle of therapy used in diseases such as hemoglobinopathies (Dover *et al.*, 1994; Olivieri *et al.*, 1998). The mechanism of action of 4-phenylbutyrate on the transcription of the *ALDR* gene is not well understood. In general, regulation of gene expression by butyrate is thought to function via inhibition of the enzyme histone deacetylase, leading to elevated levels of core histone acetylation which affect chromatin structure and transcription rates (Candido *et al.*, 1978). The transcriptional start defined by 5´ RACE-procedures corresponds to the size of the mRNA in Northern analysis and was also confirmed by cDNA amplification across intron 1. As a consequence the thyroid hormone response element (TRE)-like binding site identified is part of the transcript. mRNA
arizing from a further downstream transcriptional start might occur, although a single signal corresponding to the upstream start is obtained in Northern analysis. The central 8 bp of a 18-bp motif conserved in mouse ALDR and PMP70 genes (within otherwise non-homologous regions) are conserved in the human ALDR promoter (Fig. A1-4B) at a similar distance from the initiation codon. One might speculate this sequence to play a role in the regulation of peroxisomal ABC-transporters in general. Further investigations in particular electrophoretic mobility shift assays will show if this element is in fact a protein binding regulatory element. The putative promoter sequence reported here will allow specific studies on transcriptional regulation of the ALDR gene including testing of substances potentially suitable for induction. Furthermore a decrease in ALDR transcription due to a polymorphism in the promoter region could possibly explain the clinical heterogeneity of X-ALD. In this context the sequence data presented represent tools to investigate the role of the ALDR gene in X-ALD.

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References

The references are listed on pages 38–49.
Appendix 2

Human Adrenoleukodystrophy Protein and Related Peroxisomal ABC Transporters Interact with the Peroxisomal Assembly Protein PEX19p

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Abstract

Four ABC half transporters (ALDP, ALDRP, PMP70, and PMP69) have been identified in the mammalian peroxisomal membrane but no function has been unambiguously assigned to any of them. To date X-linked adrenoleukodystrophy (X-ALD) is the only human disease known to result from a defect of one of these ABC-transporters, ALDP. Using the yeast two-hybrid system and in vitro GST pull down assays we identified the peroxin PEX19p as a novel interactor of ALDP, ALDRP and PMP70. The cytosolic farnesylated protein PEX19p was previously shown to be involved in an early step of the peroxisomal biogenesis. The PEX19p interaction occurs in an internal N-terminal region of ALDP which we verified to be important for proper peroxisomal targeting of this protein. Farnesylated wildtype PEX19p and a farnesylation-deficient mutant PEX19p did not differ in their ability to bind to ALDP. Our data provide evidence that PEX19p is a cytosolic acceptor-protein for the peroxisomal ABC transporters ALDP, PMP70 and ALDRP and might be involved in the intracellular sorting and trafficking of these proteins to the peroxisomal membrane.
Introduction

In higher organisms proper function and assembly of peroxisomes is of vital importance as evidenced by a variety of severe human diseases caused by malfunction of these organelles (Lazarow & Moser, 1995). These include defects in many genes required for peroxisome biogenesis, termed PEX genes (Distel et al., 1996; Hettema et al., 1999). Peroxins, the proteins encoded by PEX genes, are responsible for the evolutionarily highly conserved process of post-translational trafficking of proteins to the peroxisomal membrane or into the peroxisomal matrix (Hettema et al., 1999; Subramani, 1998). The most common peroxisomal disorder is X-linked adrenoleukodystrophy (X-ALD, OMIM #300100), causing severe neurodegenerative disease with a broad spectrum of clinical manifestations (Moser et al., 1995b). Biochemical hallmarks of the disease are increased tissue and plasma concentrations of very-long-chain fatty acids (VLCFA; C > 22:0) (Lazo et al., 1989; Moser et al., 1995b). It had been generally accepted that accumulation of VLCFA in X-ALD is due to an impaired activity of very-long-chain acyl-CoA synthetase (VLCS) required for the activation of VLCFAs to their CoA thioesters (Steinberg et al., 1999a; Steinberg et al., 1999b; Watkins et al., 1998). Surprisingly, it turned out that not defects in VLCS, but mutations in the ALD gene, encoding an integral peroxisomal membrane protein (ALDP), are undoubtedly the primary genetic cause of this disease (Contreras et al., 1994; Mosser et al., 1994). ALDP belongs to the superfamily of ATP binding cassette (ABC) transporters capable of transporting a wide variety of ligands, ranging from ions to proteins, across biological membranes. In eukaryotic organisms ABC transporters typically consist of two hydrophobic transmembrane domains and two hydrophilic domains each containing a nucleotide binding fold (Klein et al., 1999). ALDP, however, is a half transporter composed of only one transmembrane domain and one nucleotide binding fold and has to form homo- or heterodimers to be functional. In humans, three other closely related peroxisomal ABC half transporters, ALDRP (ALD-related protein), PMP70 (PXMP1) and PMP69 (PXMP1-L) have been identified. (Gärtner et al., 1998a; Holzinger et al., 1997a; Kamijo et al., 1990; Shani et al., 1997). These may act as interaction or dimerization partners for ALDP (Shani & Valle, 1996; Smith et al., 1999). Using the yeast two-hybrid system it has recently been demonstrated that homo- as well as heterodimerization can occur between the carboxy-terminal halves of ALDP, ALDRP, and PMP70 (Liu et al., 1999a). It has also been suggested that a loss of ALDP dimerization plays a role in the pathogenesis of X-ALD. Nevertheless, it is unclear, how a malfunction of ALDP might be correlated to the accompanying deficient
activity of VLCS being responsible for VLCFA accumulation (Smith et al., 1999) (Kemp et al., 1998; Smith et al., 2000). Moreover, the factors that mediate trafficking and targeting of ALDP have not been investigated yet. Imanaka and co-workers had shown, that the related peroxisomal ABC half transporter PMP70 is translated in the cytosol and transported to the peroxisome by cytosolic factors using \textit{in vitro} targeting experiments (Imanaka et al., 1996). Very recent evidence suggests that the peroxin PEX19p might act as a cytosolic binding protein for PMP70 (Sacksteder et al., 2000). The observation that approximately 70% of the X-ALD mutations result in loss of ALDP immunoreactivity (Smith et al., 1999; Watkins et al., 1995) is consistent with the hypothesis that ALDP, once translated in the cytosol, needs further protein interaction to be stabilized and properly targeted to the peroxisome.

In order to further elucidate the targeting and interaction network of peroxisomal ABC half transporters, we screened for interaction partners using the yeast two-hybrid system. For that purpose, defined portions of ALDRP were tested against a cDNA library derived from human brain tissue. We provide evidence that internal N-terminal domains of ALDP, ALDRP and PMP70 strongly interact with human PEX19p, a 33kD farnesylated protein known to be involved in early steps of the peroxisomal biogenesis (Götte et al., 1998). These interactions were confirmed in \textit{in vitro} pull-down assays. As the interaction of PEX19p occurs in a region which is important for the targeting of ALDP, our results are consistent with the idea that PEX19p is an acceptor protein for ALDP and related peroxisomal ABC transporters and might be involved in their trafficking to the peroxisomal membrane.
Material and Methods

Yeast two-hybrid screen and interaction assay
PCR using standard protocols was performed to obtain specifically selected cDNA fragments. PCR primers contained appropriate restriction sites to achieve in-frame ligation to the LexA gene. Additionally, a spacer of 3 glycine codons was inserted between LexA and the fragment of interest. The reverse primer contained a termination codon. Produced PCR fragments were restriction digested and cloned into the pEG202 vector. The correctness of the constructs was confirmed by DNA sequencing. Several constructs (ALDRP1-218, ALDRP181-297, ALDRP273-489, and ALDRP365-741) were then screened against a human brain tissue cDNA library in pJG4-5 vector (CLONTech #HL4500AK). Before being used in the library screen, baits were tested in repression assays for their ability to enter the nucleus (Russell et al., 1996). The library screen was performed in the yeast strain EGY48 (Matα, trp1, his3, ura3, (lexAop)6-Leu2). Yeast transformants were selected for leucine auxotrophy (Leu2-reporter) on galactose/raffinose drop-out medium lacking leucine and for activation of β-galactosidase (lacZ-reporter). Yeast clones containing interacting proteins were identified by growth on galactose/raffinose-medium without leucine and blue color development on galactose/raffinose-X-Gal-medium. pJG4-5 plasmids of positive clones were rescued, amplified in E.coli (strain XL1-Blue MRF’), and sequenced. Determined sequences were analyzed by performing a BLAST search (Benson et al., 1999). The LexA yeast two-hybrid system (interaction trap) was kindly provided by R. Brent (Massachusetts General Hospital). Assay procedures and appropriate controls are described elsewhere (Russell et al., 1996). Using a yeast two-hybrid interaction assay, human peroxisomal ABC transporters were then tested for interaction with PEX19p. For this purpose constructs of the N-terminal region of ALDP, ALDRP, and PMP70 were cloned into the pEG202 vector and checked for interaction with PEX19p (clone pJG4-5-PEX19pΔ6) as described above.

Expression and purification of GST-fusion proteins
For in vitro binding assays the PEX19p and a CaaX-Box-mutated PEX19p (C296S-PEX19p) were cloned into the pGEXΔBamHI expression plasmid for the production of glutathione-S-transferase (GST) fusion-proteins (Leenders et al., 1996). Full length human PEX19p cDNA (GenBank #NM_002857) was recently cloned in our lab and the mutations in the CaaX motif were introduced by PCR (Kammerer et al., 1997). Expression in the E.coli strain (BL21-DE3) and affinity purification of GST-PEX19p and GST-C296S-PEX19p fusion-proteins were performed as described previously (Braun et al., 1994; Kammerer et al., 1997).

In vitro farnesylation of PEX19p at the C-terminal CaaX-box
6 µg of purified GST-PEX19p or GST-C296S-PEX19p were incubated with 25 µl TNT rabbit reticulocyte lysate supplying the essential CaaX farnesyltransferase, 2 µl reaction buffer (TNT Coupled Reticulocyte Lysate System, Promega) and 2 µl farnesyl pyrophosphate (10mM, Sigma) in a total volume of 50 µl for 2h at 30°C (Kammerer et al., 1997). Both the farnesylated wild type protein and the farnesyl-deficient C296S mutant protein were bound to GSH-Sepharose beads and used in a GST pull-down assay as described below.

In vitro translation and 35S-methionine labeling of peroxisomal ABC transporter proteins
For in vitro translation and labeling, full length ALDP, ALDRP, and PMP70, and several N-terminally truncated constructs of ALDP (Δ66, Δ186 and Δ281) were cloned into pcDNA3.1 plasmid (Invitrogen). The proteins were transcribed in vitro from the T7-promotor of pcDNA3.1, translated and labeled with 35S-methionine (Amersham) using the TNT Coupled Reticulocyte Lysate System (Promega) following standard protocols.

In vitro interaction assay (GST pull-down)
The purified GST fusion proteins were bound to glutathione-Sepharose beads (Pharmacia) in PBS for 1h at 4 °C and washed three times in PBS. For the in vitro interaction assay the GST-fusion proteins (0.5-1.5 µg protein bound to 1 µl bead suspension) were incubated with 5 µl of in vitro translated protein in 200 µl binding buffer (100 mM NaCl, 50 mM potassium phosphate pH 7.4, 1 mM MgCl2, 10 % glycerol, 0.1 % Tween 20, 1.5 % BSA) for 2h at 4 °C. Samples were pelleted and washed four times in 1 ml binding buffer without BSA. Pellets were resuspended in SDS-sample buffer and boiled for 5 min before being analyzed by SDS-PAGE according to standard protocols (Laemmli, 1970). To verify stability of the GST-fusion proteins and equal loading, gels were stained with Coomassie blue. The gels were dried and exposed to X-ray film for 24 h.

Transfection and analysis of ALDP- GFP fusion proteins
For in vivo targeting studies deletion constructs of ALDP were fused to the N-terminus of the green fluorescent protein (GFP). For this purpose ALDP1-281 and Δ281 ALDP were cloned into pEGFPN1 vector (CLONTech). Plasmids were transfected into Cos7 cells using lipofectamine
(Life Technologies) following protocols provided by the manufacturer. After 48 h transfected cells were fixed with 3% formalin/PBS for 30 min and permeabilized with 1% Triton X-100 for 5 min. Catalase was used as marker for peroxisomes. Cells were incubated with a 1:100 dilution of a polyclonal rabbit antibody against catalase (Biodesign) for 1 hour. The cells were then washed thoroughly with PBS and incubated with a 1:50 dilution of the secondary tetrarhodamine isothiocyanate (TRITC)-labeled anti mouse IgG antibody (Jackson Immunoresearch Laboratory Inc.) for 1 hour. The cells were embedded in an anti-fading reagent (Vectashield, Vector Laboratories Inc.). Fluorescence microscopy was performed using an Axiovert 135 inverted fluorescence microscope (Zeiss).

Figure A2-1: Interaction of peroxisomal ABC transporters with PEX19p in the yeast two-hybrid assay. A scheme of peroxisomal ABC half-transporter structure with the six transmembrane helices (TM) and the nuclear binding fold (NBF) is represented on the top. The N-terminal regions marked by the bar in the scheme were fused to LexA and tested against PEX19p (clone PEX19pΔ6) for interaction in a yeast two-hybrid assay (bottom). Panel 1 presents the Leu2-reporter activity (growth on galactose/raffinose media without leucine), panel 2 the negative control on glucose media without leucine, where the Leu2-reporter is inhibited. Panel 3 presents the color development caused by LacZ-reporter activity for yeasts plated on galactose/raffinose-X-Gal-medium.
Results

Identification of PEX19p as an interactor of ALD-related protein

To identify proteins, which interact with peroxisomal ABC transporters, we performed a cDNA library screen using the LexA yeast two-hybrid system with parts of ALD-related protein (ALDRP), recently cloned in our lab, as a bait (Holzinger et al., 1997b).

Prior to the library screen we verified if baits were able to enter the nucleus. Generally for large proteins and especially for complex membrane proteins a separation into several smaller baits (not bigger than 60 kDa) is necessary, probably because of impairment of nuclear import by molecular mass or by hydrophobic domains. ALDRP constructs corresponding to amino acids 1-218, 181-297, 273-489 and 365-741 (ALDRP1-218, ALDRP181-297, ALDRP273-489, and ALDRP365-741, respectively) were imported into the nucleus (data not shown).

A cDNA library from human brain tissue (Clontech) was screened with all constructs. With the N-terminal construct containing the first 218 amino acids of ALDRP (ALDRP1-218) we obtained a strong interaction with human PEX19p (data not shown). The cDNA of the isolated clone (PEX19pΔ6) encompassed nearly the complete coding sequence (missing only the first 6 amino acids out of 299) of PEX19p. The interaction between ALDRP and PEX19p was confirmed in vitro using the GST pull-down assay as described later on.

PEX19p interacts with the human peroxisomal ABC transporters ALDP, ALDRP and PMP70 in a yeast two-hybrid assay

In order to test if the closely related ABC transporters ALDP and PMP70 also interact with PEX19p as initially established for the N-terminal domain of ALDRP, a yeast two-hybrid interaction assay was performed. Therefore, the regions of ALDP and PMP70, that are homologous to the N-terminal part of ALDRP (ALDRP1-218), were cloned into the pEG202 vector (ALDP1-203 and PMP70 1-199) and used as a bait against the clone PEX19pΔ6 in the pJG4-5 vector. The ALDP and PMP70 constructs comprise, similar to ALDRP, the N-terminal region including the first two transmembrane helices but not the third and further C-terminal domains (Fig A2-1. top). Like ALDRP, both, ALDP and PMP70 interacted strongly with PEX19p. These interactions activated the LEU2 gene allowing a growth on media lacking leucine (Fig. A2-1, panel 1). The activation of LEU2 is due to a PEX19p interaction with ALDP, ALDRP or PMP70, because suppression of PEX19p expression by glucose hampers yeast growth (Fig. A2-1, panel 2). The strength of the interaction is illustrated by
blue color development within 24h due to the activation of LacZ gene when grown on X-Gal media (FIG. A2-1, panel 3).

Figure A2-2: Mapping of the PEX19p interaction domain on ALDP using a GST pull-down. A. Scheme of ALDP-constructs: A full-length (fl.) construct and the deletion constructs Δ66, Δ186 and Δ286 were generated. They were 35S-methionine labeled by *in vitro* translation using a rabbit reticulocyte lysate and tested for interaction with GST-PEX19p in a pull-down assay. The GST-PEX19p co-purified proteins were separated on 12% SDS polyacrylamide gel electrophoresis. B. Autoradiography of the gel: On the left, 20% of the total amount of the 35S-labeled proteins (input) used in the GST pull-down was loaded and illustrates the molecular masses of these proteins (left part). The labeled proteins that were co-purified with GST-PEX19p are depicted on the right. Lower molecular mass bands might correspond to alternative internal start codons or proteolytic cleavage of the *in vitro* translated proteins. No signal was obtained for a GST negative control (see Fig. A2-4).
Mapping of the PEX19p interaction domain of ALDP

To validate the observation that segments within the N-terminal domain of the peroxisomal ABC transporters ALDP, ALDRP and PMP70 are sufficient for interaction with PEX19p, we performed an *in vitro* interaction assay (GST pull-down) using ALDP as our model system. Full-length PEX19p was expressed as GST fusion protein and bound to glutathione-sepharose beads. Several deletion constructs of ALDP lacking the first 66, 186, and 281 amino acids (∆66, ∆186, and ∆281) were 35S labeled by *in vitro* translation (Fig. A2-2A) and subjected to a GST-PEX19p pull-down assay. Full-length and ∆66 ALDP constructs showed interaction with PEX19p. Deletion constructs ∆186 and ∆281, however, did not interact with PEX19p since at the same level of input only faint signals were observed (Fig. A2-2B). These results show that the binding site of PEX19p lies in a region of about 120 amino acid residues (between amino acid 67 and 186) within the N-terminal domain.

*In vivo* targeting studies of ALDP

To test the hypothesis that the region of ALDP interacting with PEX19p might be involved in the intracellular trafficking of ALDP deletion constructs were tested for their targeting to peroxisomes. For that purpose an ALDP-deletion construct (∆281 ALDP) comprising a region which did not interact with PEX19p in the pull-down assay (Fig. A2-2) was compared to a construct containing amino acids 1-281 of ALDP in its cellular localization. The proteins were labeled by N-terminal fusion to the green fluorescent protein and expressed in Cos7 cell culture. The peroxisomal localization of the constructs was determined by the co-localization of catalase, detected by immunofluorescence staining. ALDP1-281 shows a proper peroxisomal localization while ∆281 ALDP exhibits a non-peroxisomal pattern (Fig. A2-3).
**Figure A2-3: Targeting of ALDP-GFP fusion protein constructs** The cellular distribution of \(\Delta 281\) ALDP-GFP, missing the N-terminal amino acids 1-281 (A), and ALDP1-281-GFP, coding for the N-terminal amino acids 1-281 only (C), is compared with the localization of catalase detected by immunofluorescence staining (B, D) in Cos7 cells. ALDP1-281-GFP is co-localizing with catalase. \(\Delta 281\) ALDP-GFP shows a diffuse cytosolic pattern with some spots that do not co-localize with catalase.

**Farnesylation is not necessary for the in vitro interaction of PEX19p with peroxisomal ABC transporters**

Farnesylation of a C-terminal CaaX-motif of PEX19p (CLIM) was shown to be important for its biological function (Götte et al., 1998; Matsuzono et al., 1999). To test the influence of the farnesylation of PEX19p on its interaction with peroxisomal ABC transporters, a CaaX-box mutant of PEX19p (C296S-PEX19p) was compared with wildtype PEX19p in a GST pull-down after in vitro farnesylation. As shown previously, C296S-PEX19p can not be farnesylated in vitro (Kammerer et al., 1997). To conduct the assay, equal amounts of GST-PEX19p and GST-C296S-PEX19p were incubated in rabbit reticulocyte lysate under conditions that ensure attachment of the farnesyl group at the cysteine residue of the CLIM motif of PEX19p (FIG. A2-4A). Both, wild type farnesylated and mutated farnesyl-deficient protein were then tested for interaction with in vitro translated and \(^{35}\)S-labeled full-length ALDP, ALDRP and PMP70. The mutant PEX19p (C296S-PEX19p) shows no significantly different binding capacity to the peroxisomal ABC-transporters in vitro compared to wild type PEX19p (Fig. A2-4b)
Figure A2-4: Effect of *in vitro* farnesylation of PEX19p on its interaction with the peroxisomal ABC-transporters. A. Scheme of C-terminal farnesylation motif (CaaX-box) of PEX19p. Single letter notation of the consensus sequence: C- cysteine, a aliphatic amino acid, X any amino acid. A PEX19p wildtype (wt) and a CaaX-box mutated (C296S) PEX19p were expressed as GST-fusion proteins and subjected to an *in vitro* farnesylation assay as described in Methods. Farnesylated wt PEX19p and farnesyl-deficient C296S mutated PEX19p were tested for interaction with *in vitro* translated and 35S-labeled full-length ALDP, ALDRP and PMP70 in a GST pull-down. Co-purified proteins were separated on 12% SDS polyacrylamid gelelectrophoresis. B. Autoradiography: 20% of the total amount of the 35S-labeled ABC transporter proteins (input) used in the GST pull-down is shown on the left. Retention of labeled protein with GST-PEX19p and GST-C296S-PEX19p, respectively, is depicted on the right. GST only was used as a negative control. Lower molecular mass bands might correspond to alternative internal start codons or proteolytic cleavage of the *in vitro* translated proteins.
Discussion

In humans, four ABC half transporters have been localized to the peroxisomal membrane but no precise function has been unequivocally assigned to any of them. X-ALD is the only human disease known so far that results from mutations in the ALD gene. It is, however, unclear how a defective transport function of ALDP might be related to the abnormality in fatty acid metabolism observed in X-ALD as a biochemical hallmark (Kemp et al., 1998; Smith et al., 2000). One approach to unravel protein functions is to elucidate the cellular network by identifying protein interaction partners. The yeast two-hybrid system has previously been used to show that peroxisomal ABC transporters can form either homodimers or heterodimers with one of the related ABC half transporters (Liu et al., 1999a; Verleur et al., 1997). In this study the aim was to identify ALDP interaction partners beyond those required for dimerization.

Using a yeast two-hybrid screen we initially identified a strong interaction of ALDRP with a small protein first cloned and described as the human peroxisomal farnesylated protein PxF (Kammerer et al., 1997). PxF is, typical for housekeeping genes, ubiquitously expressed (Braun et al., 1994). Since PxF has subsequently been shown to be crucial for peroxisomal biogenesis (Götte et al., 1998) it is now designated PEX19p. By performing yeast two-hybrid and in vitro interaction assays we confirmed that internal N-terminal parts of ALDP, ALDRP and PMP70 directly interact with PEX19p. These findings also suggest a functional relationship between the processes of peroxisome assembly driven by peroxins such as PEX19p and those of peroxisomal membrane transport accomplished by peroxisomal ABC transporters. The cellular site of interaction seems to be outside of the peroxisome since PEX19p appears to be mainly located in the cytosol (Sacksteder et al., 2000), although immunocytochemical evidence suggests that it can also associate with the peroxisomal membrane (James et al., 1994; Kammerer et al., 1997). Very recently Pex19p has been shown to interact with a broad range of integral peroxisomal membrane proteins, but not matrix proteins (Sacksteder et al., 2000; Snyder et al., 1999b). Such data have implicated that PEX19p may act as a broad specificity cellular chaperone for newly synthesized PMPs. Recently, an inactivating mutation in PEX19 has been described that results in a complete lack of even residual peroxisomal membrane vesicles (“ghosts”) in fibroblasts of a patient with Zellweger syndrome (Matsuzono et al., 1999). Since ghosts are usually identified by anti-PMP70 antibodies, these findings indirectly demonstrate that a lack of the PEX19p
acceptor site prevents PMP70 to reach its designated location within peroxisomal vesicular structures. Lack of interaction may thereby result in mislocalization and degradation of newly synthesized PMP70 and other PMPs (Sacksteder et al., 2000).

We show here that PEX19p is interacting with ALDP and ALDRP which might contribute to the pathogenesis of X-ALD, the most common peroxisomal disorder. By using N-terminal constructs of ALDP we were able to delimit the interaction domain to an N-terminal stretch of ALDP comprising amino acid residues 67-186. The precise location of the specific interaction epitope was beyond the scope of this study since this would require experimental strategies delivering much higher resolution. Nonetheless, our in vivo targeting studies with ALDP deletion constructs suggest that the PEX19p interaction domain coincides with the region of ALDP required for targeting to the peroxisome. The data are consistent with the hypothesis that PEX19p may bind to a targeting element of ALDP thereby facilitating the intracellular trafficking of the transporter protein to the peroxisomal membrane.

In this context it is noteworthy that PEX19p is the only peroxin that can be farnesylated at a carboxyl-terminal CaaX motif (Kammerer et al., 1997). The covalent attachment of prenyl lipids, i.e. farnesyl groups or geranylgeranyl groups, by specific transferases has been shown to be indispensable for the intracellular sorting and membrane association of some proteins (Clarke, 1992; Hancock et al., 1989; Kato et al., 1992a; Tamanoi, 1993). Thus, we investigated whether in vitro farnesylation of PEX19p might influence its interaction with peroxisomal ABC transporters. Our findings were that a CaaX-box mutated and therefore farnesyl-deficient PEX19p (C296S-PEX19p) can bind peroxisomal ABC transporters as well as wildtype PEX19p. Though PEX19p farnesylation may not be a prerequisite for its in vitro interaction with peroxisomal ABC transporters it has been suggested to be essential for its overall biological action in vivo, e.g. in rescuing impaired peroxisomal assembly of PEX19p deficient fibroblasts (Matsuzono et al., 1999). Moreover, in yeast the interaction of Pex19p with the cytosolic domain of Pex3p appears to be influenced in a farnesyl-dependent manner (Götte et al., 1998; Snyder et al., 1999a). The post-translational modification of PEX19p by farnesylation might thereby provide an additional regulatory mechanism to ensure proper sorting and trafficking of PMPs (Hettema et al., 2000).

Our results might have implications in several ways. They may add to the understanding of the pathogenesis and phenotypic heterogeneity of X-ALD by demonstrating a mechanism by which newly synthesized ALDP may either be stabilized and/or translocated to the peroxisomal membrane. So far it is not clear why a surprisingly high percentage of missense mutations in the \textit{ALD} gene is resulting in a loss of ALDP immunoreactivity (Smith et al.,
APPENDIX 2: Peroxisomal ABC Transporters Interact with PEX19

1999; Watkins et al., 1995). Recent data suggest that ALDP instability may be a direct consequence of deficiency of dimerization that resides in the C-terminus of ALDP (Liu et al., 1999a). The fact that interaction with PEX19p occurs within an internal N-terminal domain of ALDP implies that a much wider range of ALD mutations could potentially result in ALDP degradation as a consequence of “interaction deficiencies”. Additional interaction domains of ALDP need to be postulated in order to explain the influence of ALDP mutations on peroxisomal ß-oxidation at the level of the VLCS protein (Smith et al., 2000; Steinberg et al., 1999a). To date we and others also have not yet obtained conclusive data whether PMP69 is sharing interaction properties similar to the three other members of the peroxisomal ABC transporter family. These questions clearly need to be addressed for further understanding of the complex picture of X-ALD pathogenesis.

The demonstration of in vitro interaction of peroxisomal ABC transporters with PEX19p also supports the idea of direct functional interaction between peroxins and other peroxisomal membrane components during peroxisome maturation. Another indirect hint for this kind of interaction is provided by the observation that a PEX2p deficiency can be restored by overexpression of peroxisomal ABC transporters (Braiterman et al., 1998; Gärtner et al., 1998b). The identification of PEX19p as an acceptor molecule for ALDP suggests that PEX19p may modulate the function of some tissues afflicted by ALD mutations and could thereby influence clinical disease manifestation.

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References

The references are listed on pages 38–49.
Appendix 3

Two Splice Variants of Human PEX19 Exhibit Distinct Functions in Peroxisomal Assembly

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Abstract

PEX19 has been shown to play a central role in the early steps of peroxisomal membrane synthesis. Computational database analysis of the PEX19 sequence revealed three different conserved domains: D1 (aa 1-87), D2 (aa 88-272), and D3 (aa 273-299). However, these domains have not yet been linked to specific biological functions. We elected to functionally characterize the proteins derived from two naturally occurring PEX19 splice variants: PEX19∆E2 lacking the N-terminal domain D1 and PEX19∆E8 lacking the domain D3. Both interact with peroxisomal ABC transporters (ALDP, ALDRP, PMP70) and with full-length PEX3 as shown by in vitro protein interaction studies. PEX19∆E8 also interacts with a PEX3 protein lacking the peroxisomal targeting region located at the N-terminus (∆66aaPEX3), whereas PEX19∆E2 does not. Functional complementation studies in PEX19-deficient human fibroblasts revealed that transfection of PEX19∆E8-cDNA leads to restoration of both peroxisomal membranes and of functional peroxisomes, whereas transfection of PEX19∆E2-cDNA does not restore peroxisomal biogenesis. Human PEX19 is partly farnesylated in vitro and in vivo. The farnesylation consensus motif CLIM is located in the PEX19 domain D3. The finding that the protein derived from the splice variant lacking D3 is able to interact with several peroxisomal membrane proteins and to restore peroxisomal biogenesis challenges the previous assumption that farnesylation of PEX19 is essential for its biological functionality. The data presented demonstrate a considerable functional diversity of the proteins encoded by two PEX19 splice variants and thereby provide first experimental evidence for specific biological functions of the different predicted domains of the PEX19 protein.
Introduction

The biogenesis of peroxisomes (reviewed by (Purdue & Lazarow, 2001; Subramani, 1998; Titorenko & Rachubinski, 2001a) is a complex process including the biosynthesis of the peroxisomal membrane, the targeting and insertion of peroxisomal membrane proteins (PMPs) into this membrane, and the import of peroxisomal matrix enzymes across the bilayer into the peroxisomal lumen. The proteins involved are termed “peroxins” and are encoded by at least 23 different PEX genes. In most cases, human PEX mutants display defects in the import of peroxisomal matrix proteins whereas the synthesis of some peroxisomal membrane vesicles and the insertion of PMPs into these membrane remnants (“peroxisomal ghosts”) remain unaffected. Only mutants in PEX3, PEX16, and PEX19 appear to lack even such peroxisomal ghosts (Matsuzono et al., 1999; Muntau et al., 2000a; South & Gould, 1999), implicating a crucial role of these proteins in the very early stage of peroxisomal membrane synthesis.

Among these PEX19 is the only peroxin known to own a C-terminal CAAX-box, thereby harboring the intrinsic property of being modified by posttranslational farnesylation. In human, PEX19 is in part farnesylated in vivo and it has been suggested that farnesylation is required for the peroxisomal localization of PEX19 (Matsuzono et al., 1999). Whether farnesylation is essential for the function of PEX19 in peroxisomal biogenesis (Matsuzono et al., 1999) or has only an ancillary effect (Sacksteder et al., 2000) remains unclear to date. PEX19 appears to be bimodally distributed between the cytoplasm and the peroxisomes, with most of the protein in the cytoplasm (Sacksteder et al., 2000). PEX19 protein has been shown to bind a wide variety of peroxisomal membrane proteins including peroxins and peroxisomal ABC transporters (Fransen et al., 2001; Gloeckner et al., 2000; Götte et al., 1998; Sacksteder et al., 2000; Snyder et al., 2000; Snyder et al., 1999a; Snyder et al., 1999b). Among these, PEX19 has been shown to interact with PEX3, another key component in early peroxisomal membrane synthesis, in yeast (Götte et al., 1998) and human (Muntau et al., 2000a; Soukupova et al., 1999; South & Gould, 1999). In yeast, this particular protein-protein interplay appears to be a prerequisite for the generation of preperoxisomal structures that can acquire and stabilize newly synthesized PMPs (Hettema et al., 2000). PEX19 harbors distinct binding sites for PEX3 and PEX16 (Fransen et al., 2001). However, these sites have not yet been linked to a specific protein domain organization of PEX19.

We have previously cloned and characterized the human PEX19 gene, which is composed of eight exons (Kammerer et al., 1997). On the transcriptional level, we identified different
PEX19 splice variants (Kammerer et al., 1997). The unspliced variant PEX19all encodes for the full-length PEX19 protein, whereas the variants PEX19ΔE2 and PEX19pΔE8 are expected to lack N- or C-terminal parts of the protein.

Tissue- or cell-specific expression of splice variants has been reported for many genes, e.g. for genes encoding cytokines or tyrosine kinases. Functional diversities of these variants, like distinct protein-protein interactions, are known (Atamas, 1997; Strehler & Zacharias, 2001; Xiong et al., 1998). For PEX19, the splice variant PEX19all has been described to be the predominant transcript in mRNA of several cells and tissues in human, although the variant PEX19ΔE2 has also been reported to account for a significant level of total human PEX19 mRNA. Interestingly, the ratio between PEX19all and PEX19ΔE2 expression was completely reversed in uterine mRNA prepared from total human uterine tissue (Kammerer et al., 1997), but a possible biological impact of these findings remained still unclear. Therefore, the characterization of PEX19 splice-variants as to their ability to interact with other peroxisomal proteins and to initiate early peroxisomal membrane synthesis could provide insights into the potential biological relevance of these PEX19 splice variants. Furthermore, the definition of functional regions of PEX19 by the characterization of truncated PEX19 splice variants could contribute to the knowledge on specific protein domains within PEX19.
Material and Methods

Computational analysis
Database searches for the prediction of the protein domain structure of human PEX19 were performed using the NCBI-BLASTP 2.2.1 program (Altschul et al., 1997) and the ProDom 2001.2 database (http://prodes.toulouse.inra.fr/prodom/doc/prodom.html).

Eukaryotic expression plasmids
Plasmids expressing full-length ALDP, ALDRP, and PMP70 have been previously described (Gloeckner et al., 2000). The plasmids pcDNA3/PEX19all, pcDNA3/PEX19ΔE2, and pcDNA3/PEX19pΔE8 encoding different PEX19 splice variants have been generated by Kammerer et al. (Kammerer et al., 1997). The plasmid encoding the first 40 amino acids of PEX3 fused to the green fluorescent protein (GFP) has been described previously (Kammerer et al., 1998). The N-terminal deletion construct missing the first 66 amino acids of PEX3 was generated by Muntau et al. (Muntau et al., 2000a).

In vitro protein-protein interaction (GST-pull-down) assay
For in vitro translation and labeling pcDNA3 (Invitrogen) derived expression plasmids were used. The proteins were transcribed in vitro from the T7-promotor of pcDNA3, translated and labeled with [35S]-methionine (Amersham) using the TNT Coupled Reticulocyte Lysate System (Promega) following standard protocols. For the generation of glutathione-S-transferase (GST) fusion proteins, different cDNAs of human PEX19 splice variants were subcloned into the prokaryotic expression plasmid pGEX-3X (Pharmacia) as described elsewhere (Kammerer et al., 1997). Expression and affinity purification using glutathione-Sepharose 4B (Pharmacia) of the fusion proteins were performed as previously described (Braun et al., 1994). The various purified GST-fusion proteins were bound to glutathione-Sepharose beads (Pharmacia) in PBS for 1 h at 4 °C and washed three times in PBS. For the in vitro interaction assay the GST-fusion proteins (0.5-1.5 μg protein bound to 1 μl bead suspension) were incubated with 5 μl of in vitro translated, [35S]-labeled protein in 200 μl binding buffer (100 mM NaCl, 50 mM potassium phosphate pH 7.4, 1 mM MgCl2, 10 % glycerol, 0.1 % Tween 20, 1.5 % BSA) for 2 h at 4 °C. The beads were then pelleted and washed four times in 1 ml binding buffer not containing BSA. Pellets were resuspended in SDS-sample buffer and boiled for 5 min before being analyzed by SDS-PAGE according to standard protocols. To verify stability of the GST-fusion proteins and equal loading, gels were stained with Coomassie blue. The gels were dried and exposed to X-ray film for 24 h.

Transfection, antibodies, and indirect immunofluorescence analysis
Plasmids were transfected into COS7 cells or into human PEX19-deficient fibroblasts using lipofectamine (Life Technologies) following protocols provided by the manufacturer. To create stable transfectants, the fibroblasts were incubated with 500 μg/ml neomycin (SIGMA). For indirect immunofluorescence analysis, stable transfectants were fixed with 3% formalin/PBS for 30 min and permeabilized with 1% Triton X-100 for 5 min. Thereafter, cells were incubated for 1 hour with a polyclonal rabbit antibody against catalase (Biodesign; 1:100 dilution) and/or a monoclonal mouse antibody against ALDP (ALD-1D6, Euromedex; 1:100 dilution). The cells were then washed 10 times with PBS and incubated for 1 hour with secondary antibodies: Rhodamine (TRITC)-conjugated goat anti-mouse IgG and/or fluorescein (FITC)-conjugated goat anti-rabbit IgG (all Jackson Immunoresearch Laboratory Inc.; 1:50 dilution). After 10 washing steps in PBS, the cells were embedded in an anti-fading reagent (Vectashield, Vector Laboratories Inc.). Fluorescence microscopy was performed using an Axiovert 135 inverted fluorescence microscope (Zeiss).
Results

Domain structure of the human PEX19 protein in relation to PEX19 splice variants

Computational database analysis of the full-length PEX19 protein sequence using the NCBI-BLASTP program and the ProDom database revealed three different conserved domains in direct succession (Fig. A3-1B): Domain D1 (ProDom-ID: PD339396) ranging from the N-terminus to amino acid 87, domain D2 (ProDom-ID: PD024170) ranging from amino acids 88 to 272, and the C-terminal domain D3 (ProDom-ID: PD329985). These domains are shared by ortholog PEX19 proteins of other species. Interestingly, the C-terminal domain is composed of amino acids that are encoded exclusively by exon 8 of human PEX19, whereas the transition from domain D1 to D2 is not linked to a special exon-exon boundary (Fig. A3-1B).

Figure A3-1: Human PEX19 splice variants and the predicted domain structure of PEX19 protein. A, Scheme of the PEX19 splice variants. The transcript PEX19all contains all eight exons, the transcript PEX19ΔE2 lacks exon 2, and the transcript PEX19pΔE8 lacks a part of exon 8. Exons (E1-E8) are indicated as gray boxes for coding or white boxes for noncoding regions. The dashed lines span regions of the transcript that are excised by splicing. The second in-frame initiation codon utilized during translation of PEX19ΔE2 is shown by a white arrow. Termination codons resulting from frame shifts are indicated by black asterisks. B, Domain structure of PEX19 and the corresponding proteins of the PEX19 splice variants. The domains D1 (ProDom-ID PD339396), D2 (ProDom-ID PD024170), and D3 (ProDom-ID PD329985) were predicted using the NCBI-BLASTP program and the ProDom database. The encoded proteins of the PEX19 splice variants including the C-terminal farnesyl group (jagged line) are shown as white boxes. The corresponding exons are numbered E1-E8. The dashed box at the C-terminus of PEX19pΔE8 is indicating amino acids that differ from the wild type PEX19 sequence because of frame shifts.
This predicted domain structure was compared with proteins expected to be encoded by different PEX19-splice variants (Kammerer et al., 1997): The unspliced transcript PEX19all contains all eight exons and encodes the full-length PEX19 protein. The PEX19ΔE2 transcript-variant lacks the complete sequence of exon 2. The possible re-initiation of translation at the second in-frame ATG results in a N-terminally truncated protein (PEX19ΔE2) that lacks the first 90 amino acids. The PEX19pΔE8 transcript-variant exhibits a deletion of the first 52 bp of exon 8. As a consequence, a C-terminally truncated PEX19 protein (PEX19pΔE8) is produced with terminal seven amino acids that are distinct from the wild type PEX19 protein sequence and lack the C-terminal farnesylation motif CLIM. The comparison of these variant PEX19 proteins to the predicted PEX19 domain structure (Fig. A3-1B) reveals that the splice variant PEX19ΔE2 lacks the N-terminal domain D1 whereas PEX19pΔE8 is composed of the domains D1 and D2 but entirely lacks the domain D3, which contains the C-terminal farnesylation consensus motif. Therefore, these two splice variants were subsequently utilized for further studies to delineate specific domain functions.

**In vitro interaction of variant PEX19 proteins with peroxisomal membrane proteins**

We performed *in vitro* interaction studies to investigate whether the N-terminally truncated protein PEX19ΔE2 lacking domain D1 and the C-terminally truncated protein PEX19pΔE8 lacking domain D3 bind to peroxisomal membrane proteins known to interact with the full-length PEX19 protein. Among those we used the [35S]-labeled peroxisomal ABC half transporters ALDP, ALDRP, and PMP70 and the peroxisomal assembly protein PEX3 in an *in vitro* GST-pull-down assay. Both the full-length PEX19 protein GST-PEX19all (Fig. A3-2A) and the truncated proteins GST-PEX19ΔE2 (Fig. A3-2B) and GST-PEX19pΔE8 (Fig. A3-2C) retained [35S]-radioactivity indicating binding to the peroxisomal membrane proteins ALDP, ALDRP, PMP70, and PEX3. GST alone did not bind to either protein (data not shown).

With regard to PEX3, retention of [35S]-radioactivity resulted in two distinct bands (Fig. A3-2A and C). This can be explained by the observation that *in vitro* translation of full length PEX3 yields two proteins of different molecular weight. The lower band is a result of the utilization of the internal start codon at position 199 of the coding region of PEX3 (Muntau et al., 2000a). The corresponding protein (Δ66aaPEX3) lacks the first 66 amino acids but retains the ability to bind to full length PEX19 (Fig. A3-2A) and to PEX19pΔE8 (Fig. A3-2C). Based on data of previous studies (Fransen et al., 2001; Soukupova et al., 1999), Δ66aaPEX3 is predicted to be devoid of its peroxisomal targeting sequence. To demonstrate that this
information is contained within the N-terminal 40 amino acids of PEX3, the first 40 amino acids of PEX3 were N-terminally fused to GFP (1-40aa PEX3-GFP) and were expressed in COS7 cells. The intrinsic GFP fluorescence subsequently revealed a punctate pattern indicative for peroxisomal localization (data not shown).

**Figure A3-2: In vitro binding of the encoded proteins of PEX19 splice variants to peroxisomal membrane proteins.** The peroxisomal membrane proteins ALDP, ALDRP, PEX3, and PMP70 were [³⁵S]-methionine labeled by in vitro translation and then tested for interaction in a GST-pull-down assay with immobilized GST-PEX19all (A and D), GST-PEX19ΔE2 (B and D), and GST-PEX19pΔE8 (C and D) fusion proteins. Eluted [³⁵S]-labeled proteins were separated on 12% SDS-polyacrylamide gel electrophoresis and exposed to autoradiography. Bands indicate protein-protein binding by the presence of the [³⁵S]-labeled PMPs being retained and eluted from the PEX19-constructs. Full length [³⁵S]-labeled ALDP, ALDRP, PEX3, and PMP70 were eluted from GST-PEX19all (A), GST-PEX19ΔE2 (B) and GST-PEX19pΔE8 (C) but not from GST alone (data not shown), indicating binding to the PEX19 variants. D. The nature of the double band representing PEX3 was evaluated in a separate in vitro binding assay. The lower band, corresponding to a 66 amino acid N-terminally truncated PEX3 protein (Δ66aaPEX3), results from the utilization of the second in frame start codon of **PEX3** cDNA. [³⁵S]-labeled Δ66aaPEX3 was eluted from GST-PEX19all and GST-PEX19pΔE8, but not from GST-PEX19ΔE2. This indicates that Δ66aaPEX3 binds to GST-PEX19all and GST-PEX19pΔE8, but not to GST-PEX19ΔE2. In contrast, full length [³⁵S]-labeled PEX3 was eluted from all three GST-PEX19 variants. GST alone did not bind to either of the PEX3 proteins. On the left, 20% of the total amount of the [³⁵S]-labeled proteins (input) used in the pull-down assay was loaded to illustrate the molecular masses of both PEX3 variants.

However, it is interesting to note that the N-terminally truncated PEX19 variant GST-PEX19ΔE2 showed only binding to a single PEX3 band (Fig. A3-2B). To demonstrate that this band corresponds to the full length PEX3 protein, we performed an in vitro binding assay comparing the binding of [³⁵S]-labeled PEX3 and Δ66aaPEX3 to the GST-immobilized PEX19 variants (Fig A3-2D). Both PEX3 translation products were detected when the retained [³⁵S]-labeled PEX3 proteins were eluted from the GST-PEX19all and GST-
PEX19pΔE8 beads. In contrast, only one PEX3 translation product was retained after elution from the GST-PEX19ΔE2 beads (Fig. A3-2D). This protein migrates at the molecular weight corresponding to the full length PEX3 protein. This indicates that the N-terminally truncated PEX19ΔE2 protein binds to the full length PEX3 protein, but not to the N-terminally truncated Δ66aaPEX3 protein.

**Figure A3-3: Functional complementation of PEX19-deficient fibroblasts by PEX19 splice variants.** PEX19-deficient fibroblasts were transfected with pcDNA3 derived cDNA-vectors expressing the human PEX19 splice variants PEX19all, PEX19pΔE8, and PEX19ΔE2. Stable transfectants were processed for indirect immunofluorescence using rhodamine-labeled antibodies to the peroxisomal membrane protein adrenoleukodystrophy-protein (ALDP; A, C, E, and G) and fluorescein-labeled antibodies to catalase (B, D, F, and H), a peroxisomal matrix protein. After transfection with the plasmid pcDNA3/PEX19all (A and B), coding for the full-length PEX19 protein, the punctate pattern for ALDP (A) and catalase (B) indicated that these proteins were co-localized either at or within restored peroxisomes. Transfection with the plasmid pcDNA3/PEX19pΔE8 (C and D), coding for a C-terminally truncated PEX19 protein lacking the farnesylation consensus sequence, restored both peroxisomal membranes and functional peroxisomes as demonstrated by a punctate immunofluorescent pattern. In contrast, transfection of the empty vector pcDNA3 (E and F) as a control did not lead to restoration of a peroxisomal punctate pattern. Transfection with the plasmid pcDNA3/PEX19ΔE2 (G and H) coding for a 90 amino acids N-terminally truncated PEX19 variant did not restore peroxisomal biogenesis as indicated by the diffuse cytosolic signal after staining for ALDP (G) and catalase (H).

**Functional complementation studies in PEX19-deficient fibroblasts**

The ability of the PEX19 splice variants to restore the formation of peroxisomal membranes was investigated by functional complementation studies in PEX19-deficient fibroblasts that entirely lack morphologically recognizable peroxisomal membranes (Matsuzono *et al.*, 1999). Transfection of PEX19all-cDNA encoding the full-length PEX19 protein restored peroxisomal biogenesis (Fig. A3-3A and B). Indirect immunofluorescence analysis was performed by staining with antibodies to the peroxisomal membrane protein ALDP (Fig. A3-
APPENDIX 3: FUNCTIONAL CHARACTERIZATION OF PEX19 SPLICE VARIANTS

3A) and to the matrix enzyme catalase (Fig. A3-3B) and yielded a co-localized peroxisomal punctate pattern. This indicates both the presence of peroxisomal membranes and of import-competent intact peroxisomes.

The splice variant PEX19pΔE8 encodes a C-terminally truncated PEX19 protein that lacks the domain D3 and therefore does not contain the farnesylation motif (CLIM). Transfection of PEX19pΔE8-cDNA revealed a co-localized punctate staining for ALDP (Fig. A3-3C) and for catalase (Fig. A3-3D) indicating the restoration of peroxisomal membranes and of functional peroxisomes in the PEX19-deficient fibroblasts. As a control, transfection of the empty vector pcDNA3 (Fig. A3-3E and F) yielded only a diffuse pattern when stained with antibodies to ALDP (Fig. A3-3E) and to catalase (Fig. A3-3F) indicating the mislocalization of these two proteins to the cytosol.

In contrast, transfection of PEX19ΔE2-cDNA (Fig. A3-3G and H), encoding a 90 amino acids N-terminally truncated PEX19 protein lacking the domain D1, yielded only a diffuse pattern when stained with antibodies to ALDP (Fig. A3-3G) and to catalase (Fig. A3-3H). This indicates a mislocalization of these two proteins to the cytosol in the absence of peroxisomal membranes or functional peroxisomes. The ability of the PEX19ΔE2-cDNA-construct to produce a protein with the expected length had been shown earlier by in vitro translation (Kammerer et al., 1997). This observation is consistent with the hypothesis that the PEX19ΔE2-cDNA-construct is fully functional. Furthermore, integration of the PEX19ΔE2-cDNA into the genome was confirmed by positive neomycin-selection of the fibroblasts. Nevertheless, transfection of PEX19ΔE2-cDNA in PEX19-deficient fibroblasts did not lead to restoration of peroxisomal biogenesis.
Discussion

The current knowledge about the domain organization of PEX19 and the exact function of these domains is limited. Computational database analysis of the human PEX19 protein sequence reveals three domains in direct succession, which are exclusively shared by ortholog PEX19 proteins of other species. However, these domains have not yet been linked to specific biological functions. The encoded proteins of two natural occurring PEX19 splice variants, each lacking exactly one of these domains, resemble this modular domain organization of PEX19. Therefore, we took advantage of these splice variants to assess their functional ability as to PMP-binding and restoration of peroxisomal membrane synthesis. The data presented provide first informations on the role of these domains in different aspects of the biological function of PEX19.

The data derived from the analysis of truncated PEX19 splice variants indicate that the C-terminal truncation of domain D3 does not disturb the ability of PEX19 to bind full length PEX3, ALDP, ALDRP, and PMP70. At least in vitro, this interaction apparently does not require the farnesylation motif at the C-terminus of PEX19. Recent other studies utilized the yeast two-hybrid system to define PEX19 protein sites that are essential for PMP binding. In these studies farnesylation of PEX19 was required for binding of PEX19 to PEX10, PEX11β, PEX12, and PEX13 but not for binding to PEX3 (Fransen et al., 2001). We show here that the interaction of PEX19 with the peroxisomal ABC half transporters ALDP, ALDRP, and PMP70 may occur independently from posttranslational farnesylation.

The interaction between PEX19 and PEX3 is considered to be crucial for the early peroxisomal membrane synthesis. Our data indicate that a truncation of 90 amino acids that correspond to the domain D1 at the N-terminus of the PEX19 protein (PEX19ΔE2) does not abolish the in vitro binding to full length PEX3. Therefore, our data support the hypothesis that a PEX19 variant composed of the domains D2 and D3 is able to interact with full length PEX3. In contrast, using the yeast two-hybrid system, the PEX3 interaction region of PEX19 has been narrowed down to approximately 50 amino acids at the N-terminus of PEX19 protein in human (Fransen et al., 2001) and Pichia pastoris (Snyder et al., 1999a). Furthermore, a truncation of 30 amino acids at the N-terminus of PEX19 abolished the interaction with PEX3 in the yeast two-hybrid system (Fransen et al., 2001). These conflicting in vitro findings could be explained by the fact that full length PEX19 is needed to perform a functional interaction with PEX3 in vivo, whereas in vitro shorter fragments of PEX19 are sufficient to bind PEX3. Alternatively, PEX19 might contain distinct binding-sights for
PEX3: One at the extreme N-terminus, as reported by Fransen et al. and Snyder et al. (Fransen et al., 2001; Snyder et al., 1999a), and one composed of the domains D2 and D3. Since domain D3 of PEX19 is dispensable for the interaction with the PMPs examined here, we conclude that a putative second PEX3-binding site within the PEX19 protein is most probably situated in domain D2.

With regard to PEX3, we show here that the N-terminal truncation of 66 amino acids of PEX3 does not abolish the binding to full-length PEX19 in vitro. Our in vitro binding data are also in agreement with recently reported “two hybrid” data, showing that N-terminally truncated Pichia pastoris (Snyder et al., 2000; Snyder et al., 1999a) and Saccharomyces cerevisiae (Götte et al., 1998) PEX3 still retains the ability to interact with PEX19. Furthermore, Fransen et al. reported that full-length PEX19 was able to bind to a N-terminally 43 amino acid truncated PEX3 protein in a blot overlay assay (Fransen et al., 2001). The N-terminal 40 amino acids of PEX3 have been shown to be sufficient to target a reporter protein to the peroxisome. The targeting information of PEX3 is thus localized in the short N-terminal part of the protein including the first transmembrane region (Soukupova et al., 1999). In all targeting signals of PMPs known today, one or more transmembrane domains are directly integrated (Brosius et al., 2001). In human PEX3, only one physical transmembrane region (amino acids 16-33) has been reported (Soukupova et al., 1999). One can therefore propose that the region of the N-terminal 40 amino acids of PEX3 harbors the one and only targeting information of the protein. Taken together, these findings support the hypothesis that PEX3 has non-overlapping functional regions that are not directly interconnected: A region containing the peroxisomal targeting information located at the extreme N-terminus of PEX3 and a spatially distinct region essential for binding to the PEX19-domains D1 or D2.

Human PEX19 protein has been reported to be partly farnesylated in vitro (Kammerer et al., 1997) and in vivo (Matsuzono et al., 1999). However, the role of farnesylation in peroxisomal biogenesis remains still unclear. Matsuzono et al. assumed that farnesylation of PEX19 is most likely essential for its biological activity because a PEX19 variant harboring a mutation in the farnesylation consensus sequence failed to restore peroxisomal biogenesis in PEX19-deficient fibroblasts (Matsuzono et al., 1999). On the other hand, Sacksteder et al. reported that PEX19 is functional in peroxisomal biogenesis even in the absence of the farnesyl moiety (Sacksteder et al., 2000). In our functional complementation studies we utilized the splice variant PEX19\(\Delta E8\) to express a truncated PEX19 protein lacking the domain D3 which includes the C-terminal farnesylation consensus sequence. We have also previously shown that this variant protein cannot be modified by a farnesyl group in vitro (Kammerer et al.,
Nevertheless, we observed that expression of this splice variant completely restored peroxisomal membrane synthesis in PEX19-deficient fibroblasts (Fig A3-3C and D). We therefore conclude that posttranslational farnesylation of the PEX19 protein is not required for its biological function regarding the induction of peroxisomal membrane synthesis.

Splice variants may display a considerable functional diversity. For example, it has been reported for several genes that different splice variants have different protein–binding partners (Strehler & Zacharias, 2001; Xiong et al., 1998). We provide evidence that the splice variants PEX19all, PEX19ΔE2, and PEX19pΔE8 exhibit similar binding-capacity to the peroxisomal ABC-transporters and to full length PEX3. On the other hand, the binding-capacity to a N-terminally truncated PEX3 variant lacking the targeting region is different for PEX19ΔE2 when compared to PEX19all and PEX19pΔE8. The interaction between PEX19ΔE2 and this truncated PEX3 variant is markedly reduced or even absent, whereas PEX19all and PEX19pΔE8 are able to bind to this variant PEX3 protein. Furthermore, PEX19pΔE8 but not PEX19ΔE2 is able to induce peroxisomal biogenesis in PEX19-deficient fibroblasts.

In conclusion the data presented here demonstrate (i) that the proteins encoded by the PEX19 splice variants PEX19ΔE2 and PEX19ΔE8 display a considerable functional diversity as shown by divergent results of in vitro protein interaction and functional complementation studies (ii) that farnesylation of PEX19 is not essential for its biological activity and (iii) that PEX19 interacts with PEX3 in a region which is spatially distinct from the peroxisomal targeting region of PEX3. Taken together, this study provides first experimental evidence for the specific biological functions of the different predicted domains of the PEX19 protein known to play a key role in the very early steps of peroxisomal biogenesis.

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References

The references are listed on pages 38–49.
Appendix 4

Genomic Organization, Expression Analysis, and Chromosomal Localization of the Mouse PEX3 Gene Encoding a Peroxisomal Assembly Protein

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Abstract

The peroxin Pex3p has been identified as an integral peroxisomal membrane protein in yeast where PEX3 mutants lack peroxisomal remnant structures. Although not proven in higher organisms, a role of this gene in the early peroxisome biogenesis is suggested. We report here the cDNA cloning and the genomic structure of the mouse PEX3 gene. The 2-kb cDNA encodes a polypeptide of 372 amino acids (42 kDa). The gene spans a region of 30 kb, contains 12 exons and 11 introns and is located on band A of chromosome 10. The putative promoter region exhibits characteristic housekeeping features. PEX3 expression was identified in all tissues analyzed, showed the strongest signals in liver and in testis, and could not be induced by fenofibrate. The data presented may be useful for the generation of a mouse model defective in PEX3 in order to clarify the yet unknown functional impact of disturbances in early peroxisomal membrane assembly.
Peroxisome biogenesis includes peroxisome proliferation, membrane biogenesis and peroxisomal matrix protein import (Subramani, 1997). Proteins involved in the assembly of peroxisomes are encoded by \( PEX \) genes and are termed peroxins (Distel et al., 1996). While at least 23 peroxins have been reported to be essential for peroxisome biogenesis, the exact functions and interactions of only a limited number of these have been determined so far. Data from yeast have suggested that the \( PEX3 \) gene is coding for an integral membrane protein essential for both peroxisomal membrane assembly and membrane protein import (Höhfeld et al., 1991; Snyder et al., 1999b). In a previous study we cloned and characterized the human \( PEX3 \) cDNA (Kammerer et al., 1998). Pex3p has been shown to interact with Pex19p in yeast (Götte et al., 1998; Snyder et al., 1999a), and in human (Soukupova et al., 1999).

\( Saccharomyces cerevisiae \) cells deficient in either Pex3p or Pex19p were reported to lack peroxisomal remnant structures, also called peroxisomal ghosts (Götte et al., 1998). These observations and the recent identification of multimeric peroxisomal assembly protein complexes are consistent with the hypothesis that these two proteins are essential players in peroxisomal membrane biogenesis and peroxisomal membrane protein localization (Snyder et al., 1999b). However, the precise contribution of these peroxins to the maturation of early peroxisomes and human disease pathogenesis is not yet known. The generation of mice with targeted disruption of early peroxin genes, such as \( PEX3 \), is expected to deliver more information about biological relevance and function. We report here the cDNA sequence and expression data as well as the analysis of the genomic structure and the chromosomal localization of the mouse \( PEX3 \) gene.

The expressed sequence tag (EST) database dbEST (Boguski et al., 1993) was probed with the human \( PEX3 \) cDNA sequence. Four mouse EST clones, m1 (IMAGp998L081096), m2 (IMAGp998H081406), m3 (IMAGp998P072169), and m4 (IMAGp998M232137) were obtained from the Resource Center of the German Human Genome Project (Lehrach, 1990). The longest clone, m1, was completely sequenced and revealed to contain 1511 bp including the 3’ portion of the gene. The cDNA was completed by 5’ RACE-PCR (Marathon-Ready mouse liver cDNA, Clontech) and showed the following features: a 5’ untranslated region of 167 bp followed by an open reading frame of 1116 bp (372 amino acids) and a 3’ untranslated sequence of 729 bp. A consensus poly(A) signal was identified 34 bp upstream of the poly(A) at position +2017 relative to the transcription start site. The usage of an alternative poly(A) site in the 3’ untranslated region was confirmed by 3’ RACE-PCR. Two further poly(A) signals at positions +1341 and +1376 were detected in several clones followed by a poly(A)
APPENDIX 4: THE MOUSE PEX3 GENE

tail 16-35 bp downstream. The position of the two alternative poly(A) sites at about 70 and 730 bp downstream of the TGA stop codon is in agreement with the data on the human PEX3 gene (Kammerer et al., 1998). Using the ClustalW program (Thompson et al., 1994) from the Baylor College of Medicine, we performed a pairwise alignment of the deduced murine Pex3p with the human Pex3p. These two proteins revealed an overall amino acid identity of 93.8%. The degree of identity to the Pex3 proteins of other species is 22.3% to Pichia pastoris (455 amino acids), 20.9% to Hansenula polymorpha (457 amino acids), 19.9% to Saccharomyces cerevisiae (441 amino acids), and 18.6% to Kluyveromyces lactis (483 amino acids). Protein similarity scores were determined using the Align program from EERIE (Nimes, France). A comparative analysis of Pex3 proteins using the TMpred server of the Bioinformatics group at ISREC (Epalinges, Switzerland) predicted two hydrophobic transmembrane regions that are located in the N-terminal half of the protein in all species.

Figure A4-1: Genomic organization of the mouse PEX3 gene. The gene consists of twelve exons (closed boxes) and eleven introns (solid lines between the exons). Exon sizes in base pairs are indicated above, intron sizes below the horizontal line. The 5' and 3' UTRs are shown as open boxes. Methods: Mouse cosmid DNA was prepared using ion exchange column purification (Midiprep Kit, Qiagen). Multiple exon-exon PCR reactions were performed using mcos3 cosmid DNA, mouse genomic DNA from 129/Ola mouse embryonic stem cells or both as a template. The resulting PCR products were sequenced from either end using the amplification primers. The sequence containing the putative promoter region was obtained by long template PCR (Expand Long Template PCR System, Boehringer Mannheim) using adaptor-ligated genomic DNA fragments (Mouse Genome Walker Kit, Clontech) according to the manufacturer’s procedure. Two successive PCR reactions were performed using the anchor-specific primer AP1 and a mouse PEX3-specific reverse primer (m128, 5’-ACT GTG CCC AGG AAG ATG C-3’) and then AP2 combined with a nested reverse primer from the coding region (m81, 5’-CCA CAT TGA TCT CAG CAT CTC C-3’). The obtained PCR products were purified from the gel (QIAquick Gel Extraction Kit, Qiagen), directly sequenced by fluorescent dye terminator technology and separated on an ABI 377 sequencer. EMBL/GeneBank accession numbers: AF152996 and AF162890 through AF162896.

To determine the exon-intron structure of the mouse PEX3 gene, a 129/Ola mouse genomic DNA cosmid library was screened with a mouse cDNA probe. One positive clone, mcos3, covering about 17 kb of the PEX3 gene including exon 3 to exon 12 was isolated. To amplify the introns, multiple exon-exon PCR reactions were performed using mcos3 cosmid DNA,
mouse genomic DNA from 129/Ola mouse embryonic stem cells or both as a template. Exons 1 and 2, which were not present on the clone mcos3, were exclusively amplified from mouse genomic DNA. Overlapping fragments spanning the complete coding region were generated and sequenced from either end using the amplification primers in order to get the intron-exon boundaries. Comparison of the obtained genomic sequence with the cDNA sequence revealed that the mouse \textit{PEX3} gene consists of 12 exons and 11 introns (Figure A4-1). The exon sizes range from 44 bp (exon 4) to 166 bp (exon 8). All exon-intron junctions conform to the GT-AG rule (Figure A4-2). Five introns (intron 4, 5, 6, 7, and 9) were fully sequenced and the exon-flanking intronic sequences of all other introns were determined. The approximate sizes of introns not fully sequenced were estimated by agarose gel electrophoresis of corresponding PCR products. The total size of the mouse \textit{PEX3} gene is approximately 30 kb (Figure A4-1).

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
5' splice site (donor) & Intron & 3' splice site (acceptor) \\
\hline
Mm & CTCGGAG & GTGAGTTGACAACA & \textbf{---In1---} & TTTAATGATTTTAG & GAGTATA \\
Hs & CTTGGAG & GTGGTGACAACCTT & \textbf{---In1---} & TTTAATGATTTTAG & GAGTATA \\
Mm & ATGACAG & GTAAGCATGGCGGC & \textbf{---In2---} & CTGTGGAAAGCAG & TGCTGTC \\
Hs & ATGACAG & GTAAGCATGGAGGA & \textbf{---In2---} & TGTTTGTATACAG & TGCTGTC \\
Mm & AAACGAG & GTGAAGGCGCTTG & \textbf{---In3---} & AATTTTTTTTTTAG & GCCTTC \\
Hs & AAACAG & GTAATCAGCAGTTT & \textbf{---In3---} & AATTTTTTTTTTAG & GCCTTC \\
Mm & ATATTTA & GTAATGCTCAATAT & \textbf{---In4---} & TCTTCTCTCTGAAG & GTCGAC \\
Hs & ATATTTA & GTAATGCTCAATAT & \textbf{---In4---} & TCTTCTCTCTGAAG & GTCGAC \\
Mm & GATACCT & GTAAGTTTAACAGA & \textbf{---In5---} & TCTGTGTTGCAG & ACCGTC \\
Hs & GATACCT & GTAAGTTTAACAGA & \textbf{---In5---} & TCTGTGTTGCAG & ACCGTC \\
Mm & GAGACG & GTAAGTTAACAGT & \textbf{---In6---} & TTTTTTTTTTTTAG & GCCTTC \\
Hs & GAGACG & GTAAGTTAACAGT & \textbf{---In6---} & TTTTTTTTTTTTAG & GCCTTC \\
Mm & TAGAAG & GTAATGCTATGCTT & \textbf{---In7---} & TCCCTTATTTTAC & TGCGAC \\
Hs & TAGAAG & GTAATGCTATGCTT & \textbf{---In7---} & TCCCTTATTTTAC & TGCGAC \\
Mm & AGCTA & GTAATGCTATGCTT & \textbf{---In8---} & TTTTTTTTTTAC & GCCATG \\
Hs & AGCTA & GTAATGCTATGCTT & \textbf{---In8---} & TTTTTTTTTTAC & GCCATG \\
Mm & TAGAAG & GTAATGCTATGCTT & \textbf{---In9---} & TTTTTTTTTTAC & GCCATG \\
Hs & TAGAAG & GTAATGCTATGCTT & \textbf{---In9---} & TTTTTTTTTTAC & GCCATG \\
Mm & TAAACAG & GTAATGGAGTGCT & \textbf{---In10---} & TAATGTGTTCTAC & TCGAT \\
Hs & TAAACAG & GTAATGGAGTGCT & \textbf{---In10---} & TAATGTGTTCTAC & TCGAT \\
Mm & TGTACG & GTAATGGAGAAGT & \textbf{---In11---} & ATGGTTGTTACAG & GATGTC \\
Hs & TGTACG & GTAATGGAGAAGT & \textbf{---In11---} & ATGGTTGTTACAG & GATGTC \\
\hline
Cs & GTAAGT & GTAAGTTAACAGT & \textbf{---In11---} & ATGGTTGTTACAG & GATGTC \\
\end{tabular}
\caption{Comparison of the exon-intron boundaries of the mouse and human \textit{PEX3} gene. The consensus sequences (CS) of the 5' and 3' intron splice sites are given below, where N is any nucleotide and, where two nucleotides are given, the one above is more common (Shapiro & Senapathy, 1987).}
\end{table}

To characterize the putative promoter region, 2 kb of genomic sequence immediately upstream of the transcriptional start sequence were obtained by long template PCR. Analysis of this 5’ flanking sequence for consensus promoter elements was performed using the
Transcription Factor Database (TFD) (Ghosh, 1991) and the Signal Scan server of BIMAS (Prestridge, 1991). As a characteristic finding for housekeeping genes, which are expressed at low levels in essentially all tissues, canonical TATA and CCAAT boxes were not found. The region close to the transcription initiation site was shown to be relatively GC-rich exhibiting a GC content of about 60% from -250 to -1. This sequence contains a putative binding site for the transcription factor Sp1 at -30 to -25, which was found with identical sequence and similar location in the orthologous human gene (Muntau, unpublished). At approximately 1.8 kb upstream of the transcription start site we detected a polymorphic CT repeat. By analyzing several subclones, repeat lengths between 17 and 27 - with the exception of 21 and 22 repeats - were identified. A visual inspection of the promoter region for possible peroxisome proliferator-responsive elements (PPRE) or oleate-responsive elements (ORE) did not reveal motifs that show a strong similarity to the consensus sequences (Karpichev et al., 1997; Zhang et al., 1992).

**Figure A4-3: Chromosomal localization of the mouse *PEX3* gene.** Specific hybridization signals were identified on band A of chromosome 10 using fluorescence in situ hybridization of metaphase chromosomes. **Methods:** Metaphase chromosome preparations were obtained from mouse bone marrow cells according to standard procedures. The cosmid clone mcos3 containing about 17 kb of the murine *PEX3* gene, was labeled with biotin-14-dUTP (Life Technologies) by nick-translation method and was detected with streptavidin-Cy3 (Amersham Life Science). Metaphase chromosomes were analyzed under an Axiopppgot microscope (Zeiss) and pictures were taken by digitizing the microscopic image with the computer program ISIS3 (Metasystems).
The chromosomal localization of the PEX3 gene was determined by fluorescence in situ hybridization (FISH) analysis. The cosmid clone mcos3 containing about 17 kb of the mouse PEX3 gene, was labeled with biotin. By hybridization of this probe to mouse metaphase chromosomes, PEX3 was localized to band A on chromosome 10 (Figure A4-3). This region has syntenic relationship with the human chromosome region 6q23-6q24 where the human PEX3 gene has been located by FISH analysis (Muntau, unpublished) and by large scale genome sequencing (NCBI, GenBank Accession number AL031320).

Figure A4-4: Expression of the PEX3 gene in various mouse and rat tissues. A Northern blot of mRNA from several murine tissues was hybridized with the 1.6-kb mouse cDNA probe. A human actin probe was used as control for the loaded amount of RNA. Fragment sizes are indicated on the right. Methods: PEX3 mRNA expression in various mouse tissues was examined using a BALB/c mouse multiple tissue Northern blot containing 2 µg poly(A)+ mRNA per lane (Clontech). A 1.6-kb cDNA fragment was isolated with NotI and XhoI from the mouse EST clone m1 and radiolabeled with [α-32P]dATP by random priming method. The blot was hybridized as described elsewhere (Braun et al., 1994) and exposed to an X-ray film for 10 days. Signals obtained were compared to those detected by a human actin cDNA probe.
PEX3 mRNA expression in various mouse tissues was examined by Northern blot analysis using a 1.6-kb mouse cDNA as a probe. A human actin probe was used as control for the loaded amount of RNA. The presence of PEX3-specific mRNA in all tissues examined with the strongest expression in liver and testis was demonstrated. In heart, brain, spleen, lung, liver, skeletal muscle, and kidney, signals at 1.6 and 2.3 kb were detected with the 2.3-kb signal being predominant in these tissues (Figure A4-4). These fragments seem to be derived from alternative polyadenylation at +1341/1376 and +2017 bp described above. In testis, in addition to the 2.3-kb transcript several shorter fragments were observed with lengths between 1.4 and 1.7 kb. This was the only tissue, in which the shorter transcripts were expressed at a higher intensity. It might be interesting to note that a marked morphological heterogeneity of peroxisomes has been described in cultured mouse testicular Leydig cells (Litwin & Bilinska, 1995). PEX3 might potentially be involved in the formation of small local networks of interconnected tubular peroxisomes observed in this study.

Pharmacological modification of expression levels of specific peroxins might be a valuable addition to treatment strategies for human peroxisomal biogenesis disorders. Fenofibrate, which was proven to be a potent peroxisomal proliferator in rodents, is a broad-spectrum lipid-lowering agent widely used in patients with coronary heart disease (Packard, 1998). To compare PEX3 mRNA expression in rats before and after fenofibrate treatment, Northern blot analysis of rat tissues using a human 2.1-kb PEX3 probe was performed. Signals obtained were compared to those detected with a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control probe. As a positive control for the effectiveness of the fenofibrate treatment, hybridization with a mouse acyl-CoA oxidase (AOX) probe was performed. As in mouse, PEX3-specific mRNA in rat was also expressed in all tissues examined. The amount of lower transcript was slightly increased in liver but overall transcripts showed a more even distribution than in mouse (Figure A4-5). The examination of rat testis tissue has still to be performed. The fenofibrate-treatment did not affect the level of PEX3 mRNA expression indicating that transcription of PEX3 is not induced by fenofibrate in rats (Figure A4-5). In contrast the level of peroxisomal acyl-CoA oxidase (AOX) mRNA used as a marker of peroxisomal proliferation was strongly enhanced in liver, kidney, intestine and heart proving the effectiveness of fenofibrate treatment (Figure A4-5).
Figure A4-5: Northern analysis in various tissues from fenofibrate-treated and untreated rats. Hybridization was performed using the 2.1-kb human PEX3 cDNA probe. Signals obtained were compared to those detected with a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control probe. The level of peroxisomal acyl-CoA oxidase (AOX) mRNA was used as marker for peroxisomal proliferation. Fragment sizes are indicated on the right. c: untreated, f: fenofibrate-treated rats. Methods: Northern blot analysis of rat tissues using a human 2.1-kb PEX3 probe was performed as previously described (Albet et al., 1997). Signals obtained were compared to those detected with a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control probe. As a positive control for the effectiveness of the fenofibrate treatment, hybridization with a mouse acyl-CoA oxidase (AOX) probe prepared from a 246-bp coding fragment was performed as described elsewhere (Albet et al., 1997).

Fenofibrate is known to strongly increase the level of peroxisome proliferator activated receptors in rats (Gebel et al., 1992). Interestingly, the mouse promoter does not exhibit canonical peroxisome proliferator-responsive elements. Assuming that this is also the case in rat, this might explain the lack of induction of rat PEX3 by fenofibrate. It has been reported
previously that fenofibrate strongly induced the expression of two other peroxisomal membrane proteins of the ABC-transporter superfamily, PMP70p and ALDRp, in rat and mouse (Albet et al., 1997; Berger et al., 1999; Holzinger et al., 1998). These data are in agreement with the hypothesis that proteins, which are directly or indirectly involved in peroxisomal metabolism, are pharmacologically inducible, whereas proteins involved in the peroxisomal biogenesis, such as Pex3p, can not be induced. Confirmation of these findings would have important consequences for the development of new treatment strategies.

Based on the findings in yeast, PEX3 is a candidate gene for human peroxisomal biogenesis disorders. PEX3 mutations have not been identified so far. Among the considerable number of PEX genes, mouse models have only been described for PEX2, which encodes a zinc-binding integral membrane protein (Faust & Hatten, 1997), and PEX5, which encodes the import receptor for most peroxisomal matrix proteins (Baes et al., 1997). However, a mouse model with targeted disruption of one of the PEX genes involved in the initial steps of peroxisomal biogenesis is not yet available. The data reported here might contribute to further elucidate the basic aspects of formation and function of peroxisomes and help to develop treatment strategies for peroxisomal biogenesis disorders, which are still leading to early death in affected children.

Acknowledgements

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References

The references are listed on pages 38–49.
Appendix 5

The Human PEX3 Gene Encoding a Peroxisomal Assembly Protein: Genomic Organization, Positional Mapping, and Mutation Analysis in Candidate Phenotypes

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Contributions to the manuscript: Outline of the project by A.A.R and S.K.; concept by A.C.M.; FISH analysis by P.L.; genomic mapping by A.H.; southern blot analysis by P.U.M.; determination of the genomic structure, sequencing of the putative promoter region, and mutation analysis by S.K.; A.C.M., and P.U.M.; writing by A.C.M.; editorial help by the co-authors
Abstract

In yeast, the peroxin Pex3p was identified as a peroxisomal integral membrane protein that presumably plays a role in the early steps of peroxisomal assembly. In human, defects of peroxins cause peroxisomal biogenesis disorders such as Zellweger syndrome. We previously reported data on the human PEX3 cDNA and its protein, which in addition to the peroxisomal targeting sequence contains a putative endoplasmic reticulum targeting signal. Here we report the genomic organization, sequencing of the putative promoter region, chromosomal localization, and physical mapping of the human PEX3 gene. The gene is composed of 12 exons and 11 introns spanning a region of approximately 40 kb. The highly conserved putative promoter region is very GC rich, lacks typical TATA and CCAAT boxes, and contains potential Sp1, AP1, and AP2 binding sites. The gene was localized to chromosome 6q23-24 and D6S279 was identified to be the closest positional marker. As yeast mutants deficient in PEX3 have been shown to lack peroxisomes as well as any peroxisomal remnant structures, human PEX3 is a candidate gene for peroxisomal assembly disorders. Mutation analysis of the human PEX3 gene was therefore performed in fibroblasts from patients suffering from peroxisome biogenesis disorders. Complementation groups 1, 4, 7, 8, and 9 according to the numbering system of Kennedy Krieger Institute were analyzed but no difference to the wild type sequence was detected. PEX3 mutations were therefore excluded as the molecular basis of the peroxisomal defect in these complementation groups.
Introduction

Peroxisomes are single-membrane-bound organelles present in all eukaryotic cells other than mature erythrocytes (van den Bosch et al., 1992). A large variety of metabolic pathways including the production and degradation of hydrogen peroxide, and many reactions that involve lipids have been assigned to the peroxisome (Tolbert, 1981). The biogenesis of functional peroxisomes including peroxisome proliferation, membrane biogenesis and peroxisomal matrix protein import, requires the interaction of numerous proteins, designated peroxins, which are encoded by PEX genes (Distel et al., 1996). At least 22 peroxins are known so far (Moser, 1999). PEX genes have first been identified by genetic analysis of peroxisome-deficient mutants of yeast (Erdmann et al., 1989). There is a remarkable degree of homology between man and yeast in respect to the factors involved in peroxisome biogenesis. Human PEX genes that are required for peroxisomal assembly have therefore been identified by genetic phenotype-complementation assays of Chinese hamster ovary (CHO) cell mutants and by searching human expressed sequence tag databases using yeast PEX gene sequences (Tateishi et al., 1997).

Data from yeast suggested that the PEX3 gene is coding for an integral peroxisomal membrane protein (PMP) essential for both peroxisomal membrane assembly and membrane protein import (Baerends et al., 1996; Snyder et al., 1999b). Pex3p has been shown to interact with Pex19p in Saccharomyces cerevisiae (Götte et al., 1998), in Pichia pastoris (Snyder et al., 1999a), and in human (Soukupova et al., 1999). The recent identification of peroxin multimeric protein complexes is consistent with the hypothesis that these two proteins are essential players in peroxisomal membrane biogenesis and PMP localization (Snyder et al., 1999b). Both proteins are thought to be involved in the early stages of peroxisomal biogenesis (Götte et al., 1998; Kammerer et al., 1997; Matsuzono et al., 1999).

In general, any defect of a human peroxin is expected to result in a lack of fully functional peroxisomes causing peroxisomal biogenesis disorders (PBD). PBD are associated with four clinical phenotypes: Zellweger syndrome (Zellweger, 1987), neonatal adrenoleukodystrophy (Ulrich et al., 1978), infantile Refsum disease (Scotto et al., 1982), and rhizomelic chondrodysplasia punctata (Spranger et al., 1971). The most important clinical symptoms are progressive neurological dysfunctions, liver disease, and eye abnormalities. Cells from patients with PBD have previously been shown to segregate into at least 12 complementation groups (Shimozawa et al., 1998). The number of PEX genes in yeast, however, suggests the existence of even more complementation groups. The relationship between the genetic defects and the clinical phenotype is not yet elucidated in detail. It is tempting to speculate that
patients even lacking peroxisomal remnants may suffer from particularly severe forms of the disease. This hypothesis is consistent with the observation of a patient with Zellweger syndrome, the most severe clinical form among peroxisomal disorders, which was shown to be caused by a PEX19 mutation (Matsuzono et al., 1999). To date, the molecular defects causing PBD in human have been identified in 10 of the 12 complementation groups, proving 10 of the corresponding PEX genes to be disease-causing.

In yeast, PEX3 deficiency leads not only to a lack of functional peroxisomes but also to the absence of peroxisomal remnant structures (“peroxisomal ghosts”) (Baerends et al., 1997; Götte et al., 1998; Höhfeld et al., 1991), whereas in most other peroxin deficiencies residual peroxisomal membrane structures can be detected. Based on these findings, PEX3 is a candidate gene for one of the peroxisomal biogenesis disorders. The corresponding phenotype is unknown so far. Interestingly, fibroblasts of some patients suffering from Zellweger syndrome and belonging to the complementation groups D, J, and G according to the Japanese classification have been shown to lack peroxisomal ghosts. These patient cells are of particular interest as candidate cells bearing a mutation in the PEX3 gene.

In a previous study we described the full-length cloning of the human PEX3 cDNA and the characterization of the human Pex3 protein (Kammerer et al., 1998). As prerequisite for the identification of the yet unknown phenotype of PEX3 deficiency we present here the genomic structure, analysis of the putative promoter region, chromosomal localization, and mapping of the human PEX3 gene. These data enabled us to design a strategy for PEX3 mutation analysis by PCR amplification and sequencing of large amplicons from genomic DNA including one or more exons each. We then evaluated fibroblasts from several patients suffering from peroxisomal biogenesis disorders assigned to different complementation groups for the presence of mutations in the human PEX3 gene.
Material and Methods

Isolation and sequencing of genomic clones
A human DNA library generated with the pCYPAC-2 vector and genomic DNA derived from cultured skin fibroblasts with an average insert size of approximately 100 kb (Ioanou et al., 1994) was screened with a 2.1-kb human PEX3 cDNA probe. This probe was excised with SacI and ApaI from the pBlueskript SK clone EST176494 (GenBank accession number AA305508) which was previously identified by our group (Kammerer et al., 1998). The probe was radiolabeled by random priming method using [α-32P]dATP and Klenow enzyme. Several positive clones were isolated by the Screening Service of the Resource Center of the German Human Genome Project (Heidelberg/Berlin) (Lehrach, 1990). The preparation of pCYPAC-2 DNA was performed using ion exchange columns (Qiagen Plasmid Midi Kit) according to the manufacturer’s protocol for P1-derived constructs. Cycle sequencing of pCYPAC-2 clone 1 (LLNLp704A02547Q3) was performed with a rhodamine dideoxy dye terminator kit (ABI/Perkin Elmer) using PEX3 specific primers and 600 ng DNA. Samples were then precipitated and separated on an ABI 377 sequencer.

Southern blot analysis of genomic clones
7µg of DNA from several pCYPAC-2 clones was digested with different restriction enzymes, separated in a 0.8% agarose gel and transferred to a positively charged nylon membrane. Hybridization with the radiolabeled 2.1-kb cDNA probe described above was performed by a modified method of Church and Gilbert as described elsewhere (Braun et al., 1994). The blots were exposed to X-ray films for 10 minutes.

Amplification and sequencing of intron-containing genomic fragments
Exon-exon PCR was performed in order to amplify human PEX3 introns using the Expand Long Template system (Boehringer Mannheim) and 200 ng of genomic leukocyte DNA or 10 ng of pCYPAC-2 clone 1 DNA, respectively. To estimate intron sizes the PCR products were separated by agarose gel electrophoresis. These products were column-purified using a Qiaquick PCR Purification Kit (Qiagen) and directly sequenced with amplification primers as described above.

Amplification and analysis of the 5' flanking region
The first 700 bp upstream of the transcription start were determined by direct sequencing of pCYPAC-2 clone 1 DNA as template. The region upstream from -700 bp was amplified using a Human Genome Walker Kit (Clontech). For this purpose, two successive PCR reactions using the Expand Long Template PCR system (Boehringer Mannheim) were performed. Products amplified with the AP1 primer and a PEX3 specific reverse primer (5'R2) were diluted 1:80 and subjected to a second PCR reaction with the AP2 primer and a nested PEX3 specific reverse primer (5'R3). The PCR products obtained were gel purified using a Qiagen Gel Extraction Kit and directly sequenced with amplification primers.

Fluorescence in situ hybridization (FISH)
Human metaphase cells were prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes according to standard procedures. In situ hybridization was performed using DNA from pCYPAC-2 clone 1, which contained the entire human PEX3 gene. The probe was labeled with biotin-14-dUTP (Life Technologies) by nick-translation and preannealed with Cot-1 DNA (Life Technologies). Detection and visualization was achieved using the avidin-fluorescein isothiocyanate/antiavidin antibody system described elsewhere (Lichter et al., 1988; Lichter et al., 1990) and chromosomes were identified by staining with 4,6-diamino-2-phenylindole dihydrochloride (DAPI).

Genomic mapping using the CEPH-Généthon YAC library
A high-density robot-spotted filter representing the CEPH-Généthon human genomic YAC library characterized with positional markers (Chumakov et al., 1995) was hybridized with a radiolabeled human PEX3 cDNA probe (Screening Service of the Resource Center of the German Human Genome Project, Heidelberg/Berlin) (Lehrach, 1990). Clones were rescreened by PCR of short fragments with exon 12-specific primers (1164F, 5'-CCC CCA ACA ACT GGA GAA ATG-3' and 1800R, 5'-CAC TTC TGT TTA CAG CAG TCT-3'). Neighboring and potentially overlapping clones were obtained from the Resource Center of the German Human Genome Project and PCR screened with the same oligonucleotide primer combinations.

Mutation analysis of the human PEX3 gene
200 ng of genomic fibroblast DNA from cultured cells of several PBD patients assigned to the complementation groups 1, 4, 7, 8, and 9 according to the numbering system of Kennedy Krieger Institute were used to amplify the complete coding region with intron-specific oligonucleotide primers (Fig. A5-1 and Table A5-2). Single exons were amplified using Taq DNA polymerase (Boehringer Mannheim). For amplification of large fragments containing several exons the Expand Long
Template system (Boehringer Mannheim) was used. The resulting PCR products were column-purified and sequenced with amplification primers, fragments spanning several exons additionally with internal intronic primers. Sequencing reaction and fragment separation was performed as described above.

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* PEX3 mutation analysis performed in this study
Results

Determination of the complete exon-intron structure

By screening a human genomic fibroblast library with a 2.1-kb cDNA probe containing the complete coding region of human PEX3, 16 positive clones were identified. The five clones that yielded the strongest signals, clone 1 (LLNLNP704A02547Q3), clone 2 (LLNLNP704I11520Q3), clone 4 (LLNLNP704A041052Q3), clone 7 (LLNLNP704K07884Q3), and clone 10 (LLNLNP704J22833Q3), were analyzed by PCR testing and Southern blot analysis (data not shown). Clone 2 and clone 7 contained only a part of the gene, whereas clones 1, 4, and 10 proved to contain the entire gene. To determine the genomic organization of the human PEX3 gene, genomic fragments were PCR amplified using cDNA-derived primers and either pCYPAC-2 clone or genomic DNA as template. Overlapping fragments spanning the complete coding region were generated and sequenced. Comparison of the obtained genomic sequence with the cDNA sequence revealed that the human PEX3 gene consists of 12 exons and 11 introns (Fig. A5-1). Exon sizes range from 44 bp to 169 bp. All exon-intron junctions conform to the GT-AG rule. Five introns (intron 4, 5, 6, 7, and 9) were fully sequenced, the exon-flanking intronic sequences of all other introns were determined and deposited with Genbank (AJ009866 to AJ009874). The approximate sizes of introns not fully sequenced were estimated by agarose gel electrophoresis of corresponding PCR products.

![Genomic organization and strategy for mutation analysis of the human PEX3 gene.](image-url)

Figure A5-1: Genomic organization and strategy for mutation analysis of the human PEX3 gene. The gene consists of twelve exons (closed boxes) and eleven introns. Exon sizes in base pairs are indicated above, intron sizes below the horizontal line. The 5' and 3' UTRs are shown as open boxes. The solid bars A, B, C1, C2, and D indicate the strategy of PCR amplification of large amplicons from genomic DNA for mutation analysis of the human PEX3 gene. A: amplification of exon 1; B: amplification of exon 2 and 3; C1: amplification of exon 4 through 9; C2: amplification of exon 10; D: amplification of exon 11 and 12. Filled arrows indicate primers used for amplification and sequencing, empty arrows indicate primers used only for sequencing (see also Table A5-2).
Characterization of the 5’ flanking region

The 1.4-kb sequence upstream of the transcription initiation site was analyzed in silico for transcription factor binding sites. The analysis of the 5’ flanking sequence for consensus promoter elements was performed using the Transcription Factor Database (TFD) (Ghosh, 1991) for an analysis with the Signal Scan server of BIMAS (Prestridge, 1991). Canonical TATA and CCAAT boxes were not found. The putative promoter sequence is very GC rich and exhibits a GC content of about 69% from -250 to -1 relative to the transcription start site. This sequence contains several predicted binding sites typically observed in GC-rich regions: an Sp1 site at -30 to -25, and three AP2 sites at -75 to -68, -116 to -109, and -221 to -214. Additionally, a putative AP1 site was identified at position -847 to -840. While the Sp1 site is identical in the orthologous mouse gene, the potential AP1 and AP2 binding sites are not highly conserved in mouse (unpublished data).

Cytogenetic localization and mapping of the human PEX3 gene

The pCYPAC-2 clone 1 containing the complete human PEX3 gene was used as a probe to hybridize metaphase chromosomes. Twin-spot signals on the long arm of both chromosomes 6 were detected and assigned to chromosome 6q23-24 (Fig. A5-2). Screening of the CEPH Généthon human genomic YAC library with the human 2.1-kb PEX3 cDNA probe described above resulted in the identification of two clones (930G4 and 930G5) that are part of the contig WC6.15. These clones are overlapping as characterized with positional markers (Whitehead Institute for Biomedical Research/MIT; http://www-genome.wi.mit.edu). PEX3-specific fragments were successfully amplified from these clones. These data placed the PEX3 gene between positional markers CHLC.GATA68F06 and CHLC.GATA41E03. Neighboring and potentially overlapping clones (967H10, 800C7, 748E1, 882D8, 773H6, 764G8, and 798D8) were obtained and PCR tested as described above. Only clone 967H10 proved positive which suggests D6S279 at 145 cM to be the closest positional marker. These results are in accordance with the results of the FISH analysis.

Mutation analysis of the human PEX3 gene

We analyzed all PEX3 exons and exon flanking regions of two patients belonging to CG-1 and one patient of CG-4, CG-7, CG-8, and CG-9, respectively. For that purpose, all exons were amplified with flanking intron primers from fibroblast genomic DNA and the resulting
PCR products subjected to cycle sequencing. No difference to the published wild type sequence was detected in all six cell lines analyzed (Kammerer et al., 1998).

Figure A5-2: Chromosomal localization of the human \textit{PEX3} gene. Plasmid DNA from pCYPAC-2 clone 1 containing the entire human \textit{PEX3} gene was labeled with biotin-14-dUTP and used as probes for in situ hybridization of metaphase chromosomes. Twin-spot signals at q23-24 on both chromosomes 6 are marked by arrows.

Table A5-2: Primers for Mutation Analysis of the Human \textit{PEX3} Gene

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer</th>
<th>5’</th>
<th>Sequence</th>
<th>3’</th>
<th>Sense</th>
<th>Fragment length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1 (A)</td>
<td>5’F3</td>
<td>GAGAGCACAGAACGGGACGA</td>
<td></td>
<td>3’</td>
<td>for</td>
<td>300 bp</td>
</tr>
<tr>
<td></td>
<td>1R</td>
<td>CTCTACATATCCCAGACTAGG</td>
<td></td>
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</tr>
<tr>
<td>Exons 2-3 (B)</td>
<td>2F2</td>
<td>GCTAATTTCTTTTTCTTTGGGCC</td>
<td>for</td>
<td></td>
<td>~ 4100 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3R3</td>
<td>GTAGTGCTTAAACAGAAATGC</td>
<td>rev</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exons 4-9 (C1)</td>
<td>4F3</td>
<td>CAGAGTTTTCTATTAGATTATCTAG</td>
<td>for</td>
<td></td>
<td>~ 6500 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5R</td>
<td>GAACCTCCTGAGGGAAAGAAC</td>
<td>rev</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>7R</td>
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<td>rev</td>
<td></td>
<td></td>
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<td></td>
<td>8F</td>
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</tr>
<tr>
<td>Exon 10 (C2)</td>
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<td>for</td>
<td></td>
<td></td>
<td>303 bp</td>
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<tr>
<td></td>
<td>10R2</td>
<td>GTTGTTATCTGGAACAAAAAGAC</td>
<td>rev</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Exons 11-12 (D)</td>
<td>11F2</td>
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<td>for</td>
<td></td>
<td>~ 4200 bp</td>
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<td></td>
<td>1255R</td>
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Discussion

Among the PEX genes known to date, PEX3 is of particular interest because of two observations: its putative implication in the very early stages of peroxisomal biogenesis (Baerends et al., 1997) and the involvement of the endoplasmic reticulum (ER) in its targeting (Baerends et al., 1996) (Kammerer et al., 1998). Recent studies on the interaction of peroxisomal membrane proteins showed that in P. pastoris Pex3p is part of a multimeric protein complex that facilitates the insertion of peroxisomal membrane proteins into the peroxisomal membrane (Snyder et al., 1999b).

In this report we present the genomic organization of the human PEX3 gene. The gene is composed of twelve exons encoding a 2.2-kb cDNA and eleven introns. The complete length of the gene is approximately 40 kb. The sequence information presented in this paper has very recently been confirmed by a PAC sequence released by the Human Genome Sequencing Project (Accession number AL031320). As a finding characteristic for housekeeping genes, sequence analysis of the putative promoter region failed to identify TATA and CCAAT boxes. Consensus binding sequences for the cellular transcription factors Sp1, AP1, and AP2, which are thought to regulate RNA polymerase II activity (Faisst & Meyer, 1992), were identified. These promoter characteristics are common in genes with ubiquitous expression and have been described for other PEX genes (Björkman et al., 1998; Kammerer et al., 1997; Purdue et al., 1997; Yahraus et al., 1996).

By in situ fluorescence hybridization, we were able to assign the cytogenetic locus of human PEX3 to chromosome 6q23-24. PEX genes are distributed over the whole genome. Human PEX6, for instance, was assigned to chromosome 6p21.1 (Fukuda et al., 1996), PEX1 to chromosome 7q21-22 (Reuber et al., 1997), PEX5 to chromosome 12p13.3 (Dodt et al., 1995), PEX10 to chromosome 8q21.1 (Warren et al., 1998), and PEX19 to chromosome 1q22 (Kammerer et al., 1997). Noteworthy is the localization of the human PEX7 gene encoding the type 2 peroxisome targeting signal (PTS 2) receptor to chromosome 6q22-24 (Braverman et al., 1997), which is the same as the one we identified for the human PEX3 gene. Although the physical distance between the two genes has not been determined so far, there could be a cluster of peroxisomal genes on chromosome 6. The results of FISH analysis in our study were confirmed by screening a human genomic YAC library that identified D6S279 to be the closest positional marker to the human PEX3 gene on chromosome 6. The mapping data might be used for segregation analysis in patients with Zellweger syndrome to exclude PEX3 gene defects as underlying cause of the disease.
Twelve complementation groups (CG) are known so far, each of which is probably defined by a distinct gene defect (Table A5-1) (Moser, 1999). Underlying mutations in the corresponding PEX genes have been identified in ten of these twelve.

In yeast, disruption of PEX3 leads to a lack of peroxisomes and remnant peroxisomal structures (“peroxisomal ghosts”) (Baerends et al., 1997). It has been hypothesized that peroxisomal ghosts may act as acceptors of matrix proteins in the peroxisomal assembly process supporting the view that peroxisomes can grow by incorporating newly synthesized matrix proteins (Yamasaki et al., 1999). PEX3 is thus a candidate gene for being involved in one of the subgroups of human peroxisomal biogenesis disorders that have yet to be characterized. So far, PEX3 has not been assigned to one of the known complementation groups. There is a high probability that human cells with a PEX3 defect also lack peroxisomal ghosts. We were therefore particularly interested in the analysis of cells from complementation group 9 as fibroblasts from all patients belonging to this complementation group very rarely have peroxisomal ghosts (Shimozawa et al., 1998). We performed mutation analysis of the human PEX3 gene in fibroblasts from patients belonging to the following complementation groups: CG-1, CG-4, CG-7, CG-8, and CG-9. A strategy of PCR amplification and sequencing of large amplicons from genomic DNA including one or more exons each was used. The sequencing technique employed has repeatedly been proven to be suitable for heterozygote detection. In all complementation groups analyzed so far, no difference to the wild type sequence was detected. This result does not completely exclude a PEX3 mutation because only exons and exon-intron boundaries were sequenced. Rare promoter or intron mutations cannot be ruled out since PEX3 cDNA complementation studies were not done. After the completion of our studies, complementation groups CG-1, CG-4, CG-7 and CG-9 have been shown to be caused by defects of PEX1 (Reuber et al., 1997), PEX6 (Fukuda et al., 1996), PEX10 (Warren et al., 1998), and PEX16 (Honsho et al., 1998), respectively. At present, among the complementation groups analyzed in our study, only for CG-8 the molecular defect has not yet been elucidated.

The most likely reason for our inability to identify a PEX3 mutation might be the fact that not all known complementation groups were available for mutation analysis. CG-G, for example, is another potential candidate for harboring a PEX3 mutation, as fibroblasts from patients belonging to this group completely lack peroxisomal ghosts (Shimozawa et al., 1998). Another possible explanation might be that not all PBD patient cell lines have been classified by complementation analysis so far. Patients not yet assigned to a complementation group would therefore not have been available for mutation analysis. Furthermore, it could be
possible that functional mutations in the PEX3 gene are prenatally lethal and are therefore not represented in the patient cell lines. On the other hand, a patient with Zellweger syndrome, which is considered to be the most severe clinical phenotype lacking all peroxisomal functions, who was assigned to CG-J lacking peroxisomal remnants was recently shown to have a PEX19 mutation (Matsuzono et al., 1999). This observation is consistent with the hypothesis that a phenotype defective in an early peroxin is not necessarily lethal. Therefore it can be expected that a PEX3 mutation will also result in a viable phenotype.

The human phenotype related to PEX3 deficiency is still unidentified. The data presented here will be helpful for the generation of a mouse model with targeted mutations in the PEX3 gene as another approach to elucidate the functional role of PEX3 in mammals and the clinical consequences arising from defects in this gene.

Acknowledgments

We wish to thank Dr. Peter Lichtner for performing FISH analysis and we are grateful to Dr. Ennes Auerswald for the opportunity to use the sequencing facility and Heide Hinz for excellent technical assistance. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (R0 727/1-1).

References

The references are listed on pages 38–49.
Appendix 6

Defective Peroxisome Membrane Synthesis
Due To Mutations in Human PEX3 Causes Zellweger Syndrome,
Complementation Group G

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Contributions to the manuscript: Outline of the project by A.A.R.; concept by A.C.M. and P.U.M.; mutation analysis by A.C.M.; cDNA cloning, immunofluorescence microscopy, functional complementation assays, and in vitro binding assay P.U.M.; somatic cell-fusion experiments by A.C.M. and P.U.M.; writing by A.C.M. and P.U.M.; editorial help by the co-authors
Abstract

The cerebro-hepato-renal syndrome of Zellweger is a severe congenital disorder associated with defective peroxisomal biogenesis. At least 23 PEX genes have been reported to be essential for peroxisome biogenesis in various species indicating the complexity of peroxisomal assembly. Cells from patients with peroxisomal biogenesis disorders have previously been shown to segregate into at least 12 complementation groups. Two patients assigned to complementation group G previously not linked to a specific gene defect were confirmed to display a cellular phenotype characterized by a lack of even residual peroxisomal membrane structures. Here we demonstrate that this complementation group is associated with mutations in the PEX3 gene encoding an integral peroxisomal membrane protein. Homozygous PEX3 mutations, each leading to C-terminal truncation of PEX3, were identified in the two patients, who both suffered from a severe Zellweger syndrome phenotype. One of the mutations involved a single nucleotide insertion in exon 7, whereas the other was a single nucleotide substitution eight nucleotides from the normal splice site in the 3' acceptor site of intron 10. Expression of wild type PEX3 in the mutant cell lines restored peroxisomal biogenesis, whereas transfection of mutated PEX3 cDNA did not. This confirmed that the causative gene had been identified. The observation of peroxisomal formation in the absence of morphologically recognizable peroxisomal membranes challenges the theory that peroxisomes arise exclusively by growth and division from preexisting peroxisomes and establishes PEX3 as a key factor in early human peroxisome synthesis.
The importance of peroxisomes in mammalian metabolism is illustrated by the existence of severe inherited metabolic diseases caused by the inability to assemble peroxisomes. Among these peroxisomal biogenesis disorders (PBD), Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum’s disease represent a continuum of clinical phenotypes (PBD [MIM 601539]), whereas rhizomelic chondrodysplasia punctata (RCDP [MIM 215100]) is characterized by distinct clinical features (Wanders, 1999). Peroxisomes have long been thought to arise exclusively by growth and division of preexisting peroxisomes (Lazarow & Fujiki, 1985) and only recently alternative hypotheses have been proposed (South & Gould, 1999). Proteins required for peroxisomal assembly are termed peroxins and are encoded by PEX genes. At least 23 peroxins have been identified in yeast, most of which are conserved among different eukaryotic organisms (Subramani, 1997). In most cases, defects in PEX genes lead to a disruption of peroxisomal matrix protein import while various peroxisomal membrane components are synthesized and accumulate in peroxisomal membrane remnants (“ghosts”). PEX3 and PEX19 mutants represent an exception to this rule as the corresponding yeast mutants have been shown to lack even these peroxisomal ghosts (Baerends et al., 1996; Götte et al., 1998). We had previously cloned the human orthologue of PEX3 (PEX3 [MIM 603164]) (Kammerer et al., 1998). Evidence suggests that human PEX3 is a peroxisomal assembly protein displaying two transmembrane helices in the N-terminal half of the protein. Immunofluorescent microscopy studies indicate that it is an integral membrane protein with its C-terminus exposed to the cytosol and its N-terminus facing the intraorganellar space (Kammerer et al., 1998; Soukupova et al., 1999).

Despite an extensive search, disease causing mutations in the human PEX3 gene have not been identified so far (Muntau et al., 2000b).

In somatic cell fusion experiments, fibroblasts from patients with peroxisomal biogenesis disorders have been shown to segregate into at least 12 complementation groups (CG) (Moser, 1999; Shimozawa et al., 1998). In this study we investigated fibroblasts from two patients (PBDG-01 and PBDG-02) previously assigned to CG-G (Gifu University nomenclature) (Poulos et al., 1995). CG-G fibroblasts lack peroxisomal ghosts as evidenced by staining with an antibody directed against the human 70-kD peroxisomal membrane protein, PMP70 (Poulos et al., 1995; Shimozawa et al., 1998) and were therefore excellent candidates for a PEX3 mutation. To our knowledge CG-G is represented so far by only two patients worldwide and cell lines from both individuals were available for this study.
Figure A6-1: Somatic cell fusion experiments. Fusion of co-cultivated fibroblasts was performed using methods described elsewhere (Roscher et al., 1989). Fusion of fibroblasts from patients PBDG-01 and PBDG-02 with fibroblasts from a patient carrying a PEX19 mutation (PBDJ-01) (Matsuzono et al., 1999) restored peroxisomes in the majority of multinucleated cells as demonstrated by a punctate immunofluorescent pattern after staining for catalase. Somatic cell fusion of fibroblasts from patient PBDG-01 with fibroblasts from patient PBDG-02 revealed only diffuse cytosolic staining with anti-catalase antibodies.

The two male infants from unrelated families were born to consanguineous Dutch and Italian parents. Patient 1 (PBDG-01) showed a marked muscular hypotonia at birth. Dysmorphic features included hypertelorism, prominent epicanthic folds, and a high, broad forehead with a round face. Seizures developed on day 1 but were controlled with treatment. His condition deteriorated rapidly and he died at 4 months of age. Patient 2 (PBDG-02) was cyanotic at birth, markedly hypotonic, and lacked deep tendon reflexes. He had a prominent midface and an antimongoloid slant of the palpebral fissures, ocular hypertelorism, small low-set ears, a prominent nose, and a high arched palate. The liver was enlarged. Seizures developed in the first 20 hours. The child required gavage feeding and died at 19 days of age of congestive heart failure. The patient’s brother had been similarly affected and died at the age of 15 days. The concentrations of very long-chain fatty acids were elevated in the plasma and fibroblasts of patient 1 and patient 2. In fibroblasts from both patients, the percentage of sedimentable
catalase, the rate of oxidation of pristanic acid, and the activities of alkyl dihydroxyacetone phosphate synthase and dihydroxyacetone phosphate acyl transferase were all reduced (Poulos et al., 1995). In summary, the clinical and biochemical features of both patients correspond to a severe Zellweger syndrome phenotype.

By performing somatic cell fusion experiments (fig. A6-1) we showed that fusion of cultured fibroblasts from patient PBDG-01 with cells from patient PBDG-02 did not rescue peroxisomal biogenesis. This confirms that the two cell lines were correctly assigned to the same complementation group and are therefore expected to bear mutations in the same gene. By contrast, fusion of cultured fibroblasts from patients PBDG-01 and PBDG-02 with those from a patient carrying a PEX19 mutation (PBDJ-01) (Matsuzono et al., 1999) restored peroxisomal biogenesis. These results indicate that complementation group G and PEX19 mutated cell lines can complement each other despite both phenotypes being devoid of detectable preexisting peroxisomal membranes.

When analyzed by immunofluorescent microscopy, both patient cell lines lacked morphologically recognizable PMP-containing peroxisomal membranes (“ghosts”). This was demonstrated by the absence of punctate staining for the peroxisomal membrane protein adrenoleukodystrophy protein (ALDP) (fig. A6-2C,D) and the membrane marker protein PMP70 (data not shown). However, these findings do not exclude the possibility that some small peroxisomal vesicles may still exist in the patients’ cells. The lack of peroxisomal staining was not accompanied by any detectable staining for these PMPs in the cytoplasm or any other cellular compartment. By contrast, fibroblasts from a Zellweger patient assigned to complementation group E (PBDE), due to the presence of a PEX1 mutation, displayed peroxisomal ghosts on immunofluorescence (fig. A6-2B,J). On Western blot analysis, the PBDE cells contained a similar amount of ALDP as fibroblasts from a healthy control, whereas ALDP was below the detection limit of our assay in the PBDG-01 and PBDG-02 fibroblasts (data not shown). The lack of immunofluorescence for ALDP and PMP70, and the reduced amount of ALDP shown on Western blot analysis, support the hypothesis that these peroxisomal membrane proteins are degraded rather than mislocalized. Analysis of the intracellular localization of another unrelated peroxisomal membrane protein, PEX14, revealed that the fate of this PMP in PBDG differs from that for ALDP and PMP70. Despite the lack of morphologically recognizable peroxisomal membrane staining, the patients’ fibroblasts gave an unequivocal signal when using antibodies to PEX14 indicating that this
protein was mislocalized to mitochondria (fig. A6-2K,L). This finding is consistent with results from the Western blots, where the abundance of PEX14 in PBDG-01 and PBDG-02 cells was not reduced when compared to cells from a healthy control or a PBDE Zellweger patient (data not shown). Taken together, our data are consistent with the hypothesis that in mutants lacking peroxisomal membranes PMPs are either degraded, as proposed for ALDP and PMP70, or mislocalized, as shown for PEX14. Mitochondrial mislocalization of PEX14 has also been reported in PEX19 deficient fibroblasts (Sacksteder et al., 2000) and a similar mitochondrial mislocalization was observed when either PEX3, PEX12, PEX13, PEX11β, or ALDP was overexpressed in these PEX19 deficient cells (Sacksteder et al., 2000). In our native PEX3 deficient cell lines, however, immunofluorescence studies using antibodies to ALDP did not lead to any detectable staining, neither in the peroxisomes nor in the mitochondria, cytoplasm or any other cellular compartment.

As the cell lines of the two patients described above were excellent candidates for a PEX3 mutation we performed PEX3 mutation analysis by direct sequencing of genomic fragments using a slight modification of the previously described technique (Muntau et al., 2000b). The primer sequences are listed in table A6-1. Patient PBDG-01 exhibited an insertion of a thymine in exon 7 at nucleotide 543 of the coding region (fig. A6-3B). This frameshift mutation is expected to result in a truncation of the C-terminal 190 amino acids of the protein (fig. A6-5A).

Sequence analysis of patient PBDG-02 did not reveal any exonic mutation. However, we identified a thymine to guanine transversion in the 3’ acceptor splice site of intron 10. While the conserved polypyrrimidine stretch in the wild type corresponds to the consensus sequence (Padgett et al., 1986), the thymine at –8 relative to exon 11 is replaced by a guanine in the patient (fig. A6-3C). To determine whether the point mutation interferes with correct splicing, RT-PCR was performed. A single PCR product was obtained missing the complete 97 base pairs of exon 11 (fig. A6-3D,E). This exon deletion causes a frameshift with a premature termination after three amino acids predicting a C-terminal truncation of the protein by 56 amino acids. Among the various types of mutations associated with splicing aberrations (Krawczak et al., 1992), 3’ splice site mutations occur less frequently than 5’ splice site mutations and in the majority of cases the invariant AG dinucleotide is involved. Mutations at positions –3 to –14 of the splice site consensus region have rarely been associated with splicing defects, but instances of mutations at position –8 leading to aberrant splicing have already been observed (Beldjord et al., 1988). Additional sequencing analysis confirmed that
the PEX3 intronic change was absent from 50 unrelated healthy individuals (data not shown). For these reasons we conclude that the –8 mutation is the cause of the aberrant splicing in the patient described here.

The mutations identified in patient PBDG-01 and patient PBDG-02 each appeared to be present in a homozygous state, consistent with the consanguinity of both parental couples. Hemizygosity with a deletion of the second allele, however, cannot be excluded as parental DNA was not available.
Figure A6-3: Mutation analyses. A, Genomic organization of the human PEX3 gene. Exons are indicated as boxes. B, Exon 7 sequencing results and predicted protein sequences of a wild type control (wt) and the patient PBDG-01. Note the homozygous thymine insertion (arrow). The frameshift causes a premature stop two codons after the insertion. C, Genomic sequencing of the exon-intron boundary of a control individual (wt) and patient PBDG-02. Note the homozygous substitution of a thymine by a guanine in the 3' splice site of intron 10 at position –8 (arrow). D, PCR amplification of PEX3 fragments from total RNA of PBDG-02 after synthesis of first strand cDNA by reverse transcription using primer 1255R (see table A6-1 for primer sequences). Amplification of a fragment containing exon 5 to exon 10 using the primers 404F and 873R did not reveal any difference between the control individual (wt) and the patient (PBDG-02). The fragment containing exon 5 to exon 11 amplified using primer 404F and the exon 11 specific primer 957R could not be amplified from the patient’s cDNA. Amplification of exon 5 to exon 12 using the primers 404F and 1120R produced an aberrantly sized PCR product from the patient’s cDNA. E, Sequence analysis of the PCR fragment containing exon 5 to exon 12 showed that the PEX3 cDNA of the patient PBDG-02 is missing 97 basepairs, which were present in wild type PEX3 cDNA. The 97-bp deletion corresponds to the sequence of exon 11 and leads to direct fusion of exon 10 to exon 12. Premature termination of the protein is predicted from the frameshift.

The functional impact of the identified mutations was subsequently investigated by complementation studies. Immunofluorescent microscopy analysis of PBDG-01 (fig. A6-4A to F) and PBDG-02 (fig. A6-4G to L) fibroblasts indicated that both cell lines were lacking intact peroxisomes as demonstrated by diffuse staining with antibodies to catalase (fig. A6-4B,H). Five days after transfection of PBDG-01 and PBDG-02 with wild type PEX3 cDNA, antibodies to ALDP (fig. A6-4C,I) and catalase (fig. A6-4D,J) showed a punctate pattern in both cell lines demonstrating rescue of organelle formation. We also investigated whether the identified PEX3 mutations affect the function of the PEX3 protein. This was particularly crucial for the splice site mutation detected in patient PBDG-02. We generated PEX3 expression vectors for each mutated PEX3 cDNA. The resulting plasmids pcDNA3-PBDG-01
and pcDNA3-PBDG-02 were transfected into PBDG-01 and PBDG-02 cells, respectively, and the cells were assayed five days later by indirect immunofluorescence. Transfection of the mutated PEX3 constructs failed to restore peroxisomal biogenesis in the cells (fig. A6-4E,F,K,L). In summary, these results demonstrate that the mutations identified in the PEX3 gene disrupt the function of PEX3.

![Figure A6-4: Functional complementation of patient fibroblasts.](image)

The observation of peroxisomal rescue despite the absence of preexisting peroxisomal membranes is at odds with the favored model of peroxisomal biogenesis where peroxisomes arise exclusively by budding from and/or fission of preexisting peroxisomal vesicles (Lazarow & Fujiki, 1985). Instead, it lends support to the existence of an alternative pathway
of peroxisome biogenesis. There is recent evidence from yeast and human studies that this pathway is likely to involve the interaction of three peroxins, namely PEX3, PEX16, and PEX19 (Sacksteder et al., 2000; South & Gould, 1999). In this regard, it is interesting to note that restoration of peroxisomes after expression of PEX3 in the complementation group G fibroblasts occurred very slowly over several days. The step-wise nature of peroxisome synthesis in mutants deficient in peroxisomal membranes has been observed previously for a PEX16 deficient cell line (South & Gould, 1999), and a PEX19 deficient cell line (Matsuzono et al., 1999) with PMP-containing vesicles becoming visible first, followed by import of peroxisomal matrix proteins. These findings suggest that peroxisomal membrane vesicles form prior to the import of matrix proteins.

How might the PEX3 mutations disrupt early peroxisomal biogenesis? The precise role of PEX3 in the complex processes leading to the assembly of peroxisomes has still to be elucidated. If not from preexisting vesicles, peroxisomes may arise from some other endomembranes of the cell (South & Gould, 1999). Although not proven, it has been hypothesized that PEX3 may be sorted to the peroxisome via the endoplasmic reticulum (ER) (Baerends et al., 1996). It was therefore proposed that PEX3 mediates the early steps of peroxisome formation from the ER (Baerends et al., 1996; Kammerer et al., 1998; Kunau & Erdmann, 1998; Titorenko & Rachubinski, 1998) and mutations in the PEX3 gene could potentially interfere with this process. PEX3 has previously been shown to interact with PEX19 and with PEX16 in yeast (Götte et al., 1998) and in humans (Soukupova et al., 1999; South & Gould, 1999). A PEX3 mutation might lead to disruption of the interaction between these early peroxins. In yeast, this protein-protein interplay appears to be a prerequisite for generating pre-peroxisomal structures that can acquire and stabilize newly synthesized PMPs (Hettema et al., 2000). Thus, a lack of interaction due to a PEX3 mutation could disturb peroxisomal assembly by leading to degradation or mislocalization of other PMPs.

To address this question, we performed PEX3 interaction studies to see whether a PEX3 mutation could lead to a disruption of the interaction between PEX3 and PEX19. For this purpose we performed a pull down assay after in vitro translation of wild type PEX3 cDNA and mutated PEX3 cDNA from patient PBDG-01. In an in vitro binding assay we investigated the ability of wild type PEX3 and the truncated PEX3 protein from patient PBDG-01 to bind to an immobilized GST-PEX19 fusion protein (fig. A6-5B). Wild type PEX3 eluted from the GST-PEX19 fusion protein yielded a strong signal indicating that wild type PEX3 binds PEX19. However, no signal was detected when the interaction of mutated PEX3 with PEX19
was analyzed despite using an excess amount of mutated protein compared to the wild type protein. *In vitro* translation of the wild type sequence yielded two proteins of differing molecular weight (fig. A6-5B,C). To demonstrate that the molecular weight of the lower band in the PEX3 wild type lanes was a result of the utilization of the internal start codon at position 199 of the coding region, we generated a construct lacking the first start codon and the following 63 bp of the PEX3 gene (∆66bp). *In vitro* translation of this construct using the internal start codon yields a protein corresponding to the lower band of wild type PEX3 (fig. A6-5C). In summary, results of the *in vitro* binding assays reported here are consistent with the hypothesis that the interaction between mutated PEX3 and PEX19 is markedly reduced or absent. While this does not necessarily reflect the situation *in vivo*, the potential functional impairment might contribute to impaired peroxisomal membrane synthesis in cells from patient PBDG-01.

**Figure A6-5: PEX3 in vitro binding assays.** A, Schematic diagram of wild type PEX3 and truncated PEX3 of patient PBDG-01. The mutation in PBDG-01 results in a C-terminally truncated PEX3 protein while predicted transmembrane regions (TM) remain unaffected. B, Pull down assays of *in vitro* translated PEX3 using immobilized GST-PEX19 were performed as previously described (Gloeckner et al., 2000). The left hand gel was loaded with 10% of the 35S-methionine-labelled PEX3 translation products used as input for the binding assays. To ensure that even weak binding of the mutated PEX3 would be detected, the amount of mutated PEX3 was chosen so that it clearly exceeded the amount of wild type PEX3. Wild type PEX3 (wt) was bound by GST-PEX19, whereas no detectable signal was obtained when the interaction of the mutated PEX3 protein (PBDG-01) with PEX19 was analyzed (central gel). GST alone did not bind to either protein (right hand gel). Several repetitions of the assay yielded similar results. C, To demonstrate that the molecular weight of the lower band in the PEX3 wild type lanes is a result of the utilization of the internal start codon at position 199 of the coding region, we generated a construct lacking the first start codon and the following 63 bp of the PEX3 gene (∆66bp). *In vitro* translation of this construct using the internal start codon yielded a protein corresponding to the lower band of wild type PEX3.
The molecular and biochemical evidence shown here leaves little doubt that PEX3 mutations are responsible for Zellweger syndrome in complementation group G patients. Both patients display a very severe Zellweger syndrome phenotype with all tested peroxisomal functions being deficient. A correlation between the severity of the clinical phenotype and biochemical activities such as plasmalogen synthesis (Lazarow & Moser, 1995; Roscher et al., 1985) as well as the extent of morphological changes such as the number of residual peroxisomes in tissues (Arias et al., 1985) has previously been described for PBD patients. Therefore, it is not surprising that we observed a severe clinical phenotype associated with mutations in the PEX3 gene, which encodes a protein considered to be important in the very early steps of peroxisomal biogenesis.

We have shown that, by expressing wild type PEX3, intact peroxisomes are formed in the PEX3 mutant cell lines, which had lacked detectable peroxisomal membrane structures. Similar observations have recently been made for human cells bearing mutations in either PEX16 or PEX19 (Matsuzono et al., 1999; South & Gould, 1999). The combined data provide evidence for the essential role of these three early peroxins in a pathway for human peroxisome formation that does not require morphologically detectable preexisting peroxisomal structures. Besides supplying the basis for molecular diagnostics, the identification of PEX3 mutations in the human provides an important model for elucidating a fundamental mechanism in human organelle biosynthesis.

Acknowledgements

The initial cell fusion analysis, which defined the complementation group for patients PBDG-01 and PBDG-02 was carried out by N. Shimozawa, Y. Suzuki, T. Orii and their colleagues at the Gifu University School of Medicine. The PEX19 deficient cell line was kindly provided by R. Wanders. We thank W. Just and G. Dodt for providing antibodies. We thank T. Kattenfeld and F. Lagler for excellent technical assistance. We are grateful to T. Meitinger and A. Holzinger for helpful suggestions and discussions. This work was in part supported by a grant from the Deutsche Forschungsgemeinschaft (R0 727/1-1).

Electronic-Database Information

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for PBD [MIM 601539], for RCDP [MIM 215100], for PEX3 [MIM 603164]).
References

The references are listed on pages 38–49.

Table A6-1: Primers for mutation analysis and RT-PCR of the human *PEX3* gene

<table>
<thead>
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<th>Method and Exon</th>
<th>Primer Name(^a)</th>
<th>Sequence (5’→3’)</th>
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<tr>
<td>Exon 1</td>
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<td>GCTCCATTGTCAACAGATC</td>
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\(^a\) F: forward; R: reverse
Abbreviations

AAA ATPase ATPases associated with various cellular activities
ABC transporter ATP binding cassette transporter
ALD(P) adrenoleukodystrophy (protein)
ALDR(P) adrenoleukodystrophy related (protein)
CaaX-box C-terminal farnesylation consensus sequence
CG(s) complementation group(s)
CoA coenzyme A
COP coat protein complexes
DHAP(AT) dihydroxyacetonephosphate (acyltransferase)
ER endoplasmic reticulum
GFP green fluorescent protein
Graf GTPase regulator associated with focal adhesion kinase
GST glutathione-S-transferase
HK33 housekeeping gene encoding a 33kDa protein; now termed PEX19
IRD infantile Refsum disease
MCFA medium chain fatty acids
NALD neonatal adrenoleukodystrophy
NBF nucleotide binding fold
PAS2 Pichia pastoris peroxisome-assembly mutant No. 2, now termed PEX3
PAS3 Saccharomyces cerevisiae peroxisome-assembly mutant No. 3, now termed PEX3
PBDs peroxisomal biogenesis disorders
PBDG-01/02 Zellweger patients 1/2, assigned to CG-G
PER9 Hansenula polymorpha peroxisome-assembly mutant No. 9, now termed PEX3
PEX peroxisomal assembly gene
PEX peroxisomal assembly protein
PEX3 peroxisomal assembly protein 3
PEX19 peroxisomal assembly protein 19
PEX19AE2 PEX19 splice variant lacking exon 2
PEX19pAE8 PEX19 splice variant lacking parts of exon 8
PMP69 69 kDa peroxisomal membrane protein
PMP70 70 kDa peroxisomal membrane protein
PPAR peroxisome proliferator-activated receptor
PMP(s) peroxisomal membrane protein(s)
PRNK PYK2-related non-kinase
PTS peroxisomal targeting sequence
PX1/2 Saccharomyces cerevisiae peroxisomal ABC-transporter 1/2
PxT peroxisomal farnesylated protein, now termed PEX19
PYK2 proline-rich tyrosin kinase 2
RCDP rhizomelic chondrodysplasia punctata
SH3 src-homology domain 3
TM(D) transmembrane (domain)
TPR tetratricopeptide
Ub ubiquitin
VLCFA very-long-chain fatty acids
X-ALD X-linked Adrenoleukodystrophy
ZS Zellweger syndrome
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List of Publications


Oral Presentations


Citable Abstracts


Poster Presentations

Mayerhofer PU, Maier EM, Kattenfeld T, Berger J, Roscher AA, and Holzinger A (2001) No evidence for adrenoleukodystrophy-related protein (ALDRP) acting as modifier gene in a large X-linked adrenoleukodystrophy kindred with various phenotypes. 3rd FEBS Advanced Lecture Course: „ATP-Binding Cassette (ABC) Proteins: From Genetic Disease to Multidrug Resistance“; Gosau, Austria


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