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Pex5p, the Peroxisomal Cycling Receptor, Is a Monomeric Non-globular Protein*

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In mammals, targeting of newly synthesized peroxisomal matrix proteins to the organelle requires Pex5p, the peroxisomal cycling receptor. Pex5p is a multidomain protein involved in a complex network of transient protein-protein interactions. Besides interacting directly with most peroxisomal proteins en route to the organelle, Pex5p has also binding domains for several components of the peroxisomal docking/translocation machinery. However, our knowledge of how binding of a cargo protein to Pex5p influences its properties is still rather limited. Here, we describe a protease assay particularly useful for identifying and characterizing protein-protein interactions involving human Pex5p. Binding of a PTS1-containing peptide/protein to Pex5p as well as the interaction of this peroxin with the Src homology domain 3 of Pex13p could be easily demonstrated using this assay. To address the possible effects of these Pex5p-interacting peptides/proteins on the assumed quaternary structure of Pex5p, we have analyzed the hydrodynamic properties of human Pex5p using size exclusion chromatography, sucrose gradient centrifugation, and sedimentation equilibrium centrifugation. Our results show that Pex5p is a monomeric protein with an abnormal shape. The implications of these findings on current models of protein translocation across the peroxisomal membrane are discussed.

Peroxisomal matrix proteins are synthesized by cytosolic ribosomes and posttranslationally imported into the organelle. Specific targeting of these proteins to the peroxisome is promoted by one of two receptors, Pex5p or Pex7p (reviewed in Refs. 1–4). By far the vast majority of these proteins use Pex5p as their import receptor. Proteins belonging to this family possess a tripeptide with the sequence SKL (or a variant) at their C termini, the so-called peroxisomal targeting signal type 1 (PTS1)[1] (5–7). The Pex5p domain involved in this interaction is now well characterized. It comprises six TPR domains present in the C-terminal half of the protein (6, 8–13).

PTS1-containing proteins interact with Pex5p while still in the cytosol (reviewed in Refs. 1–4). The recognition of this cytosolic Pex5p-cargo protein complex by the peroxisomal docking/translocation machinery is the next step in the protein transport pathway mediated by Pex5p. Pex13p and Pex14p as well as the RING finger peroxins Pex2p, Pex10p, and Pex12p are components of this docking/translocation machinery (14–16). The third step in this pathway has been the subject of much controversy. According to some researchers, Pex5p is translocated completely across the peroxisomal membrane together with the cargo it transports (17–19). The other perspective is that the Pex5p protein is inserted into the peroxisomal membrane without crossing it completely. In this model, the cargo protein-binding domain of Pex5p reaches the lumen of the organelle where it releases the cargo protein (reviewed in Refs. 20 and 21). Regardless of the true mechanism by which matrix proteins are translocated across the peroxisomal membrane, it is consensually accepted that Pex5p returns to the cytosol to catalyze further rounds of transportation. This last step (the recycling event) is probably the only one requiring energy in the form of ATP hydrolysis (22).

The data supporting this general mechanism, the so-called cycling receptor model, are now numerous (18, 23, 24). However, it is evident that our knowledge on many of the "details" of this pathway is still quite poor, leaving room for controversies between researchers in the field. These controversies are not restricted to the translocation step as already stated above. Another point of discussion regards the nature of the Pex5p molecule(s) that interact(s) with the docking/translocation machinery. In principle, formation of a heterodimeric complex involving a Pex5p molecule on one side and one cargo protein on the other should suffice to ensure specific targeting of proteins to the peroxisomal compartment. However, this simple idea has been challenged by a more complex model in which Pex5p is seen as a homotetramer and thus capable of binding up to four PTS1-containing cargo proteins (21). Because many peroxisomal matrix proteins are oligomers, it was proposed that multivalent interactions would be established between Pex5p and the proteins en route to the peroxisomal matrix, leading to the generation of huge protein complexes. Presumably, these large protein complexes would be formed at the surface of the organelle, resulting in an increase in local concentration of the cargo proteins that would facilitate their translocation across the peroxisomal membrane. More recently, a refined version of this "pre-implex model" has been proposed (25). It was suggested that in *Hansenula polymorpha* cargo proteins are actually translocated across the peroxisomal membrane by tetrameric Pex5p. After releasing the cargo pro-

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[1] The abbreviations used are: PTS, peroxisomal targeting sequence; GST, glutathione *S*-transferase; TPR, tetratricopeptide repeat; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); SH3, Src homology domain 3; DTT, dithiothreitol.

teins in the matrix of the peroxisome, Pex5p would monomerize and return to the cytosol. Interestingly, a model in which Pex5p also oscillates between a tetrameric and a monomeric/dimeric form has been described recently in *Leishmania donovani* (26). In this case, however, it was proposed that *L. donovani* Pex5p mono- or dimerizes upon binding PTS1-containing proteins and tetramerizes when leaving the peroxisomal compartment.

In this work, we have studied the properties of human Pex5p when bound to a PTS1-containing peptide/protein. Data suggesting the existence of PTS1-induced conformational alterations on human Pex5p were obtained when *in vitro* synthesized and recombinant Pex5p were subjected to partial proteolysis. To determine whether binding of a PTS1-containing peptide/protein modulates in some way the assumed oligomeric state of human Pex5p, we have characterized the hydrodynamic properties of this peroxin. In agreement with previous data, human Pex5p was found to behave as a 270-kDa globular protein upon size exclusion chromatography (27, 28). Strikingly, however, this species represents monomeric Pex5p and not homotetramers, as assumed previously. The abnormal behavior of Pex5p on size exclusion chromatography is just the result of a high frictional ratio, a property that seems to derive from its N-terminal half. The implications of these findings for the mechanism of protein translocation across the peroxisomal membrane are discussed.

MATERIALS AND METHODS

Synthesis of ^{35}S-labeled Pex5p Proteins—The cDNAs encoding full-length human Pex5p (the large isoform; Ref. 29) or the C-terminal-truncated version comprising amino acid residues 1–324 of Pex5p (ΔC1-Pex5p) preceded by the T7 RNA polymerase promotor were obtained as described previously (30, 31). To obtain a cDNA encoding a N-terminal-truncated version of Pex5p lacking the first 204 amino acid residues (ΔN204-Pex5p), the plasmid pGEM-4-Pex5 (31) was subjected to PCR using the primers 5′-gcggtcgacatggtggatgaccccaaattggcta-3′ and 5′-gcggtcgactcactggggcaggccaaacata-3′. The amplified fragment was cloned into pGEM®-T Easy Vector according to the manufacturer's instructions (Promega). The recombinant plasmid was digested with SalI, the insert was cloned into the SalI site of pGEM-4 (Promega), and the resulting plasmid was linearized by cutting it with NheI. These Pex5p-encoding DNAs were subjected to *in vitro* transcription using T7 RNA polymerase (Roche Applied Science). ^{35}S-labeled proteins were synthesized using the translation kit Retic Lysate IVT™ (Ambion) in the presence of Redivue™ L-[^{35}S]methionine (specific activity >1000 Ci/mmol) following the manufacturer's instructions.

Recombinant Proteins—For expression of the fusion protein His$_6$-PEX5p, the plasmid pGEM-4-Pex5 (31) was used as template in a PCR reaction using the forward primer 5′-ccggcatgcgcaatgcgggagctggtgg-3′ and the reverse primer 5′-gcggtcgactcactggggcaggccaaacat-3′. The amplified DNA fragment was cloned into pGEM®-T Easy Vector according to the manufacturer's instructions (Promega). The recombinant plasmid was digested with SphI and SalI, and the insert was cloned into pQE-30 (Qiagen), resulting in the pQE-Pex5 plasmid. Expression was performed in the M15 strain of *Escherichia coli*. 100-ml cultures were induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 2 h at 37 °C. Pelleted cells were cooled on ice and lysed by sonication in 1.5 ml of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% (w/v) Triton X-100, 1 mM EDTA-NaOH, pH 8.0, 1 mM DTT, 0.1 mg/ml phenylmethylsulfonyl fluoride, and 1:500 (v/v) mammalian protease inhibitor mixture (Sigma). Cell debris were removed by centrifugation (15 min at 10,000 × *g*), and the clarified supernatant was incubated with 100 μl (bed volume) of HIS-Select™ nickel affinity gel (Sigma) for 2 h at 4 °C. The gel was washed three times with 1.5 ml of 50 mM sodium phosphate, pH 8.0, 150 mM NaCl, and the His$_6$-PEX5p was eluted by washing the beads three times with 600 μl of 50 mM sodium phosphate, pH 8.0, 150 mM NaCl, 50 mM imidazole. The eluted protein was concentrated to ~50 μl using a Vivaspin 10,000 MWCO PES concentrator (Vivascience), diluted to 600 μl with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA-NaOH, pH 8.0, 1 mM DTT, and concentrated again. This procedure was repeated two more times. Aliquots of the His$_6$-PEX5p recombinant protein were frozen in liquid nitrogen and stored at −70 °C.

To test whether the introduction of this purification tag at the N terminus of human Pex5p interferes with its function, the plasmid pQE-Pex5 was subjected to an expression PCR protocol (32). In the first PCR the upper primer 5′-gggagagccaccatgagaggatcgcatcac-3′ and the lower primer 5′-gcgtaattaagcttggctgcaggtc-3′ were used. In the second PCR the same lower primer was used together with the primer 5′-gaattctaatacgactcactatagggagagccaccatg-3′. The amplified fragment was subjected to *in vitro* transcription/translation as described above, and the ^{35}S-labeled His$_6$-tagged Pex5p was used in *in vitro* import experiments exactly as described before (23). These experiments revealed that His$_6$-tagged Pex5p is inserted into the peroxisomal membrane originating Stage 2 Pex5p (when ATP was included in the import reactions) or Stage 2 plus Stage 3 Pex5p (when ATPγS was used). Furthermore, addition of a GST fusion protein containing the TPR domains of Pex5p to the import medium blocked the insertion of this His$_6$-tagged protein into the organelle membrane (data not shown), as described previously for the untagged human Pex5p (see Refs. 23 and 30). These data suggest that the modified N terminus does not inactivate Pex5p. The synthesis and purification of GST-LKS (GST ending with a nonfunctional PTS1-like sequence), GST-SKL (GST containing a PTS1 signal at the C terminus), and the histidine-tagged SH3 domain of human Pex13p (amino acid residues 236–403; Pex13p-SH3) were described before (30, 33).

Protease Assay—Protease treatment of Pex5p was performed in buffer A (50 μl of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1 mM DTT). One μl of the reticulocyte lysates containing the ^{35}S-labeled Pex5p proteins or 5 μg of His$_6$-PEX5p recombinant protein were used per reaction. Peptides (see below) and GST-LKS or GST-SKL were added to 2 μM final concentration. Pex13p-SH3 was used at a final concentration of 1.5 μM. After 10 min at 37 °C, the samples were placed on ice and treated for 15 min with 0.5 μg/ml (for radioactive proteins) or 1 μg/ml (for recombinant Pex5p) of proteinase K. The protease was inactivated with phenylmethylsulfonyl fluoride (0.5 mg/ml), and the proteins were precipitated with trichloroacetic acid (10% (w/v)), washed with acetone, and analyzed by SDS-PAGE.

Sucrose Gradient Centrifugation—20 μg of recombinant Pex5p or 3 μl of the reticulocyte lysates containing radioactive Pex5p or ΔC1-Pex5p were incubated in 200 μl of buffer A in the presence of 2 μM GST-LKS or GST-SKL for 10 min at 37 °C. Trace amounts (~2 μg) of bovine IgGs (6.9 *s*), bovine serum albumin (4.3 *s*), soybean trypsin inhibitor (2.3 *s*), and cytochrome *c* (1.9 *s*) were added to the samples as internal standards (numbers in parenthesis represent the sedimentation coefficients). These mixtures were then applied onto the top of a discontinuous sucrose gradient (2.0 ml of 4%, 1.8 ml of 8%, 1.7 ml of 12%, 1.5 ml of 16%, 1.2 ml of 20%, 1 ml of 25%, and 1 ml of 30% (w/v) sucrose in buffer A supplemented with 2 μM GST-LKS or GST-SKL. After centrifugation at 38,000 rpm for 20 h at 4 °C in a TST41.14 swing-out rotor (Sorvall), 13 fractions of 0.8 ml were collected from the bottom of the tube and analyzed by SDS-PAGE. The sedimentation coefficients of the Pex5p proteins were estimated by interpolation from the linear plots of the *s* values of the standard proteins *versus* their gradient fractionation volume.

Size Exclusion Chromatography—Human Pex5p recombinant protein (20 μg of protein) or *in vitro* synthesized proteins (1 μl of the corresponding reticulocyte lysates) were incubated in 200 μl of buffer A containing 40 μM peptides (see below) or 10 μM GST-SKL or GST-LKS for 10 min at 37 °C. Protein samples were injected into a Superose 12 HR 10/30 column (Amersham Biosciences) running with 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM EDTA-NaOH, pH 7.5, at 0.5 ml/min. The inclusion of 1 mM DTT in this running buffer did not change the elution profile of the Pex5p proteins (see Ref. 28; data not shown). The column was calibrated with the following standards (numbers in parenthesis represent the Stokes radii): thyroglobulin (8.5 nm), ferritin (6.1 nm), aldolase (4.8 nm), bovine serum albumin (3.6 nm), and soybean trypsin inhibitor (2.3 nm). Fractions of 500 μl covering the relevant Stokes radius range were always collected, subjected to trichloroacetic acid precipitation, and analyzed by SDS-PAGE. In some experiments with the recombinant protein the A_{280} profile of the chromatography revealed the existence of one or two extra protein peaks (corresponding to globular proteins with molecular masses of 130 and 60 kDa) in addition to the "270 kDa" species. Analysis of the corresponding SDS gels showed that only the "270 kDa" peak correlated with His-tagged Pex5p; the other peaks were due to the presence of Pex5p proteolytic fragments (data not shown). These Pex5p preparations were discarded. The Stokes radii of the Pex5p proteins were determined as described (34).

Sedimentation Equilibrium Centrifugation—Recombinant Pex5p (200 μg) was subjected to size exclusion chromatography as described above. Fractions containing the "270-kDa" protein peak were pooled and concentrated to 1 mg/ml using a Vivaspin 10,000 MWCO PES

concentrator (Vivascience). Sedimentation equilibrium analysis was performed at the National Centre for Macromolecular Hydrodynamics at the University of Nottingham (UK) using a Beckman XL-A analytical ultracentrifuge equipped with scanning absorption optics.

The same analysis was performed using a different version of human Pex5p recombinant protein purified as described before (28). In this protein there is a tobacco etch virus protease cleavage site between the N-terminal His tag and the Pex5p sequence. Sedimentation equilibrium analysis of this recombinant protein (0.4 mg/ml in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 15 mM $MgCl_2$, 1 mM DTT) was performed in a Beckman XL-A analytical ultracentrifuge at European Molecular Biology Laboratory, Heidelberg, Germany.

Miscellaneous—Molecular masses of native proteins (M) and frictional ratios (f/f_o) were calculated from their Stokes radii and sedimentation coefficients as described in Ref. 35 using Equations 1 and 2

$$M = 6\pi\eta Nas/(1 - \upsilon\rho) \quad \text{(Eq. 1)}$$

and

$$f/f_o = a/(3\upsilon M/4\pi N)^{1/3} \quad \text{(Eq. 2)}$$

where η is the viscosity of the medium, N is the Avogadro's number, a is the Stokes radius, s is the sedimentation coefficient, υ is the partial specific volume of the protein, and ρ is the density of the medium.

Partial specific volumes for Pex5p proteins and GST-SKL were calculated using the program SEDNTERP v1.08 (www.jphilo.mailway-.com/default.htm). Edman degradation of Pex5p proteolytic fragments was performed by HHMI/Keck Biotechnology Resource Laboratory (New Haven, CT). The peptides CRYHLKPLQSKL (Pep-SKL) and CRYHLKPLQLKS (Pep-LKS) were synthesized by Sigma Genosys. The peptide YQSKL was a kind gift from Dr. W. Nastainczyk, University of Saarland, Germany.

RESULTS

Controlled digestion of a protein using proteases may reveal important insights on its structure. In many cases, such assays can even be used to characterize ligand-induced conformational alterations on the protein being studied (*e.g.* Ref. 36). Naturally, such strategy can also be applied to the identification of putative ligands of a given protein even if the ligands being tested are proteins themselves. In this case, however, positive results may only be obtained if the protein under study is much more sensitive to the protease than the ligands added to the assay, something that can be easily determined empirically.

Using this rationale we have developed a simple procedure to identify and characterize protein-protein interactions involving human Pex5p. As shown below, because of its high sensitivity to proteases (see also Ref. 37) Pex5p is a suitable protein to use in this kind of assay. The procedure consists of incubating human Pex5p (obtained either from *in vitro* transcription/translation reactions or from heterologous expression in *E. coli*) with the test proteins/peptides, followed by limited proteolysis with proteinase K (see "Materials and Methods" for details).

Binding of a PTS1-containing Peptide/Protein to Human Pex5p Changes the Accessibility of Proteinase K to a Region of the Pex5p Protein That Precedes Its PTS1-binding Domain—We started our analysis by characterizing the interaction of Pex5p with PTS1-containing proteins/peptides. *In vitro* synthesized ^{35}S-Pex5p was incubated with either a PTS1-containing peptide (pep-SKL) or with a negative control peptide (pep-LKS; see "Materials and Methods") and subjected to treatment with a very low concentration (0.5 μg/ml) of proteinase K. After inactivation of the protease the samples were analyzed by SDS-PAGE and autoradiography. As shown in Fig. 1*A*, *lanes 1* and *2*, different proteolytic patterns are observed suggesting that Pex5p bound to pep-SKL displays a different susceptibility to proteinase K. The most evident differences regard the appearance of a 40-kDa doublet protein band when pep-SKL is used in these experiments and a 35-kDa protein band that is particularly abundant when the control peptide is used. The 40-kDa doublet band was also observed when a shorter PTS1-containg peptide (YQSKL) was used in these experiments (data not shown). Exactly the same results were obtained when GST-SKL and GST-LKS were substituted for pep-SKL and pep-LKS, respectively (Fig. 1*A*, *lanes 3* and *4*).

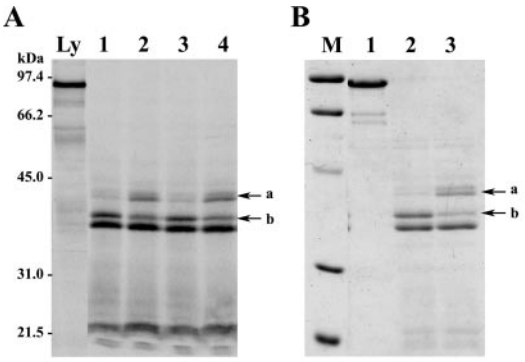

FIG. 1. **Binding of a PTS1-containing peptide/protein to Pex5p changes its proteolytic profile.** *A*, *in vitro* synthesized Pex5p was incubated in the presence of pep-LKS (*lane 1*), pep-SKL (*lane 2*), GST-LKS (*lane 3*), or GST-SKL (*lane 4*) for 10 min at 37 °C. After treatment with proteinase K (see "Materials and Methods") the samples were analyzed by SDS-PAGE and autoradiography. The 40- and the 35-kDa bands are indicated by *arrows a* and *b*, respectively. *Lane Ly*, 0.5 μl of the Pex5p-containing reticulocyte lysate. Numbers at left side indicate the molecular mass of the protein markers. *B*, recombinant Pex5p was incubated in the presence of either pep-LKS (*lane 2*) or pep-SKL (*lane 3*) and subjected to treatment with proteinase K. A Coomassie blue-stained gel is shown. *Arrows a* and *b* indicate the 40- and 35-kDa protein bands, respectively. *Lane 1*, untreated recombinant protein (2 μg). *M*, molecular mass standards as shown in *panel A*.

To characterize the Pex5p-derived fragments described above, we repeated the protease assay but this time using chemical amounts of recombinant human Pex5p. The results presented in Fig. 1*B* demonstrate that recombinant Pex5p displays the behavior of the *in vitro* synthesized peroxin. N-terminal sequencing of the 40-kDa doublet protein band generated a mixture of two peptide sequences, as expected (for practical reasons, no efforts were made to resolve the two protein bands in order to obtain independent sequences). The sequences were easily ascribed to amino acid residues 291–296 and 295–300 of human Pex5p. Edman degradation of the 35-kDa protein band also resulted in a mixture of two peptide sequences, indicating the presence in this band of two Pex5p fragments that probably also differ at their C termini. One of these fragments starts with the sequence WLSDYD (residues 308–313) and the other with TYDKDG (residues 319–324) of human Pex5p. Taken together these data indicate that binding of a PTS1-containing peptide/protein to Pex5p changes the accessibility of proteinase K to peptide bonds linking amino acid resides 307–308 and 318–319 of human Pex5p. These amino acid residues precede the first TPR domain of human Pex5p (11) by 30 and 19 amino acid residues, respectively.

Characterization of the Pex5p-Pex13p Interaction—Pex13p is a component of the peroxisomal docking/translocation machinery that contains a C-terminal SH3 domain facing the cytosolic side of the peroxisomal membrane (reviewed in Refs. 1–4). It has been demonstrated that Pex5p from lower eukaryotes interacts directly with the SH3 domain of Pex13p (38–41). In mammals, however, this subject has remained obscure. First, using yeast two-hybrid technology both positive and negative results have been described for this pair of proteins (39, 42). Second and most importantly, all attempts to detect the existence of a direct interaction between mammalian Pex5p and the SH3 domain of Pex13p using several *in vitro* assays have failed

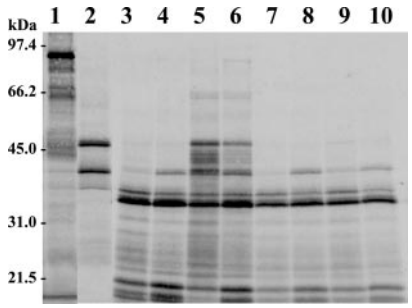

FIG. 2. **Proteolytic profiles of Pex5p in the presence of Pex13-SH3.** *In vitro* synthesized Pex5p (*lanes 3–6*) or ΔN204-Pex5p (*lanes 7–10*) were incubated with either pep-LKS (*lane 3*, *5*, *7*, and *9*) or pep-SKL (*lanes 4*, *6*, *8*, and *10*) in the absence (*lanes 3*, *4*, *7*, and *8*) or presence of Pex13-SH3 (*lanes 5*, *6*, *9*, and *10*). After proteinase K treatment, the samples were analyzed by SDS-PAGE and autoradiography. *Lanes 1* and *2*, reticulocyte lysates containing human Pex5p and ΔN204-Pex5p, respectively. *In vitro* synthesis of ΔN204-Pex5p results in two major products. The faster migrating band is probably the result of translation initiation at an internal ATG. Numbers at left side indicate the molecular mass of the protein markers.

(33, 43). It should be emphasized that mammalian Pex5p does interact with the complete version of Pex13p, as demonstrated recently by pulldown experiments (43). Interestingly, the Pex5p domain mapped in those experiments corresponds to the SH3-binding domain of Pex5p from lower eukaryotes. However, when GST fusion proteins containing different domains of Pex13p were applied to these assays positive results were only obtained with a recombinant protein comprising amino acid residues 1–135 of human Pex13p (43).

As an attempt to clarify this issue, two GST fusion proteins containing either amino acid residues 1–135 or 255–403 of human Pex13p were produced and used in our protease assay. In both cases, negative results were obtained (data not shown). Obviously, as stated above, negative results in this assay may result from the fact that the ligands being tested are particularly sensitive to the protease. Nevertheless, the possibility that the presence of a GST moiety at the N terminus of the SH3 domain of Pex13p interferes with the binding of this fusion protein to Pex5p was still considered. Thus, an N-terminal histidine-tagged version of the SH3 domain of human Pex13p (Pex13-SH3) was used in these assays. As shown in Fig. 2, proteinase K treatment of ^{35}S-Pex5p preincubated with Pex13-SH3 results in major differences in the proteolytic pattern of Pex5p. Interestingly, the degree of Pex13-SH3-induced protection is particularly evident in the absence of PTS1-containing peptide (compare *lanes 5* and *6*), corroborating the idea that Pex13p binds with a higher affinity to free Pex5p than to cargo protein-loaded Pex5p (40, 43). To verify the specificity of the observed interaction, we included in these experiments a truncated version of Pex5p lacking amino acid residues 1–204 (ΔN204-Pex5p). This protein lacks the Pex13p-interacting domain defined previously (43) but is still functional in binding the PTS1-containing peptide (compare *lanes 3* and *4* with *lanes 7* and *8*). No Pex13-SH3-induced protection effect was observed in this case (*lanes 9* and *10*). We conclude that the SH3 domain of human Pex13p interacts specifically with Pex5p and thus that the type of interaction between these two peroxins was conserved during evolution.

Human Pex5p Is a Monomeric Protein—Considering the growing number of reports claiming that Pex5p from several organisms oscillates between a tetrameric and a monomeric state (see "Discussion"), the protein-protein interactions described above cannot be rationalized in a simple way. For instance, the observed effect of a PTS1-containing peptide/protein upon the sensitivity of human Pex5p to proteinase K could simply result from a change in the quaternary structure of Pex5p, as suggested recently for Pex5p from *L. donovani* (26). To clarify this issue we have determined the molecular mass of the Pex5p proteins used in this work. Two different approaches were used. In the first, we used the strategy developed by Siegel and Monty (35). In this method the sedimentation coefficient (determined by sucrose gradient centrifugation analysis) and the Stokes radius of a protein (determined by size exclusion chromatography) are used to calculate its molecular mass. In the second, we used equilibrium sedimentation analysis. Here, the molecular mass of a protein is determined directly and is a function of both its sedimentation and diffusion coefficients.

We started these studies by determining the sedimentation coefficient of human Pex5p. Particular attention was given to the resolution properties of the sucrose gradients; protein samples in a small volume (200 μl) were carefully applied onto the top of the gradients. GST-SKL or the corresponding negative control, GST-LKS, was included in the sucrose solutions (2 μM each) used to make the gradients. In each experiment, two sucrose gradients were run in parallel. One, containing GST-SKL, was loaded with Pex5p preincubated with GST-SKL; the other, containing GST-LKS, received the same amount of Pex5p preincubated with GST-LKS.

The results obtained with recombinant human Pex5p are shown in Fig. 3*A*. In the absence of a PTS1-containing protein ("plus GST-LKS" conditions), recombinant Pex5p (predicted molecular mass of 72.3 kDa) behaves as a 40-kDa globular protein (sedimentation coefficient of 3.68 ± 0.18 *s*), sedimenting well above bovine serum albumin (MW 66.2; sedimentation coefficient of 4.31 *s*). This abnormal sedimentation behavior indicates that Pex5p is not a globular/spherical protein. A different sedimentation coefficient is observed when this analysis is performed in the presence of GST-SKL. Under these conditions, Pex5p behaves as a 90-kDa globular protein (sedimentation coefficient of 5.0 ± 0.27 *s*), sedimenting slightly below albumin, indicating that Pex5p formed a complex with GST-SKL. It is important to note that the vast majority of the recombinant Pex5p protein displays this behavior, indicating the absence of significant amounts of misfolded Pex5p in our protein preparations.

The sedimentation properties of *in vitro* synthesized human Pex5p were also analyzed. In the experiment described here, a truncated version of human Pex5p (ΔC1-Pex5p) that lacks the PTS1-binding domain of the peroxin was also included as a negative control. As shown in Fig. 3*B*, *in vitro* synthesized Pex5p displays the sedimentation properties of the recombinant protein: an increase in the sedimentation coefficient is observed when GST-SKL is used in these experiments. As expected, the sedimentation behavior of ΔC1-Pex5p is the same independent of the GST fusion protein used in these analyses. In the presence of GST-SKL or GST-LKS, ΔC1-Pex5p (predicted molecular mass of 37.1 kDa) was found to sediment together with soybean trypsin inhibitor, displaying a sedimentation coefficient of 2.4 ± 0.13 *s*.

We next subjected these Pex5p proteins to gel filtration chromatography. The results obtained with the recombinant protein are shown in Fig. 4*A*. Pex5p preincubated with GST-LKS or pep-LKS elutes from the column after ferritin (MW 440; Stokes radius of 6.1 nm) and before aldolase (MW 158; Stokes radius of 4.8 nm). A Stokes radius of 5.33 ± 0.21 nm was estimated for this recombinant protein. Basically the same value is obtained when Pex5p is preincubated with pep-SKL. In contrast, when Pex5p is incubated with GST-SKL prior to chromatography, a small decrease in the elution volume of the recombinant protein is observed, suggesting that a Pex5p-GST-SKL complex was formed and resisted the separation proce-

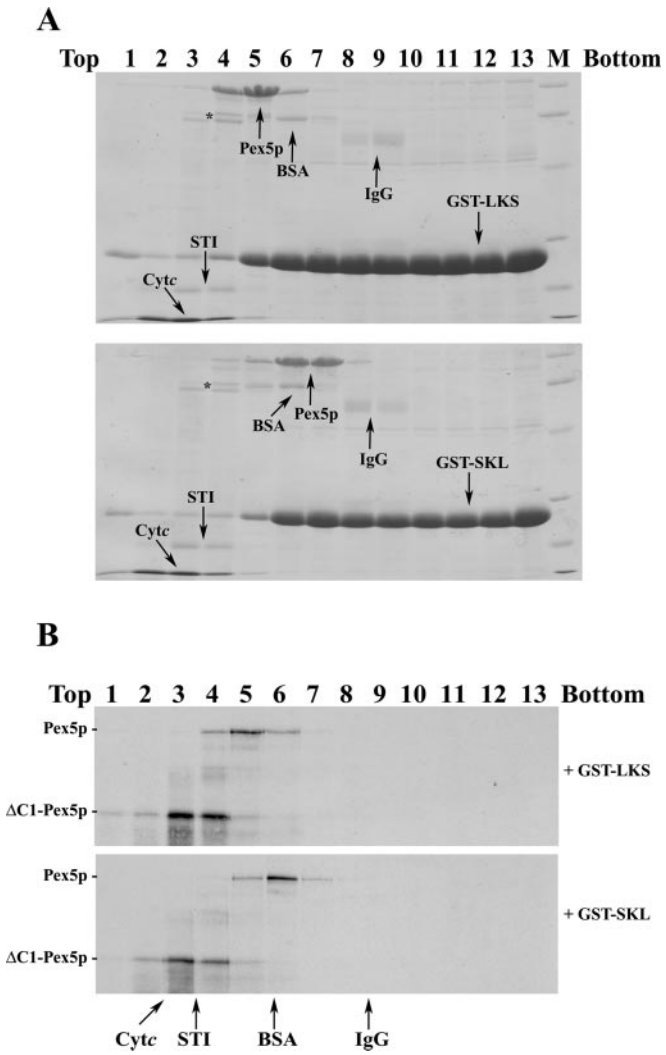

FIG. 3. **Sucrose gradient sedimentation analysis of Pex5p.** *A*, recombinant human Pex5p was preincubated with either GST-LKS (*upper panel*) or GST-SKL (*lower panel*). A mixture of proteins with known sedimentation coefficients was added to both samples before applying them onto the top of the sucrose gradients containing either GST-LKS (*upper panel*) or GST-SKL (*lower panel*). After centrifugation, fractions were collected from the bottom of the tube and analyzed by SDS-PAGE. Pex5p, the GST fusion proteins, and the sedimentation standards are indicated by *arrows*. The internal sedimentation standards used were: cytochrome *c* (*Cytc*), soybean trypsin inhibitor (*STI*), bovine serum albumin (*BSA*), and immunoglobulins (*IgG*). Only the heavy chains of IgGs are visible in the gels; the light chains, which co-sediment with the heavy chains, are partially masked by the GST fusion proteins. The *asterisk* indicates two protein bands present in some preparations of recombinant Pex5p. Considering that these two polypeptides are recognized by an anti-Pex5p antibody (data not shown) and that they were purified exploring the presence of a N-terminal histidine tag, they probably represent C-terminal-truncated Pex5p molecules. The similarity between their sedimentation behavior and the one displayed by ΔC1-Pex5p (see *panel B*) is evident. Molecular mass standards (from *top to bottom*): 97.4, 66.2, 45.0, 31.0, 21.5, 14.4 kDa. *B*, *in vitro* synthesized Pex5p and ΔC1-Pex5p were preincubated with either GST-LKS (*upper panel*) or GST-SKL (*lower panel*). A small amount of sedimentation standards (see above) was added to both samples. The mixtures were loaded onto the top of the sucrose gradients containing either GST-LKS (*upper panel*) or GST-SKL (*lower panel*). After centrifugation, fractions were subjected to SDS-PAGE, blotted onto a nitrocellulose membrane, and exposed to an x-ray film. The internal sedimentation standards (indicated at the bottom of the figure) were visualized on the Ponceau S-stained membrane.

dure. SDS-PAGE analysis of the chromatography fractions confirmed this interpretation (see Fig. 4*B*). A stoke radius of 5.86 ± 0.31 nm was estimated for this protein complex.

Analysis of ^{35}S-labeled Pex5p by size exclusion chromatography revealed essentially the same behavior. Preincubation of the ^{35}S-labeled Pex5p with either pep-SKL or pep-LKS does not change the elution profile of the *in vitro* synthesized protein. In both cases a behavior similar to the one displayed by the recombinant protein is observed (data not shown). The same result is obtained when the *in vitro* synthesized protein is preincubated with GST-LKS before being subjected to chromatography (Fig. 4*C*). In contrast, preincubation of ^{35}S-labeled Pex5 with GST-SKL leads to a small decrease in the elution volume of the radioactive protein. Interestingly, ΔC1-Pex5p elutes from the column immediately after Pex5p. As expected, the behavior of this truncated protein remains unchanged regardless of the GST fusion protein used in the preincubation step. A Stokes radius of 5 ± 0.25 nm was estimated for ΔC1-Pex5p.

Having determined the sedimentation coefficients and the Stokes radii for these Pex5p proteins, we calculated their molecular masses using Equation 1 (see "Materials and Methods"). Values of 80.5 ± 4.9, 48.3 ± 3.6, and 124.1 ± 9.4 kDa were obtained for Pex5p, ΔC1-Pex5p, and the Pex5p-GST-SKL protein complex, respectively. We have also determined the frictional ratios (see Equation 2 under "Materials and Methods") for human Pex5p and its N-terminal half, ΔC1-Pex5p. Values of 1.94 ± 0.08 and 2.28 ± 0.12, respectively, were obtained. Thus, human Pex5p is a monomeric protein with an abnormal shape, a property that seems to stem from the conformation of its N-terminal half (see "Discussion").

Exactly the same conclusion regarding the molecular mass of recombinant human Pex5p was obtained upon sedimentation equilibrium centrifugation analysis. It is important to note that in this technique the protein under analysis is neither diluted (quite the opposite) nor subjected to high hydrostatic pressures. The experimental molecular mass determined for this protein was 73.8 ± 2.6 kDa, a value in perfect agreement with its predicted molecular mass (72.3 kDa). No evidence for the existence of dimers, tetramers, or higher order oligomers could be found in this experiment.

This analysis was also performed with a different Pex5p recombinant protein produced and purified as described elsewhere (28). In this case a molecular mass of 73.3 ± 1.0 kDa was determined. Again, no evidence for the existence of Pex5p oligomers could be found in this analysis. In summary, these results indicate that human Pex5p is a monomeric protein and remains in a monomeric state when bound to a PTS1-containing protein.

DISCUSSION

Over the last years a number of reports have been published stating that recombinant Pex5p from several organisms is a tetrameric protein (25–28). These conclusions have been used to develop and support the so-called pre-implex model (21). According to this hypothesis, tetrameric Pex5p would bind up to four PTS1-containing proteins. Because many of these cargo proteins are themselves oligomers, it was proposed that large Pex5p-cargo protein complexes would be formed at the peroxisomal membrane. This phenomenon would create a high local concentration of peroxisomal proteins at the outer surface of the organelle membrane, which in turn could facilitate protein translocation across the peroxisomal membrane.

The propensity of recombinant human Pex5p to originate aggregates is a well known property of this protein (27, 28). Indeed, incubation of recombinant human Pex5p in low ionic strength buffers (28) or the isolation of this protein in the absence of reducing agents can lead to heterogeneous (oligo-

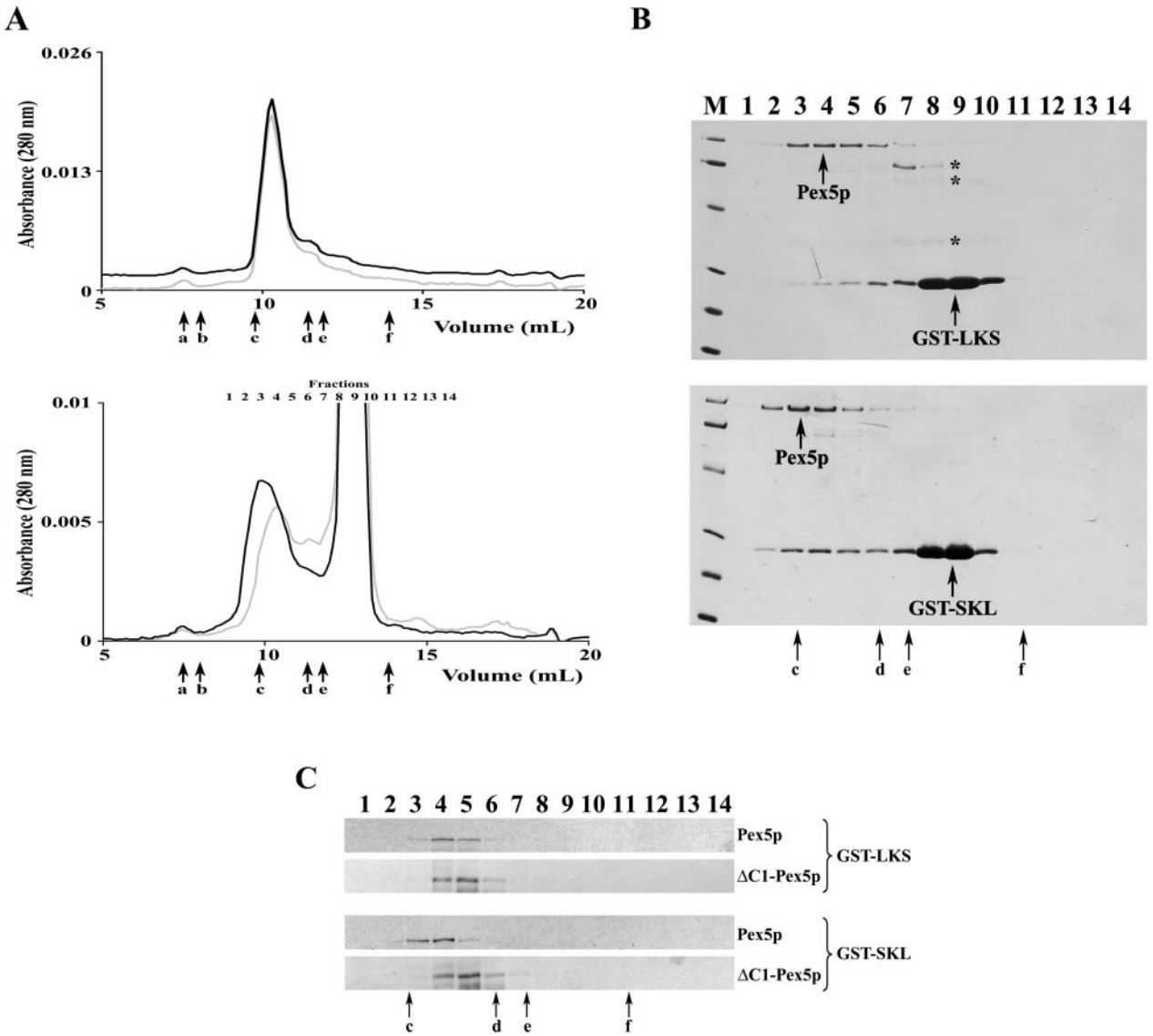

FIG. 4. **Size exclusion chromatography of human Pex5p.** *A*, recombinant Pex5p was preincubated with either pep-SKL (*black line*) or pep-LKS (*gray line*) and analyzed by size exclusion chromatography (*upper panel*). The same analysis was performed with recombinant Pex5p preincubated with either GST-SKL (*black line*) or GST-LKS (*gray line*). The corresponding chromatograms are shown in the lower panel. Numbers at the top of the chromatogram (*lower panel*) indicate the fractions collected and analyzed by SDS-PAGE. The Coomassie Blue-stained gels are shown in *panel B*. The *asterisks* indicate three *E. coli* contaminating proteins present in this GST-LKS preparation. *C*, *in vitro* synthesized human Pex5p or ΔC1-Pex5p was incubated with either GST-LKS or GST-SKL and analyzed by size exclusion chromatography. Collected fractions were subjected to SDS-PAGE and analyzed by autoradiography. The void volume (*a*) and the elution volumes of tyroglobulin (*b*), ferritin (*c*), aldolase (*d*), bovine serum albumin (*e*), and soybean trypsin inhibitor (*f*) are indicated.

meric) protein preparations.[2] Obviously, these are not the type of Pex5p-Pex5p interactions residing at the base of the preimplex model. The model explicitly assumes the existence of biologically relevant Pex5p tetramers. What is the experimental evidence behind this premise? One of the first observations suggesting that Pex5p could be a tetramer of identical subunits was obtained when negatively stained recombinant human Pex5p preparations were analyzed by electron microscopy (28). Those studies revealed the existence of square-shaped particles with approximate dimensions of 13 × 13 nm. The data presented in this work do not provide any clues regarding the nature and origin of these square-shaped particles. However, they do indicate that these particles do not represent the Pex5p protein as it exists in solution: as shown here, Pex5p is a monomeric protein.

The assumption that Pex5p is a spherical protein has also been made by researchers, one of us included, trying to infer the molecular mass of recombinant Pex5p using size exclusion chromatography (26–28). In this context, the results available for Pex5p from *L. donovani* are particularly interesting. This protein also displays an abnormal behavior upon size exclusion chromatography, an observation that led to the conclusion that Pex5p from this organism is a tetramer (44). However, as emphasized in a later work, binding assays using a PTS1-containing peptide failed to demonstrate the existence of those tetramers (26). Instead, the binding data reported were compatible with the existence of a monomeric Pex5p population. Thus, it was hypothesized that binding of PTS1-containing proteins to *L. donovani* Pex5p results in its monomerization (26). If the data described here for human Pex5p are extrapolated to the *L. donovani* protein, then these conflicting results have a simple explanation: *L. donovani* Pex5p is a monomeric protein with an asymmetric shape. It should be noted that the

[2] J. Costa-Rodrigues, A. F. Carvalho, C. Sá-Miranda, and J. E. Azevedo, unpublished results.

similarities between the experimental results described for these two Pex5p proteins are not limited to the behavior of the full-length versions upon size exclusion chromatography. The results regarding the N-terminal halves of the two Pex5p proteins are also in perfect agreement with each other. Our conclusion is that the N-terminal half of human Pex5p is a monomeric protein displaying a high frictional ratio. The conclusion regarding the N-terminal domain of *L. donovani* Pex5p was that it adopts a hexameric structure (26).

Finally, similar observations regarding the tetrameric nature of *H. polymorpha* Pex5p have also been described. The authors inferred the molecular mass of Pex5p from the diffusion constant of the protein at pH 7.2, as determined by fluorescence correlation spectroscopy (25). Again, the premise used to calculate the molecular mass of *H. polymorpha* Pex5p was that the protein is spherical in shape. The data presented here for the human peroxin strongly suggest that this may not be the case. Interestingly, the hydrodynamic data described by the same group for a Pex5p-Pex8p protein complex at pH 7.2 are compatible with the existence of a heterodimeric protein complex displaying a globular shape (25). Based on our data we would propose that upon binding Pex8p, *H. polymorpha* Pex5p suffers large conformational alterations. Determination of the sedimentation coefficients for *H. polymorpha* Pex5p alone and in the presence of Pex8p should clarify this issue.

All considered, we have to admit that the experimental evidence supporting the existence of a tetrameric Pex5p and thus of the pre-implex model is, at the very least, questionable. The data described here for the human protein are clearly not compatible with such a model.

The high frictional ratio observed for the N-terminal half of Pex5p indicates that this domain does not adopt a globular folding. Thus, it is likely that this Pex5p domain adopts an extended conformation and/or is natively unfolded (see Ref. 45 for a review on natively unfolded domains). The resolution of this issue will require biophysical data regarding the structure of this Pex5p domain.

The hydrodynamic properties of the N-terminal domain of Pex5p are in contrast with the characteristics of the C-terminal half of Pex5p. Indeed, all the available data indicate that this domain displays "normal" hydrodynamic properties as can also be predicted by the tridimensional structure of the human TPR domains (11, 28, 44). Considering the high frictional ratio of human Pex5p observed in this work, it is thus evident that the N-terminal domain of Pex5p dominates the hydrodynamic properties of the full-length protein. This domain structure explains why relatively small deletions at the N-terminal half of Pex5p can result in dramatic alterations of its Stokes radius as observed by size exclusion chromatography (Ref. 26).[2]

How are the C- and N-terminal halves oriented to each other? The fact that the Stokes radius of ΔC1-Pex5p is almost similar to the one displayed by the full-length protein could suggest that the two halves of the protein interact laterally and not in a head-to-head configuration. Although such a structural model is still speculative, it should be noted that data indicating that the N- and C-terminal halves of Pex5p interact with each other have already been reported (46). If we assume this domain arrangement, then the long-range PTS1-induced conformational alterations of Pex5p described here and by others (40, 43) can be rationalized in an easier way. The alternative would be to consider that binding of a PTS1 cargo protein to Pex5p also induces conformational alterations in regions of the Pex5p protein located more than 100 amino acid residues away as is the case for the Pex13p-binding domain of Pex5p (43). This possibility, however, is not supported by the observation that Pex5p-truncated versions lacking the TPR domains are still good substrates for the peroxisomal docking/translocation machinery and even for the ATPase catalyzing the export of Pex5p back into the cytosol (30, 47, 48). Thus, as suggested recently, it is possible that the peroxisomal targeting domain of Pex5p is a target of negative regulation exerted by the TPR domains (30). One prediction of this model is that the TPR domains are not static structures. Binding of a PTS1-containing protein to Pex5p should induce significant conformation alterations in these domains in order to expose the Pex5p peroxisomal targeting domain. We are currently testing this possibility.

To conclude, we would like to note that the protease assay described here represents an alternative approach to study of protein-protein interactions involving Pex5p. In contrast to other *in vitro* binding assays (*e.g.* blot overlays or pulldown experiments), the assay used in this work does not involve a washing procedure. Thus, if the appropriate negative controls are included, weak protein-protein interactions (as is probably the case with the Pex13p-Pex5p interaction) should be detected with a higher success rate.

REFERENCES

1. Eckert, J. H., and Erdmann, R. (2003) *Rev. Physiol. Biochem. Pharmacol.* **147,** 75–121
2. Purdue, P. E., and Lazarow, P. B. (2001) *Annu. Rev. Cell Dev. Biol.* **17,** 701–752
3. Sparkes, I. A., and Baker, A. (2002) *Mol. Membr. Biol.* **19,** 171–185
4. Weller, S., Gould, S. J., and Valle, D. (2003) *Annu. Rev. Genomics Hum. Genet.* **4,** 165–211
5. Gould, S. J., Keller, G. A., Hosken, N., Wilkinson, J., and Subramani, S. (1989) *J. Cell Biol.* **108,** 1657–1664
6. Lametschwandtner, G., Brocard, C., Fransen, M., Van Veldhoven, P., Berger, J., and Hartig, A. (1998) *J. Biol. Chem.* **273,** 33635–33643
7. Miura, S., Kasuya-Arai, I., Mori, H., Miyazawa, S., Osumi, T., Hashimoto, T., and Fujiki, Y. (1992) *J. Biol. Chem.* **267,** 14405–14411
8. Brocard, C., Kragler, F., Simon, M. M., Schuster, T., and Hartig, A. (1994) *Biochem. Biophys. Res. Commun.* **204,** 1016–1022
9. Dodt, G., Braverman, N., Wong, C., Moser, A., Moser, H. W., Watkins, P., Valle, D., and Gould, S. J. (1995) *Nat. Genet.* **9,** 115–125
10. Fransen, M., Brees, C., Baumgart, E., Vanhooren, J. C., Baes, M., Mannaerts, G. P., and Van Veldhoven, P. P. (1995) *J. Biol. Chem.* **270,** 7731–7736
11. Gatto, G. J., Jr., Geisbrecht, B. V., Gould, S. J., and Berg, J. M. (2000) *Nat. Struct. Biol.* **7,** 1091–1095
12. Klein, A. T., Barnett, P., Bottger, G., Konings, D., Tabak, H. F., and Distel, B. (2001) *J. Biol. Chem.* **276,** 15034–15041
13. Terlecky, S. R., Nuttley, W. M., McCollum, D., Sock, E., and Subramani, S. (1995) *EMBO J.* **14,** 3627–3634
14. Agne, B., Meindl, N. M., Niederhoff, K., Einwachter, H., Rehling, P., Sickmann, A., Meyer, H. E., Girzalsky, W., and Kunau, W. H. (2003) *Mol. Cell* **11,** 635–646
15. Albertini, M., Girzalsky, W., Veenhuis, M., and Kunau, W. H. (2001) *Eur. J. Cell Biol.* **80,** 257–270
16. Reguenga, C., Oliveira, M. E., Gouveia, A. M., Sa-Miranda, C., and Azevedo, J. E. (2001) *J. Biol. Chem.* **276,** 29935–29942
17. Szilard, R. K., Titorenko, V. I., Veenhuis, M., and Rachubinski, R. A. (1995) *J. Cell Biol.* **131,** 1453–1469
18. Dammai, V., and Subramani, S. (2001) *Cell* **105,** 187–196
19. van der Klei, I. J., Hilbrands, R. E., Swaving, G. J., Waterham, H. R., Vrieling, E. G., Titorenko, V. I., Cregg, J. M., Harder, W., and Veenhuis, M. (1995) *J. Biol. Chem.* **270,** 17229–17236
20. Azevedo, J. E., Costa-Rodrigues, J., Guimaraes, C. P., Oliveira, M. E., and Sa-Miranda, C. (2004) *Cell Biochem. Biophys.* **41,** 451–468
21. Gould, S. J., and Collins, C. S. (2002) *Nat. Rev. Mol. Cell. Biol.* **3,** 382–389
22. Oliveira, M. E., Gouveia, A. M., Pinto, R. A., Sa-Miranda, C., and Azevedo, J. E. (2003) *J. Biol. Chem.* **278,** 39483–39488
23. Gouveia, A. M., Guimaraes, C. P., Oliveira, M. E., Reguenga, C., Sa-Miranda, C., and Azevedo, J. E. (2003) *J. Biol. Chem.* **278,** 226–232
24. Dodt, G., and Gould, S. J. (1996) *J. Cell Biol.* **135,** 1763–1774
25. Wang, D., Visser, N. V., Veenhuis, M., and van der Klei, I. J. (2003) *J. Biol. Chem.* **278,** 43340–43345
26. Madrid, K. P., De Crescenzo, G., Wang, S., and Jardim, A. (2004) *Mol. Cell. Biol.* **24,** 7331–7344
27. Harper, C. C., Berg, J. M., and Gould, S. J. (2003) *J. Biol. Chem.* **278,** 7897–7901
28. Schliebs, W., Saidowsky, J., Agianian, B., Dodt, G., Herberg, F. W., and Kunau, W. H. (1999) *J. Biol. Chem.* **274,** 5666–5673
29. Braverman, N., Dodt, G., Gould, S. J., and Valle, D. (1998) *Hum. Mol. Genet.* **7,** 1195–1205
30. Gouveia, A. M., Guimaraes, C. P., Oliveira, M. E., Sa-Miranda, C., and Azevedo, J. E. (2003) *J. Biol. Chem.* **278,** 4389–4392
31. Oliveira, M. E., Reguenga, C., Gouveia, A. M., Guimaraes, C. P., Schliebs, W., Kunau, W. H., Silva, M. T., Sa-Miranda, C., and Azevedo, J. E. (2002) *Biochim. Biophys. Acta* **1567,** 13–22
32. Kain, K. C., Orlandi, P. A., and Lanar, D. E. (1991) *BioTechniques* **10,** 366–374
33. Fransen, M., Terlecky, S. R., and Subramani, S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 8087–8092
34. Laurent, T. C., and Killander, J. (1964) *J. Chromatogr.* **14,** 217–230

35. Siegel, L. M., and Monty, K. J. (1966) *Biochim. Biophys. Acta* **112,** 346–362
36. Manciu, L., Chang, X. B., Buyse, F., Hou, Y. X., Gustot, A., Riordan, J. R., and Ruysschaert, J. M. (2003) *J. Biol. Chem.* **278,** 3347–3356
37. Gouveia, A. M., Reguenga, C., Oliveira, M. E., Sa-Miranda, C., and Azevedo, J. E. (2000) *J. Biol. Chem.* **275,** 32444–32451
38. Elgersma, Y., Kwast, L., Klein, A., Voorn-Brouwer, T., van den Berg, M., Metzig, B., America, T., Tabak, H. F., and Distel, B. (1996) *J. Cell Biol.* **135,** 97–109
39. Gould, S. J., Kalish, J. E., Morrell, J. C., Bjorkman, J., Urquhart, A. J., and Crane, D. I. (1996) *J. Cell Biol.* **135,** 85–95
40. Urquhart, A. J., Kennedy, D., Gould, S. J., and Crane, D. I. (2000) *J. Biol. Chem.* **275,** 4127–4136
41. Erdmann, R., and Blobel, G. (1996) *J. Cell Biol.* **135,** 111–121
42. Fransen, M., Brees, C., Ghys, K., Amery, L., Mannaerts, G. P., Ladant, D., and Van Veldhoven, P. P. (2002) *Mol. Cell. Proteomics* **1,** 243–252
43. Otera, H., Setoguchi, K., Hamasaki, M., Kumashiro, T., Shimizu, N., and Fujiki, Y. (2002) *Mol. Cell. Biol.* **22,** 1639–1655
44. Jardim, A., Liu, W., Zheleznova, E., and Ullman, B. (2000) *J. Biol. Chem.* **275,** 13637–13644
45. Dyson, H. J., and Wright, P. E. (2002) *Curr. Opin. Struct. Biol.* **12,** 54–60
46. Harano, T., Nose, S., Uezu, R., Shimizu, N., and Fujiki, Y. (2001) *Biochem. J.* **357,** 157–165
47. Costa-Rodrigues, J., Carvalho, A. F., Gouveia, A. M., Fransen, M., Sa-Miranda, C., and Azevedo, J. E. (2004) *J. Biol. Chem.* **279,** 46573–46579
48. Dodt, G., Warren, D., Becker, E., Rehling, P., and Gould, S. J. (2001) *J. Biol. Chem.* **276,** 41769–41781