Structural Insights into Cargo Recognition by the Yeast PTS1 Receptor

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Background: Peroxisomal proteins are recognized in the cytosol by the import receptor Pex5p.

Results: Photo-cross-linking and mass spectrometry reveal the binding interface of Pex5p and its cargo protein Pcs60p.

Conclusion: Pex5p-cargo interaction extends beyond signal sequence recognition and exhibits a bivalent binding mode.

Significance: These data indicate a two-step concept of peroxisomal cargo recognition with initial tethering and subsequent lock-in.

The peroxisomal matrix protein import is facilitated by cycling import receptors that shuttle between the cytosol and the peroxisomal membrane. The import receptor Pex5p mediates the import of proteins harboring a peroxisomal targeting signal of type I (PTS1). Purified recombinant Pex5p forms a dimeric complex with the PTS1-protein Pcs60p in vitro with a $K_D$ of 0.19 µM. To analyze the structural basis for receptor-cargo recognition, the PTS1 and adjacent amino acids of Pcs60p were systematically scanned for Pex5p binding by an in vitro site-directed photo-cross-linking approach. The cross-linked binding regions of the receptor were subsequently identified by high resolution mass spectrometry. Most cross-links were found with TPR6, TPR7, as well as the 7C-loop of Pex5p. Surface plasmon resonance analysis revealed a bivalent interaction mode for Pex5p and Pcs60p. Interestingly, Pcs60p lacking its C-terminal tripeptide sequence was efficiently bivalent interaction mode for Pex5p and Pcs60p. Interestingly, Pcs60p lacking its C-terminal tripeptide sequence was efficiently

Peroxisomes are ubiquitous, single membrane-bound cell organelles, which carry out a wide variety of metabolic processes in eukaryotic organisms. The maintenance of peroxisomes depends on the formation of the peroxisomal membrane and the subsequent import of both membrane and matrix proteins (1, 2). All peroxisomal matrix proteins are nuclearily encoded, synthesized on free ribosomes, and imported post-translationally into the organelle (3). Peroxisomal protein import differs from protein import into other organelles, such as mitochondria and the endoplasmic reticulum, in that folded and even oligomeric proteins are transported across the membrane (4). Peroxisomal matrix protein import is a cycling multistep process, which commences in the cytosol with the recognition of newly synthesized proteins designated for import into peroxisomes by peroxisomal import receptors. In the following, the assembled receptor-cargo complex is targeted to the peroxisomal membrane and docks to the peroxisomal protein translocation machinery. A highly dynamic transient protein-conducting channel is formed, and the cargo is translocated into the peroxisomal lumen in a so far unknown manner (5, 6). Finally, the receptor is released from the peroxisomal membrane to the cytosol for the next round of import (7).

Like sorting of proteins to other cellular compartments, protein targeting to peroxisomes depends on signal sequences. Most peroxisomal matrix proteins contain a conserved C-terminal peroxisomal targeting signal 1 (PTS1), which originally was defined as the tripeptide serine-lysin-leucine or variants thereof (8). As it turned out that additional adjacent amino acids might be crucial for receptor-cargo interaction, PTS1 was redefined as C-terminal dodecamers (9).

The abbreviations used are: PTS1, peroxisomal targeting signal type 1; AGT, alanine-glyoxylate aminotransferase; $p$BP, $p$-benzoyl-$l$-phenylalanine; ITC, isothermal titration calorimetry; SEC, size exclusion chromatography; SPR, surface plasmon resonance spectroscopy; TPR, tetratricopeptide repeat; NTA, nitrilotriacetic acid; RU, response unit; PNS, post-nuclear supernatant.

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The PTS1 receptor Pex5p displays a dual localization in the cytosol and at peroxisomes, and it guides newly synthesized PTS1 proteins from the cytosol to the peroxisomal matrix, acting as a cycling receptor (10). The dysfunction of the PTS1 receptor leads to devastating diseases such as the Zellweger syndrome or neonatal adrenoleukodystrophy. Patients develop severe signs and symptoms during the newborn period or in early childhood leading to premature death (11, 12). Functional and structural data revealed that Pex5p includes two separated, functionally distinct domains. The N-terminal domain is intrinsically disordered and capable to mediate all transport steps of the receptor cycle, including docking and pore formation as well as dislocation from the peroxisomal membrane (13–15). The C-terminal domain of the receptor consists of an array of tetratricopeptide repeat (TPR) domains and directly binds the PTS1 motif (16). The crystal structure of the human PEX5 C-terminal domain in complex with a PTS1-containing model peptide or sterol carrier protein 2 (SCP2) revealed that two TPR triplets, TPR1-TPR3 and TPR5-TPR7, are arranged into a ring-like structure upon binding (16–19). TPR4, which is only partly separated from both TPR triplets, TPR1-TPR3 and TPR5-TPR7, is ligated, resulting in a ring-like structure upon cargo binding (16, 17). The PTS1 motif is bound in a central cavity of this structure (16–19). TPR4, which is only partly ordered and thus displays a non-canonical conformation, separates both TPR triplets, supporting its role as a conformational mobile unit allowing conformational flexibility within the TPR motifs of PEX5 (16, 17, 20, 21).

To gain insight into the recognition of PTS1 proteins by Pex5p in yeast, the interaction of the recombinant receptor and its cargo protein Pcs60p was analyzed in detail. The PTS1 receptor forms a complex with Pcs60p in vitro with a binding constant of 0.19 μM. To determine contact sites, we performed in vitro photo-cross-linking studies, the amber codon TAG was used as a target to incorporate p-benzoyl-L-phenylalanine (pBpa) into the protein during translation. To this end, the TAG present in wild-type Pcs60p facilitates expression of wild-type Pcs60p with an N-terminal glutathione S-transferase (GST) tag. For construction of Pcs60p variants for in vitro photo-cross-linking, coding regions for amino acids 536–543 were separately changed to TAG by PCR mutagenesis with plasmid pNH1. For in vitro photo-cross-linking, the amber codon TAG was used as a target to incorporate p-benzoyl-L-phenylalanine (pBpa) into the protein during translation. To this end, the TAG present in wild-type Pcs60p was exchanged to the ochre codon TAA by QuikChange® site-directed mutagenesis according to the manufacturer’s instructions (Stratagene, Amsterdam, Netherlands). The obtained plasmid pSH01 facilitates expression of wild-type Pcs60p with an N-terminal glutathione S-transferase (GST) tag. For construction of Pcs60p variants for in vitro photo-cross-linking, the amber codon TAA by QuikChange® site-directed mutagenesis according to the manufacturer’s instructions (Stratagene, Amsterdam, Netherlands). The obtained plasmid pSH01 facilitates expression of wild-type Pcs60p with an N-terminal glutathione S-transferase (GST) tag. For construction of Pcs60p variants for in vitro photo-cross-linking, the amber codon TAG was used as a target to incorporate p-benzoyl-L-phenylalanine (pBpa) into the protein during translation.

Although wild-type Pcs60p displays a bivalent interaction mode, the PTS1 lacking truncated Pcs60p variant exhibits a monovalent interaction with its receptor.

**Experimental Procedures**

**Plasmid Constructions**—Plasmids and sequences of oligonucleotides used are listed in Tables 1 and 2. Plasmids for expression of wild-type *Saccharomyces cerevisiae* Pcs60p and variants thereof in *Escherichia coli* were constructed as follows. The Pcs60 encoding region was obtained by PCR using primers RE2975 and RE2976 and genomic DNA as template. The PCR product and the vector pGEX-4T-3 (GE Healthcare, Munich, Germany) were both hydrolyzed with BamHI and XhoI and ligated, resulting in plasmid pNH1. For in vitro photo-cross-linking studies, the amber codon TAG was used as a target to incorporate p-benzoyl-L-phenylalanine (pBpa) into the protein during translation.

**Table 1**

| Plasmid | Description | Source or Ref. | Oligonucleotides |
|---------|-------------|---------------|------------------|
| pKN5/0-2 | pET9d_His-ScPex5p | This study | RE2975/RE2976 |
| pNH01 | pGEX-4T-3_Pcs60p | This study | RE3617/RE3618 |
| pSH01 | pGEX-4T-3_Pcs60pK540 | This study | RE3619/RE3620 |
| pSH03 | pGEX-4T-3_Pcs60pM536A | This study | RE4179/RE4180 |
| pSH04 | pGEX-4T-3_Pcs60pR543 | This study | RE4181/RE4182 |
| pSH12 | pGEX-4T-3_Pcs60pS537 | This study | RE4233/RE4234 |
| pSH23 | pGEX-4T-3_Pcs60pS541 | This study | RE4235/RE4236 |
| pSH24 | pGEX-4T-3_Pcs60pS542 | This study | RE4237/RE4238 |
| pSH25 | pGEX-4T-3_Pcs60pL543 | This study | RE4299/RE4300 |
| pSH26 | pGEX-4T-3_Pcs60pS544 | This study | RE4516/RE4517 |
| pSH27 | pET9d_His-ScPex5p-M536A | This study | RE5885/RE5886 |
| pSH47 | pGEX-4T-3_Pcs60pS545 | This study | RE5887/RE5888 |
| pSH48 | pGEX-4T-3_Pcs60pA8 | This study | RE5890/RE5891 |
| pSH63 | pGEX-4T-3_Pcs60p substitution | This study | RE4919/RE4920 |
| pSH62 | pGEX-4T-3_Pcs60p insertion | This study | RE4891/RE4892 |
| pSH56 | pRS416_Pcs60p | This study | RE4885/RE4886 |
| pSH57 | pRS416_Pcs60pΔ8 | This study | RE4909/RE4910 |
| pSH60 | pRS416_Pcs60p substitution | This study | RE4923/RE4934/RE4955/RE4956 |
| pSH59 | pRS416_Pcs60p insertion | This study | RE4931/RE4932 |
| pEVL-RS | tRNA and tRNA synthetase | Peter Schultz; The Scripps Research Institute, La Jolla, CA |

**Plasmids used in this study**

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| pKN5/0-2 | pET9d_His-ScPex5p | This study | RE2975/RE2976 |
| pNH01 | pGEX-4T-3_Pcs60p | This study | RE3617/RE3618 |
| pSH01 | pGEX-4T-3_Pcs60pK540 | This study | RE3619/RE3620 |
| pSH03 | pGEX-4T-3_Pcs60pM536A | This study | RE4179/RE4180 |
| pSH04 | pGEX-4T-3_Pcs60pR543 | This study | RE4181/RE4182 |
| pSH12 | pGEX-4T-3_Pcs60pS537 | This study | RE4233/RE4234 |
| pSH23 | pGEX-4T-3_Pcs60pS541 | This study | RE4235/RE4236 |
| pSH24 | pGEX-4T-3_Pcs60pS542 | This study | RE4237/RE4238 |
| pSH25 | pGEX-4T-3_Pcs60pL543 | This study | RE4299/RE4300 |
| pSH26 | pGEX-4T-3_Pcs60pS544 | This study | RE4516/RE4517 |
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| pSH47 | pGEX-4T-3_Pcs60pS545 | This study | RE5887/RE5888 |
| pSH56 | pRS416_Pcs60p | This study | RE5890/RE5891 |
| pSH57 | pRS416_Pcs60pΔ8 | This study | RE5890/RE5891 |
| pSH60 | pRS416_Pcs60p substitution | This study | RE4919/RE4920 |
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| pEVL-RS | tRNA and tRNA synthetase | Peter Schultz; The Scripps Research Institute, La Jolla, CA |
acids are underlined), PCS60 insertion (K-SSRNK-ASA-SKL, inserted amino acids are underlined), and PCS60Δ8 (deletion of the last eight amino acids) were created with pSC20-FB6 as template by QuikChange® PCR. All constructs were verified by sequencing.

**Protein Purification**—Expression of recombinant proteins (His6-Pex5p, His6-Pex5p-M536A, GST-Pcs60p, and GST-Pcs60p variants) was performed in E. coli BL21 (DE3) cells (24). Cells expressing Pcs60p variants for *in vitro* photo-cross-linking experiments were in addition co-transformed with pEVOL-RS (kindly provided by P. Schultz, La Jolla, CA) to allow pBpa incorporation during translation (25). Cells were harvested and resuspended in a 10-fold volume of binding buffer I (50 mM Tris, 300 mM NaCl, 0.1 mM DTT, 20 mM imidazole, pH 8.0) for His6-Pex5p and His6-Pex5p-M536A or binding buffer II (22 mM NaH2PO4·2H2O, 2.8 mM NaH2PO4, 150 mM NaCl, pH 7.5) for GST-Pcs60p variants thereof. Both buffers contained protease inhibitors (8 mM aprotinin, 0.3 mM aprotinin, 0.16 mg/ml benzamidine, 1 mM bestatin, 10 mM chymostatin, 5 mM leupeptin, 15 mM pepstatin, 1 mM PMSF, 0.21 mg/ml NaF) and 25 µg/ml DNase I. Cells were broken using a French press, and the obtained lysates were clarified by centrifugation at 14,000 rpm for 45 min (rotor SS-34, Thermo Scientific, Schwerte, Germany).

The supernatant contained the soluble His6-Pex5p or His6-Pex5p-M536A and was loaded onto a nickel-NTA column (HisTrap HP, GE Healthcare) of an ÄKTAprime™ system (GE Healthcare). After washing with binding buffer I, proteins were eluted with an imidazole gradient up to 500 mM. Samples were incubated at an A280 nm of 0.1, and expression of the recombinant proteins was induced by adding 0.4 mM isopropyl 1-thio-β-D-galactopyranoside when reaching an A600 nm of 0.5 to 0.6. Time of expression for His6-Pex5p and GST-Pcs60p variants (WT, ΔSKL, substitution, insertion, Δ8) was 4 h at 30 °C. GST-Pcs60p variants for *in vitro* photo-cross-linking approaches were expressed for 20 h at 30 °C in the presence of 1 mM pBpa (Bachem, Bubendorf, Switzerland) and 2% arabinose.

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were combined in defined amounts (10 nmol of His₁₀-Pex5p and 6 nmol of Pcs60p in 500 μl) and incubated for 1 h on ice to allow complex formation. 500 μl of the samples were loaded onto a Superose™6 PC 10/300 GL column (GE Healthcare) and equilibrated with buffer (22 mM Na₂HPO₄·2H₂O, 2.8 mM NaH₂PO₄, 150 mM NaCl, pH 7.5), and subsequently, 500-μl fractions were collected. Calibration of the column was performed with the HMW-Gel Filtration Calibration kit (GE Healthcare) containing thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), and ovalbumin (44 kDa). To estimate the molecular mass, multivariate light scattering (MiniDawn Treos, Wyatt, Dernbach, Germany) was performed during size exclusion chromatography (SEC).

Photo-activated Cross-linking—For cross-linking reactions, purified proteins were transferred to 96-well microtiter plates. Samples were irradiated at 365 nm by using a handheld UV lamp (115 V, 60 Hz, 0.2 A; Spectronics, Westbury, NY) for 60 min at room temperature. Samples were removed from the wells, diluted with SDS sample buffer, and subjected to SDS-PAGE on 12.5% gels.

Mass Spectrometric Analyses—Following SDS-PAGE and colloidal Coomassie staining, protein bands were cut out, subjected to in-gel digestion using trypsin, and analyzed by nano-HPLC/ESI-MS/MS using an UltiMate 3000 RSLCnano/LTQ-Orbitrap ELITE system (Thermo Fisher Scientific, Bremen, Germany) as described (26). The analysis of LC-MS/MS datasets with the aim to identify cross-linked peptides was performed as described previously (27) with only slight modifications. For the search of potentially photo-cross-linked products, pairs of peptides, each according to the enzyme specificity of trypsin with up to two missed cleavages, were generated from amino acid sequences of the recombinant proteins, and cross-links between the given site of pBpa and any amino acid position in the target peptide were considered. For the amino acid position at which pBpa (encoded as U) was incorporated by site-directed mutagenesis, a relative mass of 251.09463 both in single and cross-linked peptides was considered. For each candidate cross-linking product, the computed mass had to match the measured precursor mass. In addition, the p value, giving the probability of a random match and calculated from comparison of the list of theoretical fragment ion masses with observed masses in the MS/MS spectrum according to the Andromeda score (28), had to be below 1 × 10⁻⁶. The reported site of photo-cross-linking within the target peptide corresponds to the match with the lowest p value.

ScPex5p(301–612)-Pcs60p-Peptide Modeling—Homology model of ScPex5p(301–612) was created using the RaptorX server (29). The final model used the template 2J9Q chain A gave an overall score of 312 and a p value of 9.2 × 10⁻⁷. To create an initial model for the Pcs60p C-terminal octapeptide, the human PEX5-SCP2 complex (Protein Data Bank code 2C0L) was used as a template. The C-terminal peptide sequence of SCP2 was mutated to that of Pcs60p and merged with the ScPex5p(301–612) model into one single Protein Data Bank file with Coot (30). This new file was uploaded to the Flex PepDock server (31) to create a model of the ScPex5p(301–612) in complex with the extended eight-residue-long PTS1 of Pcs60p. The first 10 docking solutions, of which the best one gave an overall Rosetta score of −56,584, were visually analyzed using PyMOL.

ITC Measurements—All proteins were dialyzed against 50 mM HEPES, pH 7.5, 300 mM NaCl, concentrated, and degassed shortly before performing measurements. ITC measurements were conducted at 25 °C on a MicroCal VP-ITC with 41–42 μM full-length His₁₀-Pex5p as a sample and 340–400 μM wild-type Pcs60p as a titration ligand and 20–30 μM full-length Pex5p and 300–622 μM Pcs60pΔSKL, respectively. Pcs60p constructs were injected in volumes of 10 μl each in a total of 27 steps, resulting in a 2-fold excess of Pcs60p at the end of each titration experiment, and 2–6-fold excess of Pcs60pΔSKL, respectively. Ligand heating effects by dilution were subtracted, and data were fitted using MicroCal Origin 5.0.

Surface Plasmon Resonance Measurements—Real time kinetic interaction analyses of Pcs60p variants with His₁₀-Pex5p were performed using a BIACore2000 instrument (GE Healthcare) at 25 °C analysis temperature. His₁₀-Pex5p (ligand) was covalently immobilized site-directed at physiological pH on NTA (32) or randomly at pH 4.5 on CM5 sensor chip surfaces. Immobilization was performed in analysis buffer A (10 mM HEPES, 150 mM NaCl, 50 μM EDTA, 0.05% Tween 20, pH 7.4) in the absence of DTT for NTA chips and supplemented with 1 mM DTT and 0.1% Tween 20 for CM5 chips. NTA chip surface was conditioned by three consecutive injections of 10 mM NaOH (0.5 min, 50 μl/min respectively) followed by capturing of Ni²⁺ ions (0.5 mM NiCl₂ in analysis buffer, 1 min injection, 10 μl/min). Subsequently the Ni²⁺ ions containing chip surface was activated with a mixture of 200 mM 3-dimethylaminopropyl-3-ethylcarbodiimide and 50 mM N-hydroxysuccinimide for 7 min (10 μl/min). His₁₀-Pex5p was diluted to 5 μg/ml in analysis buffer A and injected at a flow rate of 10 μl/min until the desired surface density was reached (~1100 RU). His-tagged Pex5p was immobilized on the surface of a CM5 sensor chip (GE Healthcare) via amine coupling using acetate buffer, pH 4.5, at a flow rate of 5 μl/min. The remaining unreacted esters were quenched with 1 M ethanolamine (pH 8.5, 7 min, 10 μl/ml), and Ni²⁺ and non-covalently bound His₁₀-Pex5p were removed from the NTA chips by injecting 250 mM EDTA in analysis buffer A, pH 7.4 (2 min, 10 μl/min). To remove non-bound proteins, CM5 chips were washed extensively with 0.5% Triton X-100 and then 0.5% CHAPS and 1 mM NaCl. Reference surfaces were prepared as ligand-containing surfaces except for the His₁₀-Pex5p injection step. Final immobilization levels typically were 900–1100 RU. Immediately after immobilization, the sensor chip and the BIACore microfluidics were primed with analysis buffer B containing DTT (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05–0.1% Tween 20, 1 mM DTT, pH 7.4). Interaction analyses of all Pcs60p variants with His₁₀-Pex5p were conducted in buffer B under comparable conditions at a flow rate of 20–30 μl/min. The same concentration range (serial 2-fold dilutions, 2048–0.13 nm) of each Pcs60p variant was analyzed in duplicate. Surface regeneration of NTA chips was achieved by three consecutive injections of buffer B supplemented with 0.3 mM MgCl₂ and 0.1% Triton X-100 (30 s each). The surface of CM5 chips was regenerated by 0.5 mM NaCl and 0.1% Triton X-100 (30 s each).
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X-100 (30 s each). Data processing (e.g. double referencing) and evaluation were performed by globally fitting applying a two-state reaction model or a Langmuir 1:1 binding model using the BlAevaluation software version 3.1 or 4.1.1. To assess the best fit, residual plot of the different fits were inspected. For a good fit, the residuals will only scatter randomly around ±2 RU, which corresponds to the short term noise in the detection system. This allows a visual inspection of the fitted curves overlaid on the experimental data, indicating the closeness of the fit to the data obtained.

Cell Fractionation and Protease Protection Assay—For in vivo studied yeast, wild-type strain UTL-7A (33) or its isogenic deletion strain Δpcs60 (23) were used. Spheroplasting of yeast cells, homogenization, and differential centrifugation at 25,000 × g of homogenates were performed essentially as described (34). In particular, post-nuclear supernatants (PNS) were prepared, adjusted to 2 mg per 3 ml, and loaded onto a 0.5 M sucrose cushion, and organelles were sedimented for 20 min by 25,000 × g (SS-34 rotor). For the protease protection assay, the post-nuclear supernatants were divided in two portions, and 30 μl of 20% Triton X-100 was added to one of them. Subsequently, 50 μl of protease K (10 mg/ml) was added, and samples were taken at 0, 5, 15, 30 (4 °C), and 45 min (at room temperature), precipitated with TCA, and subjected to immunoblot analysis.

Immunodetection—Immunoreactive complexes were visualized using anti-rabbit IgG IRDye800CW-conjugated combination with the Odyssey® infrared imaging system (LI-COR Bioscience, Bad Homburg, Germany). Polyclonal rabbit antibodies were raised against Pex5p (35), Pcs60p (23), fructose-1,6-bisphosphatase (Fbp1p (36)), and Porin (37).

Miscellaneous—Proteins in polyacrylamide gels were visualized by Coomassie staining according to Ref. 38. For mass spectrometric analysis, SDS samples were prepared by SDS-PAGE (39–41) and colloidal staining (42). Yeast transformations were performed according to Gietz and Woods (43).

Results

In Vitro Reconstitution of the Pcs60p-Pex5p Complex—Pcs60p is a peroxisomal oxalyl-CoA synthetase that belongs to the family of AMP-binding proteins and is associated with the luminal side of the peroxisomal membrane (23, 44). Consistent with the intra-peroxisomal localization, Pcs60p contains the consensus sequence for a PTS1 at its extreme C terminus (23). The PTS1 was demonstrated to be crucial for peroxisomal targeting of Pcs60p and thus is supposed to bind the PTS1 receptor Pex5p.

In vitro binding studies were performed to gain insight into the mode of interaction between Pcs60p and the PTS1 import receptor Pex5p. To this end, Pex5p was fused to an N-terminal hexahistidyl tag (His6), and Pcs60p was decorated with an N-terminal GST tag and a thrombin cleavage site between tag and protein. The genes coding for the two fusion proteins were expressed separately in E. coli, and soluble fractions were subjected to purification of the proteins by affinity chromatography. To this end, His6-Pex5p was bound to nickel-NTA-agarose and eluted by an imidazole gradient (Fig. 1A, upper panel). GST-Pcs60p was bound to glutathione-agarose and proteolytically released from the GST domain by thrombin cleavage (Fig. 1A, lower panel).

Expression and purification of both proteins were analyzed by SDS-PAGE and Coomassie staining. The analysis revealed that both proteins were purified to apparent homogeneity with a yield of ~4 mg and 2.4 mg/g of cells for His6-Pex5p and Pcs60p, respectively.

To estimate the oligomeric state of the recombinant proteins, Pex5p and Pcs60p were individually subjected to SEC. Obtained fractions of the column were separated on SDS-PAGE, and proteins were visualized by Coomassie staining (Fig. 1B, lower panel). Pex5p peaked in fractions corresponding to a size of ~160 kDa. Analysis of the peak fraction by multilayer light scattering revealed a molecular mass of 79 kDa (Table 3). Thus, the monomeric Pex5p displays a higher mobility as expected for the 69-kDa protein (45). SEC of Pcs60p revealed two peaks at 160 and 40 kDa (Fig. 1B). Thus, the estimated mobility of the 40-kDa peak is close to the calculated size of 60 kDa for monomeric Pcs60p (23), and the second peak represents a dimer or trimer of the protein. This conclusion was confirmed by light scattering, revealing 85 kDa for the first and 181 kDa for the second peak (Table 3). Further analyses including ITC, SPR, and small angle x-ray scattering confirmed the tendency of Pcs60p to oligomerize at higher protein concentrations (data not shown). To analyze Pex5p-Pcs60p complex formation, both proteins were incubated together in defined amounts for 1 h on ice. Subsequently, samples were subjected to SEC and analyzed by SDS-PAGE. In comparison with the single proteins, Pcs60p and Pex5p together display a clear mobility shift to fractions of about 440 kDa (Fig. 1B, SEC) or 384 kDa (Table 3, multilayer light scattering), showing the formation of a heteromeric complex. Signal intensity measurement of the peak fraction revealed a ratio of cargo and receptor of ~1:1 (Fig. 1B).

Site-specific in Vitro Cross-linking of the Pex5p-Pcs60p Complex—To analyze the contact sites between the PTS1 receptor Pex5p and its cargo Pcs60p, we applied site-specific in vitro photo-cross-linking (46). In particular, we genetically incorporated the photo-cross-linking amino acid pBpa into recombinant Pcs60p. pBpa incorporation occurs at the position of the amber codon TAG, using an orthogonal tRNA/aminoacyl-tRNA synthetase pair. The pBpa-containing proteins efficiently form stable complexes with their partners upon UV irradiation (46, 47). To prepare Pcs60p as a suitable target for this approach, we first mutated the native TAG codon of Pcs60p to TAA by site-directed mutagenesis to prevent read-through of the stop codon and unwanted incorporation of pBpa at this position of the protein (Fig. 2). Next, the base triplets encoding the C-terminal amino acid residues 536–543 of Pcs60p were individually changed to amber codons (TAG, Fig. 2). Thus, the cross-linking pBpa was introduced at the C-terminal PTS1 of Pcs60p and at regions in close proximity to the PTS1.

Expression of the Pcs60p variants was carried out in the presence of an evolved Methanococcus jannaschii tRNA/aminocycl-tRNA synthetase pair, 2% arabinose and 1 mM pBpa in E. coli strain BL21 (DE3). Wild-type and mutant Pcs60p were expressed as GST fusion proteins and isolated by affinity chromatography on glutathione-agarose with thrombin cleavage.
FIGURE 1. Formation of a recombinant His$_6$-Pex5p/-Pcs60p complex. A, profile of the purification of recombinant His$_6$-Pex5p (upper panel) and GST-Pcs60p (lower panel) by affinity chromatography. The protein profiles shown are from equal portions of cells prior to induction (T$_0$) and isopropyl 1-thio-β-D-galactopyranoside-induced (T$_4$) cells. Samples of the homogenate (H), the sediment (P), and the supernatant (S) after differential centrifugation, wash fraction (W), and obtained fractions after elution of bound proteins from the nickel-NTA-agarose or GSH-agarose column with increasing imidazole concentrations (E1–E21 as for His$_6$-Pex5p) or cleavage with thrombin (E for Pcs60p) were subjected to SDS-PAGE and stained with Coomassie Blue. In comparison with the starting material, loaded eluates of His$_6$-Pex5p and Pcs60p were concentrated 70- or 50-fold, respectively. B, size exclusion chromatography of recombinant His$_6$-Pex5p-Pcs60p complex. Shown are the absorption profiles obtained by the Unicorn software (GE Healthcare) and Coomassie-stained gels. As indicated, recombinant Pcs60p, His$_6$-Pex5p, or pre-formed complex of both were subjected to size exclusion chromatography (Superose 6 10/300 GL). Fractions were collected and analyzed by SDS-PAGE and Coomassie staining. His$_6$-Pex5p peaked at a size of ~160 kDa and Pcs60p at 160 and 40 kDa, indicating that it forms an oligomer and a monomer. Combined and pre-incubated proteins form a complex that peaked at a size of ~440 kDa with a 1:1 ratio of Pcs60p and Pex5p. Molecular mass markers are indicated.
Identification of Pcs60p/Pex5p Contact Sites—To identify interlinks between Pex5p and its PTS1 cargo Pcs60p, the cross-linked products of the Pcs60p variants Ser-537, Arg-538, Asn-539, Ser-541, and N541ΔSKL as well as the corresponding bands of monomeric proteins were analyzed by mass spectrometry (MS). To this end, the bands were cut out of the SDS gel (Fig. 3A, lower right panel), and proteins were subjected to in-gel trypic digestion prior to MS analysis. Fig. 4A depicts the identification of the cross-linked product between pBpa at position Asn-539 of Pcs60p and the peptide 529YNLAVSSMNIGCFK442 from the TPR7 domain of Pex5p based on its accurate precursor mass observed in the MS1 spectrum (Fig. 4A, upper left graph) and matches of fragment ion masses in the MS2 spectrum (Fig. 4A, lower graph). As a result of this analysis, the site of the cross-link was mapped to Met-536 (Fig. 4A, upper right scheme). Furthermore, Met-536 was also identified as the cross-linking site for Pcs60p variants carrying pBpa at positions Ser-537, Arg-538, Asn-539, and N539ΔSKL, whereas only pBpa at position Ser-541 cross-linked to Val-533 of Pex5p (Fig. 4B). The cross-linking MS data obtained are summarized in Table 4 and illustrated in Fig. 5.

Although only one Pex5p peptide bound to Pcs60p was found for the pBpa mutant Ser-537, and additional peptides were identified for the other Pcs60p variants. The Arg-538 variant bound to a second peptide, which is part of the 7C-loop and the C-terminal helical bundle (564GDVGSLLNTYDVETLK583) of Pex5p. The respective binding region comprised Leu-569–Leu-570 or Asp-575–Thr-580. Furthermore, the variant Asn-539 bound to a peptide of the first helix of the C-terminal helical bundle (583RVFIAMNR590, target site Arg-583), whereas the Ser-541 variant bound to a region of TPR6 (197LGSALANSNR206, target site Ala-502) (Table 4 and Fig. 5). The Asn-539 variant lacking the C-terminal tripeptide SKL bound to the same region of the first helix of the C-terminal helical bundle (target sites Arg-583 and Glu-579) as the Asn-539 variant comprising the PTS1 (Table 4).

Interestingly, many variants did interact with Met-536 of TPR7. The postulated reactive radius for benzophenones like pBpa is about 3.1 Å (48), and it has been suggested to exhibit a preferential affinity to methionine in a range of 8–11 amino acids (49). Indeed, we observed by ITC and SPR measurements that the cross-linker pBpa strengthens the interaction between Pcs60p and Pex5p. To avoid false-positive interactions in our cross-linking study caused by binding of pBpa to methionine, we therefore generated a Pex5p-M536A point mutant in which the methionine was replaced by alanine. Cross-linking experiments were carried out with all Pcs60p variants. Except for Ser-541, which did not reveal a cross-link, the same results as for wild-type Pex5p were obtained with the Pex5p-M536A variant (Fig. 3B). These data suggest that there is no major effect of M536A on the affinity of the pBpa mutants of Pcs60p to the PTS1 receptor protein Pex5p. Analysis of interaction sites by MS measurements revealed that, except for the peptide 529YNLAVSSMNIGCFK442, all cross-links essentially remained unchanged (Table 4).

Based on the data of the combined photo-cross-linking and MS approach, a model of the cargo-loaded PTS1 receptor was generated (Fig. 6). As a starting point, the known three-dimensional structures of human and trypanosome Pex5p was appropriated (17, 50) as well as the known structures of PTS1 signals of human SCP2 and AGT in comparison with bakers’ yeast Pcs60p (Fig. 6A). Our model of the interaction between the C-terminal region of Pcs60p with Pex5p shows Met-536 of Pex5p at a central position within the substrate cavity and fac-

**Table 3**

| Protein       | Mass (kDa) | Molecular mass calculated by MALS | Oligomerization       |
|---------------|------------|----------------------------------|-----------------------|
| Pcs60p        | 60         | 85 kDa ± 2%                      | Monomer               |
|               | 181 kDa ± 0.2% |                                  | Dimers/trimers        |
| Pex5p         | 69         | 79 kDa ± 0.2%                    | Monomer               |
| Pex5p/Pcs60p  | 82         | 135 kDa ± 0.2%                   | Pcs60p                |
|               | 82 kDa ± 0.2% |                                  |                       |
|               | 384 kDa ± 2%  |                                  | Complex (oligomer)    |

SDS-PAGE analysis showed no difference between the purified amber mutant Pcs60p and the wild-type protein (Fig. 3A, upper left panel). Isolated Pcs60p variants were incubated with purified recombinant His6-tagged Pex5p and subjected to UV irradiation for 0 min as negative control or for 60 min. Upon UV treatment, neither wild-type nor mutated Pcs60p variants displayed significant mobility changes in the absence of Pex5p, indicating that the proteins do not form intramolecular cross-links (Fig. 3A, lower left panel). In contrast, cross-linking products formed when Pcs60p was incubated with Pex5p. Prior UV treatments for both Pex5p and Pcs60p appeared as distinct bands of about 70 and 60 kDa, respectively (Fig. 3A, upper right panel). An additional band of about 130 kDa was detected after UV treatment for some Pcs60p variants (Fig. 3A, lower right panel). The size of 130 kDa corresponds well with the estimated size of a dimer of Pex5p and Pcs60p, indicating that these bands represent cross-linked receptor-cargo complexes. This was confirmed by immunoblot analysis using specific antisera against Pex5p and Pcs60p (Fig. 3B). Thus, incorporation of the photo-cross-linking amino acid pBpa at certain positions of Pcs60p allows for the efficient cross-linking of the protein with its receptor Pex5p upon UV treatment. In particular, efficient photo-cross-linking was observed when the pBpa was introduced at positions adjacent to the C-terminal PTS1 of Pcs60p (SS37, RS38, NS39; Fig. 3A). A significant reduction (SS41, Fig. 3A) or absence (KS42, LS43, Fig. 3A) of Pex5p/Pcs60p cross-links was observed when these crucial residues of the PTS1 were replaced by pBpa. In this respect, it has to be considered that the photo-cross-linking of the two proteins requires that the interacting regions exhibit a certain spatial proximity. However, if the original spacing is too small, as expected for the binding of the PTS1, binding and cross-linking might be sterically hindered. We observed that positioning of pBpa at Asn-539 of Pcs60p did result in the most efficient cross-linking to Pex5p. Surprisingly, a Pcs60p-construct lacking the PTS1 in a Asn-539 background (N539ΔSKL) was cross-linked to Pex5p to a higher extent compared with the version harboring its PTS1 (Fig. 3A).
Arg-538 of Pcs60p at a C/H9251 distance of 5.8 Å (Fig. 6B). In contrast, Ser-541 of Pcs60p is situated in close proximity to Val-533 of Pex5p (C/H9251 distance of 6.0 Å), which is located one helix turn toward the inside of the cavity. This is in agreement with the identification of a cross-link from Ser-541 to Val-533 and not Met-536 (see Fig. 4B and Table 4). It is also important to note that another methionine (Met-588) is located ~1.5 helix turns apart from Arg-583 of Pex5p, which constitutes the main

[Diagram showing Pcs60p variants for cross-linking studies.]

FIGURE 2. Pcs60p variants for cross-linking studies. Schematic of wild-type Pcs60p and coding sequence with focus on the C-terminal PTS1-comprising region. The Pcs60p variants used in this study are shown with their 3'-coding region (base pairs) and the deduced amino acid sequence (amino acids). Introduced TAG amber codons, which allow pBpa incorporation, are underlined. Asterisks indicate pBpa position at the protein level.

FIGURE 3. In vitro photo-cross-linking of Pcs60p and His6-Pex5p as well as His6-Pex5p-M536A. A, purified recombinant variants of Pcs60p were subjected to UV irradiation in the absence (left panel) or presence (right panel) of purified equimolar amounts of His6-Pex5p. Samples were taken prior to (0 min) and 60 min after UV treatment and subjected to SDS-PAGE. Proteins were visualized by Coomassie staining. Pex5p/Pcs60p cross-linking products are indicated (pc complex). B, verification of the obtained cross-linking bands by immunoblot analysis using specific antisera against Pex5p and Pcs60p. C, purified recombinant His6-Pex5p-M536A was subjected to UV irradiation in the absence (left panel) or presence (right panel) of purified equimolar amounts of Pcs60p variants. Samples were taken prior to (0 min) and 60 min after UV irradiation and subjected to SDS-PAGE. Proteins were visualized by Coomassie staining. Pex5p-M536A/Pcs60p cross-linking products are indicated (pc complex).
cross-linking target site in the M536A mutant. The Met-588 did not cross-link to the photo-activated pBpa, which is in agreement with the model. The orientation and the principal binding mode of the Pcs60p peptide is similar to that of the other known PTS1 motifs (Fig. 6B).

**Analysis of Equilibrium Binding Properties of Pex5p Interacting with Wild-type Pcs60p and the Mutant Pcs60p ΔSKL**—For a more detailed analysis on the interaction of Pex5p with the Pcs60p cargo protein, ITC measurements were performed. To this end, Pex5p, Pcs60p, and Pcs60p ΔSKL were purified as described above and dialyzed against HEPES buffer, filtered, and degassed. The receptor Pex5p was used as sample and wild-type and mutant form of Pcs60p as the titration ligand.

The ITC data show that Pex5p binds to wild-type Pcs60p with an equilibrium dissociation constant \( K_D \) of 0.19 \( \text{M} \) (Fig. 7A), indicative of a high affinity binding. Thus, the binding affinity of the yeast Pex5p to its PTS1 cargo Pcs60p is in the same range as those observed for human Pex5p to PTS1 sequences of its cargo pipecolic acid oxidase (240 nM) or acyl-CoA oxidase 2 (257 nM) (51). Upon deletion of the PTS1 signal, the truncated Pcs60p did still interact with Pex5p with a calculated \( K_D \) of 7.7 \( M \) (Fig. 7B).

**Analysis of the Interaction of Pex5p and Pcs60p by Surface Plasmon Resonance Measurements**—For a more detailed analysis of the kinetics of receptor-cargo binding, SPR measurements were performed. His-tagged Pex5p was immobilized on the surface of a CM5 or NTA sensor chip, and wild-type or mutants of Pcs60p were applied with varying concentrations (Fig. 8).

The analyzed variants comprised the wild-type Pcs60p with the C-terminal ending K-SSRNK-SKL, the protein lacking its...
C-terminal tripeptide SKL (Pcs60pΔSKL). Kinetic global fit analysis was performed using the BIAevaluation software 4.1.1. For wild-type Pcs60p, two distinct binding events were recorded (Fig. 8A, Pcs60p, left panel). At low concentration (0.13–16 nM), a high affinity (saturable) binding site was detected, which was explained best with the two-state reaction model with a calculated $K_D$ of 2.4 nM (Fig. 8, Pcs60p, right panel). At higher concentration, a low affinity binding site was detected, which was not saturable, and the estimated $K_D$ value of this site was in the low micromolar range (Fig. 8A, Pcs60p, left panel). Deletion of the C-terminal tripeptide SKL did result in the disappearance of the high affinity site, and the low affinity site was still present, with an estimated $K_D$ of about 2 μM (Fig. 8A, Pcs60pΔSKL, left panel). Deletion of the C-terminal eight amino acids of Pcs60p, and thus deletion of the tripeptide sequence and the adjacent five amino acids, did virtually completely abolish binding of Pcs60p to Pex5p. The comparison of wild-type Pcs60p and the Pcs60pΔ8 mutant is shown in Fig. 8B. This result indicates the existence of two binding sites, a high affinity site comprising the tripeptide SKL and a low affinity site, which depends on the adjacent five amino acids. Cooperation of these binding sites might contribute to the equilibrium $K_D$ of 190 nM as determined by ITC measurements.

For further characterization of the binding interface between Pex5p and Pcs60p, we analyzed the region adjacent to the tripeptide SKL in more detail (Fig. 9). To this end, the wild-type C-terminal sequence was modified by an insertion of additional three amino acids in front of the SKL sequence to K-SSRNKASA-SKL (Pcs60p-insertion) and by substitution of the five amino acids in front of the SKL sequence to K-AAEAE-SKL (Pcs60p substitution). The binding properties of the mutant variants were compared with wild-type and the deletion mutants. The obtained binding curves were evaluated using the BIAevaluation software 3.1. The best fit for wild-type Pcs60p interacting with Pex5p was obtained with the bivalent analyze fit, confirming the two-step binding process of Pex5p and wild-type Pcs60p (Fig. 8). Remarkably, the substitution of five amino acid residues adjacent to the PTS1 of Pcs60p (Pcs60p substitution) resulted in a lower binding affinity ($K_D$ 2.8 μM) and an indicated 1:1 interaction with Pex5p. This underlines the importance of this region for interaction of Pcs60p and Pex5p. The result for an insertion of three amino acids in front of the SKL (Pcs60p insertion) revealed a differentiated picture. Although the interaction mode could not be conclusively assigned as monovalent or bivalent, efficient binding ($K_D$ 160 nM) was still observed. This suggests that the spatial separation of this region from the tripeptide SKL does not impair its recognition by Pex5p. Again, no interaction was observed for the Pcs60p variant with a deletion of the extreme C-terminal eight amino acid residues (Pcs60pΔ8).

**In Vivo Pcs60p Localization Studies**—To evaluate the impact of the signal sequence alterations on the peroxisomal targeting of Pcs60p (Fig. 9), the plasmid-encoded Pcs60p variants were investigated in the Δpcs60 mutant strain (Fig. 10). The different strains, including the non-transformed wild-type as positive control, were grown on oleic acid. PNS were prepared and subjected to subcellular fractionation resulting in an organellar pellet enriched for peroxisomes and mitochondria and a supernatant enriched for cytosolic proteins. As judged by immunoblot analyses, wild-type Pcs60p was predominantly found in the pellet fraction (Fig. 10A). Deletion of the C-terminal SKL (Pcs60pΔSKL) leads to clear cytosolic mislocalization of Pcs60p, demonstrating the well known requirement of the C-terminal tripeptide for proper peroxisomal localization. Substitution of the five amino acids adjacent to the SKL (Pcs60p substitution) also abolished the peroxisomal targeting of Pcs60p, thereby underlining the importance also of this region for peroxisomal targeting. This result is in agreement with the reduced affinity of this variant to Pex5p (Fig. 9C). In accordance with the in vitro binding studies, the amino acid insertion between C-terminal SKL and the adjacent region (Pcs60p insertion) still efficiently targeted Pcs60p to peroxisomes.

To discriminate whether the sedimented Pcs60p is not only targeted to peroxisomes but is actually translocated across the membrane, we analyzed its accessibility to exogenously added proteasome K in a protease protection assay (Fig. 10B). Accordingly, PNS were incubated with proteasome K in the absence and presence of detergent, and samples were subjected to SDS-PAGE and immunoblot analysis at different time points. Wild-type Pcs60p as well Pcs60p harboring the insertion (Pcs60p insertion) were not degraded in the absence of detergent, indicating its localization in the peroxisomal matrix and thus protection by the membrane. Accordingly, both proteins were only degraded in the presence of detergent, which destroys membrane integrity (Fig. 10B). The small sedimented amount of the variant lacking the C-terminal tripeptide (Pcs60pΔSKL) as well as of the substitution variant (Pcs60p substitution) was not protected against the protease, indicating that they were not imported into peroxisomes. The results confirm the requirement for both the C-terminal SKL and its adjacent region for proper peroxisomal import of Pcs60p.
Formation of the Pex5p-Pcs60p Complex—In this study, we analyzed the recognition and binding of cargo protein by the peroxisomal import receptor Pex5p by photo-cross-linking combined with high resolution MS, ITC, and surface plasmon resonance. We focused on *S. cerevisiae* Pex5p and its binding to Pcs60p, which contains a typical PTS1 and was shown to be imported in a Pex5p-dependent manner (23). Our data revealed that the purified recombinant Pcs60p forms monomers and oligomers at higher protein concentrations (Fig. 1 and Table 3). Interestingly, the monomeric Pex5p was found in SEC fractions of 160 kDa, well above the expected molecular mass of 69 kDa. This peculiar migration behavior of Pex5p upon gel filtration adds to the different observations made for human PEX5. Based on experimental approaches, including SEC, electron microscopy, and fluorescence anisotropy, Pex5p was reported to form a tetramer (52, 53). However, applications of biophysical and biochemical approaches, including differential scanning calorimetry, circular dichroism, attenuated total reflection Fourier transform infrared spectroscopy, determination of the sedimentation coefficient by equilibrium sedimentation centrifugation and of the Stokes radius by gel filtration as well as calculation of the resulting molecular mass, led to the conclusion that the human PEX5 is a monomeric non-globular protein with its N terminus being natively unfolded (15, 54). Observations made for the *Hansenula polymorpha* Pex5p by fluorescence analysis revealed that the protein is a monomer at pH 6.0 and a tetramer at pH 7.2 (55, 56). According to SEC and SDS-

**FIGURE 6.** Model of ScPex5p interacting with its PTS1 cargo protein Pcs60p. A, model of ScPex5p(301–612). The regions of the three Pex5p peptides identified in cross-linking experiments (cf. Fig. 5) are highlighted in violet, blue, and cyan. B, homology model of the C-terminal part of Pcs60p bound to Pex5p. Pcs60p contact residue sites (underlined in A) on the three Pex5p peptides are highlighted in red. For methionine on position 536, the side chain is also shown. Pcs60p residues that were positive in the *in vitro* photo-cross-linking experiments are highlighted in orange and labeled. Asn-539 from Pcs60p, which provided the strongest interaction signal, is colored in cyan. The remaining Pcs60p residues shown are in green.

**FIGURE 7.** ITC analysis of Pex5p interacting with wild-type Pcs60p (A) and Pcs60pΔSKL (B). ITC measurements were conducted in a buffer containing 50 mM HEPES, 300 mM NaCl at 25 °C on a MicroCal VP-ITC with 41–42 μM full-length Pex5p as a sample and 340–400 μM wild-type Pcs60p and 20–30 μM full-length Pex5p and 300–622 μM Pcs60pΔSKL, respectively. The upper panels represent the thermogram showing the amount of heat released after each injection of a volume of 10 μL. The lower panels represent the heat of reaction per injection as a function of the cargo/Pex5p ratio.
PAGE under non-reducing conditions, Pex5p of *Leishmania donovani* and *Pichia pastoris* forms a tetramer of two dimers (57, 58). Thus, the unusual migration behavior of yeast Pex5p upon SEC in light of the clear indication that the protein is monomeric by light scattering adds to the peculiar behavior of Pex5p from other species, and in analogy to its human orthologue it is best explained by the assumption that yeast Pex5p is monomeric with an unfolded N-terminal half. The data indicate that Pex5p and Pcs60p form a heteromeric complex in a 1:1 ratio.

**Identification of Pex5p/Pcs60p-binding Sites**—A combinatorial approach of site-specific photo-cross-linking and MS was applied to identify contact sites between Pcs60p and its receptor Pex5p. The cross-linking pBpa was introduced at the C-terminal PTS1 of Pcs60p and at regions in close proximity. The cross-linking products appeared as 130-kDa bands on a SDS-PAGE for Pcs60p variants Ser-537, Arg-538, Asn-539, Ser-541, as well as N539ΔSKL (Fig. 3A). MS analyses of these protein bands revealed distinct interactions of the different Pcs60p variants to Pex5p peptides (Table 4). Taken together, our cross-linking study shows that the adjacent region of the C-terminal tripeptide sequence makes direct contact to the corresponding region of Pex5p and thus contributes to the binding interface between the two proteins. The analysis provides structural insights and location of a binding interface between receptor and cargo to TPR6, TPR7, and the first helix of the C-terminal helix bundle of Pex5p (Fig. 5). The data allowed the generation of a model of the cargo-loaded yeast PTS1 receptor (Fig. 6) based on the known three-dimensional structures of human and trypanosome Pex5p (17, 50). Besides protein and species-dependent amino acid variations of the PTS1 (9), the structure of the signal sequence seems to be very similar, as shown for SCP2 (sterol carrier protein 2), and the YQSKL peptide (16, 17, 59, 60). Sites of interaction of human PEX5 with the PTS1 of SCP2 and AGT revealed five conserved asparagines at the α-helices of TPR3, TPR6, and TPR7 of Pex5p (21, 59). A similar observation was made for trypanosomal Pex5p (50). By a deletion library of Pex5p in combination with a yeast two-hybrid screen with the peroxisomal Mdh3p (malate dehydrogenase 3) as an interaction partner, the loops of TPR2 and TPR3 as well as TPR6 were predicted as direct interaction sites with the PTS1 (61).
Analysis of the Peroxisomal Targeting Signal of Pcs60p—Pcs60p contains the C-terminal amino acids SKL, a typical peroxisomal signal sequence, which certainly interacts with Pex5p. However, upon replacement of residues of the PTS1 tripeptide by pBpa, the formation of UV-generated cross-links was drastically reduced (S541, Fig. 3A) or virtually absent (K542 and L543, see Fig. 3A). This can be explained by the assumption that the distance between the tightly associated amino acids of the PTS1 with the corresponding regions of Pex5p is too close for the reactive radius of the cross-linked or the cross-linking might be sterically hindered.

Deletion of the PTS1 signal of Pcs60p prevents its import into peroxisomes (23). The easiest explanation is that upon deletion of the targeting signal, no interaction with the PTS1
receptor occurs. However, surprisingly, we found that the Pcs60p mutant Asn-539 was efficiently cross-linked to Pex5p independent of the presence or absence of the PTS1 (Fig. 3A).

In both cases the cross-linked target sites were virtually identical (Table 4 and Fig. 5). These data indicate that beside the PTS1-dependent site, a second contact site exists between Pex5p and Pcs60p. Accordingly, SPR measurements revealed a bivalent interaction mode with the existence of a high affinity and a low affinity site for the Pcs60-Pex5p interaction (Fig. 8). The existence of a second binding site beside the C-terminal tripeptide was confirmed by the observation that the low affinity binding site present upon deletion of the tripeptide SKL disappeared upon further deletion of adjacent amino acids (Fig. 9). Thus, our results indicate two modes of interactions between Pcs60p and Pex5p. One binding site is SKL-dependent and involves the direct binding of the PTS1 tripeptide to corresponding amino acids of Pex5p. The second binding site occurs in an SKL-independent manner and involves amino acids adjacent to the PTS1. The binding affinity of this interaction was lower but still exhibited a $K_d$ of 7.7 $\mu M$ as determined by ITC and confirmed by SPR. However, Pcs60p lacking its PTS1 sequence is no longer imported into peroxisomes in vivo (23), indicating that the lower affinity site is not sufficient for peroxisomal targeting. Nevertheless, substitution of the five amino acids adjacent to the SKL also abolished import, underlining that the importance of this low affinity binding region is essential for the functionality of the targeting signal. For other cargo proteins, $K_d$ values in the micromolar range are sufficient for peroxisomal import (59). Thus, alternatively, the presence of the PTS1 might trigger conformational changes in Pex5p that render it competent for subsequent import. Conformational changes upon PTS1 binding have been described for human PEX5 (62). The existence of additional binding sites beside the PTS1 is not exceptional. For instance, Cat2p (carnitine acetyltransferase 2) has an N-terminal mitochondrial targeting signal and a PTS1 at the C terminus. However, by deletion of both signals, Cat2p still interacts with its PTS1 receptor and is imported into peroxisomes (63). A second intrinsic recognition site is also discussed for Mdh3p (64). Moreover, yeast catalase A contains two peroxisomal targeting signals, each sufficient to direct reporter proteins to peroxisomes (65). However, for none of these yeast proteins, the receptor interface has been defined in structural terms. Crystal structures of human PEX5-cargo complexes also revealed secondary binding sites, which were shown to increase the binding affinity to PEX5 (19, 59, 62). Although the interacting region of the cargo SCP2 is far apart from the PTS1-binding site, the second PEX5-binding site of AGT is next to its C terminus and thereby resembles that of Pcs60p. Like the ancillary binding region of AGT, the secondary binding site of Pcs60p

FIGURE 10. Analysis of peroxisomal import of Pcs60p variants by cell fractionation and protease protection assay. Plasmids coding for indicated Pcs60p variants were expressed in $\Delta$pcs60 cells. Wild-type cells and $\Delta$pcs60 transformed with wild-type Pcs60p served as controls. A, test for peroxisomal targeting of Pcs60p variants by differential centrifugation. Cytosolic proteins in the supernatant and proteins associated with the organellar sediments were analyzed by immunoblotting. Mitochondrial porin and cytosolic fructose-1,6-bisphosphatase (Fbp1p) served as controls for proper separation. T, total; S, supernatant; P, pellet. B, analysis of peroxisomal import of Pcs60p variants by protease protection assay. Postnuclear supernatants of indicated yeast strains were incubated with proteinase K in the absence or presence of Triton X-100, and samples were analyzed at indicated time points by immunoblotting.
is essential but not sufficient for protein import (Fig. 10). It has been suggested that additional binding regions of AGT promote the non-autonomous binding of the non-canonical targeting sequence KKL (59, 62). However, Pcs60p carries the strong targeting signal SKL, which binds with high affinity to the yeast Pex5p receptor site but still requires the adjacent region for peroxisomal targeting.

What could be the role of an additional low affinity binding region of Pcs60p? It is easily conceivable that the low affinity binding site might be part of a so-called “extended PTS1” as postulated by Fodor et al. (59). In fact, the importance and influence of amino acids upstream of the PTS1 for recognition and import by Pex5p has been reported (9, 66, 67). Based on sequence analysis and modeling, it has been proposed that a dodecameric sequence of the 12 C-terminal residues of such a substrate protein are implicated in PTS1 signal recognition (68). The authors suppose that the region at position −3 to −6 upstream of the C-terminal PTS1 tripeptide interacts with the surface of Pex5p. Our studies showed that the amino acids adjacent to the PTS1 of Pcs60p are efficiently cross-linked to Pex5p, especially upon deletion of PTS1 (Pcs60pNAŠKL), and that this region interacts with Pex5p independent of the presence of the C-terminal tripeptide sequence. Our data indicate that substitution of the adjacent region with other amino acids abolished the targeting signal, although an insertion of three amino acids between the conserved tripeptide and the adjacent region did not impair receptor binding and peroxisomal targeting. These observations suggest that the region upstream of the PTS1 tripeptide can be spatially separated, supporting the notion that the “extended PTS1” of Pcs60p contains distinct modules.

In the future, it will be interesting to elucidate whether the bivalent binding mode reflects a sequential event with a PTS1-independent initial contact (tethering), followed by high affinity binding of the PTS1 (lock-in). Such a sequential tethering and lock-in would provide a novel concept on the dynamics of cargo recognition by the PTS1 receptor Pex5p.

Author Contributions—S. H. constructed all plasmids, performed and analyzed the experiments, and wrote the paper. F. D. and S. F. performed and analyzed the mass spectrometry analysis. K. F. and S. F. performed and analyzed the ITC experiments. K. F. and S. F. and analyzed the SPR data evaluation.

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