Members of the E2D (UbcH5) Family Mediate the Ubiquitination of the Conserved Cysteine of Pex5p, the Peroxisomal Import Receptor

Claudia P. Grou§, Andreia F. Carvalho§,1,2, Manuel P. Pinto§,1,2, Sebastian Wiese§, Heike Piechura§, Helmut E. Meyer§, Bettina Warscheid¶, Clara Sá-Miranda¶, and Jorge E. Azevedo§,3

From the 1Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, Rua do Campo Alegre, 823, 4150-180 Porto, Portugal, the 2Instituto de Ciências Biomédicas de Abel Salazar (ICBAS), Universidade do Porto, Largo Professor Abel Salazar, 2, 4009-003 Porto, Portugal, and the 3Medizinisches Proteom-Center, Ruhr-Universitaet Bochum, Universitaetsstrasse 150, 44780 Bochum, Germany

According to current models of peroxisomal biogenesis, newly synthesized peroxisomal matrix proteins are transported into the organelle by Pex5p. Pex5p recognizes these proteins in the cytosol, mediates their membrane translocation, and is exported back into the cytosol in an ATP-dependent manner. We have previously shown that export of Pex5p is preceded by (and requires) monoubiquitination of a conserved cysteine residue present at its N terminus. In yeasts, and probably also in plants, ubiquitination of Pex5p is mediated by a specialized ubiquitin-conjugating enzyme, Pex4p. In mammals, the identity of this enzyme has remained unknown for many years. Here, we provide evidence suggesting that E2D1/2/3 (UbcH15a/b/c) are the mammalian functional counterparts of yeast/plant Pex4p. The mechanistic implications of these findings are discussed.

Peroxisomal matrix proteins are synthesized in cytosolic ribosomes and post-translationally targeted to the peroxisome. Correct targeting of these proteins to the organelle requires an intricate machinery comprising more than 10 different proteins (1, 2). One central component of this machinery is Pex5p, a partially cytosolic, partially peroxisomal protein that interacts directly with newly synthesized peroxisomal matrix proteins containing a peroxisomal targeting signal (PTS)4 type 1 (3). In mammals and plants but not in yeasts/fungi, Pex5p is also in charge of targeting PTS2-containing proteins to the organelle matrix (4). In this case, however, the Pex5p-PTS2 interaction is not direct but rather bridged by Pex7p, the so-called PTS2 receptor (5).

According to current models, after binding cargo proteins in the cytosol, Pex5p interacts with the peroxisomal docking/translocation machinery, a multisubunit protein complex comprising mostly transmembrane proteins (1, 2). The core subunits of this machinery are Pex13p, Pex14p, and the RING peroxins Pex2p, Pex10p, and Pex12p (6–8). The interaction of the Pex5p-cargo protein complex with this machinery ultimately results in the partial insertion of Pex5p into the peroxisomal membrane with the concomitant translocation of cargo proteins into the matrix of the organelle (9, 10). Interestingly, this step is ATP-independent, suggesting that the driving force for protein translocation derives from the strong protein-protein interactions that Pex5p establishes with some components of the docking/translocation apparatus (11–13).

Pex5p is not consumed in this pathway. It is instead exported in an ATP-dependent way back into the cytosol to promote further rounds of protein transportation (11–13). For some time, it was assumed that this energy-requiring process comprised only one step: the dislocation of Pex5p from the docking/translocation complex by the mechanoenzymes Pex1p and Pex6p, two members of the ATPases associated with various cellular activities (AAA) family of proteins (11, 13). However, recent data uncovered another ATP-dependent step, this one involving the ubiquitin-conjugating cascade (UCC). Indeed, data suggesting that both yeast and mammalian Pex5p have to be ubiquitinated to be recognized by the export machinery were provided (14, 15). Interestingly, ubiquitin is conjugated to Pex5p not through the usual isopeptide bond but rather via a thiol ester bond involving a conserved cysteine present at the N terminus of the peroxin (14, 16). Although thiol ester bonds are at the heart of the chemistry used by the enzymes of UCC, they are, at least apparently, quite uncommon in ubiquitin-substrate conjugates (17). In fact, besides Pex5p, only one other protein, a major histocompatibility complex class I molecule, was reported to be ubiquitinated in this way (18).

The UCC acting on yeast Pex5p during its normal and transient passage through the peroxisomal membrane is now fairly

References

1. The abbreviations used are: PTS, peroxisomal targeting signal; UCC, ubiquitin-conjugating cascade; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; ATP6, adenosine 5’-O-(thiotriphosphate); PNS, postnuclear supernatant; GST-Ub, glutathione S-transferase-ubiquitin; E-64, N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutyramide; NEM, N-ethylmaleimide; DTT, dithiothreitol; MOPS, 4-morpholinopropanesulfonic acid; HPLC, high pressure liquid chromatography.

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characterized (13, 15, 16, 19–22). After the ATP-dependent activation of ubiquitin catalyzed by UBA1, the only ubiquitin-activating enzyme (E1) present in yeast (23), ubiquitin is transferred to Pex4p (UBC10) (24, 25). This ubiquitin-conjugating enzyme (E2) is a peripheral component of the peroxisomal membrane in yeasts and probably also in plants. In the absence of Pex22p, a peroxisomal intrinsic membrane protein, Pex4p is unstable, suggesting that the two proteins form a structural/functional unit (26–29). The identity of the ubiquitin ligase(s) (E3) acting at the terminal step of this cascade is still unknown. It seems likely, however, that this role is played by the RING peroxins(s) of the docking/translocation machinery, as supported by the observation that yeast Pex4p interacts with Pex10p (30).

Much less is known regarding the UCC acting on mammalian Pex5p. Actually, the only known facts about this issue are that Pex5p is ubiquitinated at the peroxisomal membrane after the ATP-independent membrane insertion step, that this ubiquitination is a prerequisite for its export, and that ubiquitinated Pex5p is exported into the cytosol in an ATPγS-sensitive process (14). Several factors may explain this lack of knowledge. Perhaps the most important is related to the fact that the main approach that has been used to identify mammalian peroxins (i.e., homology searches using yeasts/fungi sequences as queries) has failed in this case. Indeed, no in silico evidence for a mammalian PEX22 gene could be found in several recent studies (31, 32). For Pex4p, a different problem applies. Mammals have more than 30 ubiquitin-conjugating enzymes (reviewed in Ref. 33). However, none of these proteins seem to display a particularly close sequence relationship to the yeast/fungi/plants Pex4p (27). Proteomic studies aiming at defining the protein repertoire of mammalian peroxisomes have been of no help in this subject either (34–36). No evidence for a peroxisome-associated E2 was found, and none of the novel peroxisomal proteins identified in those studies displays even a remote relationship to the yeast/plant Pex22p. Clearly, other approaches are needed to address this problem.

During the last years, we have been using an in vitro system to characterize the Pex5p-mediated import pathway in mammals. Two different versions of this in vitro system have been employed. In one version, a postnuclear supernatant (PNS) from rat liver is used as the source of peroxisomes and other required components. In this version, all the steps of the Pex5p-mediated import pathway can be monitored. These include 1) the cargo-dependent but ATP-independent insertion of Pex5p into the peroxisomal docking/translocation machinery (12, 37), 2) the stage 2/stage 3 ATPγS-insensitive transition, which we now know reflects ubiquitination of the conserved cysteine at the N terminus of Pex5p (14, 38, 39), and 3) the subsequent ATP-dependent (and ATPγS-sensitive) export step of ubiquitinated Pex5p back into the cytosol (12, 39, 40). In the other version, highly purified peroxisomes from rat liver are used in the in vitro reactions. Here, cargo-dependent but ATP-independent insertion of Pex5p into the docking/translocation machinery is still possible. However, because the system lacks the required amounts of ubiquitin and/or components of the UCC, transition of stage 2 Pex5p into stage 3 Pex5p is no longer possible, and the peroxin becomes arrested at the docking/translocation machinery (12). In this work, we have explored the properties of this incomplete version to identify the rat liver ubiquitin-conjugating enzyme(s) mediating the peroxisomal ubiquitination of the conserved cysteine residue of Pex5p.

**EXPERIMENTAL PROCEDURES**

**Peroxisome Preparation**—Isolation of highly pure peroxisomes from rat liver by differential centrifugation and Nycodenz gradient purification was performed as described (41), with minor modifications. The SIE buffer (0.25 M sucrose, 5 mM imidazole, pH 7.4, 1 mM EDTA-NaOH, pH 7.4) was supplemented with 0.1 mg/ml phenylmethylsulfonyl fluoride and 1:500 (v/v) mammalian protease inhibitor mixture (Sigma). The purified peroxisomes were carefully resuspended in SEM buffer (0.25 M sucrose, 20 mM MOPS-KOH, pH 7.4, 1 mM EDTA-NaOH, pH 7.4) containing 2 μg/ml N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide (E-64), frozen in liquid N2, and stored at −70 °C.

**Cytosol Preparation, Fractionation, and Purification of E2s**—Livers from male rats fasted overnight were homogenized in 5 volumes of ice-cold 0.25 M sucrose, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA-NaOH, pH 7.5, supplemented with 2 μg/ml E-64. A cytosolic fraction was obtained from this homogenate after the following sequential centrifugations: 10 min at 600 g, 20 min at 12,300 g, and 1 h at 100,000 g. Cytosolic Fraction I and Fraction II were obtained according to Ciechanover et al. (42). Covalent affinity chromatography was performed according to Ref. 42 with modifications. One hundred and fifty mg of cytosolic proteins (at 30 mg/ml) were subjected to precipitation with 16% (v/v) polyethylene glycol 4000 (added from a 50% (w/v) stock solution in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl) for 20 min on ice. The pellet obtained after centrifugation at 10,000 × g for 20 min was resuspended in 5 ml of 50 mM Tris-HCl, pH 7.5, 5 mM ATP, 10 mM MgCl2, and 0.2 mM DTT. The clarified sample was applied at a 0.1 ml/min flow rate to a 0.8-ml ubiquitin-agarose column (10 mg/ml; BIOMOL), previously equilibrated with 5 volumes of buffer A (50 mM Tris-HCl, pH 7.5, 2 mM ATP, 5 mM MgCl2, 0.2 mM DTT). The column was washed at 0.5 ml/min as follows: 3 volumes of buffer A, 6 volumes of 50 mM Tris-HCl, pH 7.5, and 3 volumes of 50 mM Tris-HCl, pH 7.5, 1 mM KCl. Elution was performed at 0.1 ml/min flow rate with 3 volumes of a high pH/DTT buffer (50 mM Tris-HCl, pH 9.0, 20 mM DTT). The eluate was immediately neutralized with a 100 mM Tris-HCl, pH 7.5, 33 μg/ml bovine serum albumin solution. The wash and elution fractions were subjected to three cycles of concentration/dilution with 20 mM Tris-HCl, pH 7.5, supplemented with 0.2 mM DTT, using Vivaspin 500 columns (3,000 molecular weight cutoff polyethersulfone; Vivascience), pretreated for 30 min at room temperature with 600 μl of 0.1% (w/v) bovine serum albumin to minimize protein loss by adsorption. In the subsequent steps, only Eppendorf® protein LoBind microcentrifuge tubes were used. Concentrated eluates (100–150 μl) were incubated with 30 μl (bed volume) of DEAE-cellulose (DE52, Whatman) for 30 min at 18 °C. The beads were washed two times for 10 s with 100 μl of 20 mM Tris-HCl, pH 7.5, and the bound proteins were eluted twice with 50 μl of 20 mM Tris-HCl, pH 7.5, 0.5 mM KCl, for 5 min. Throughout the entire purification procedure, aliquots of all fractions were withdrawn for SDS-PAGE analysis and in
**E2D-mediated Ubiquitination of Mammalian Pex5p**

*In vitro* import experiments to test for E2 activity. With the exception of the cytosolic fraction, which was kept frozen at −70 °C for 5–10 days, the entire procedure, activity assays included, was always performed in a single day.

**Synthesis of ³⁵S-labeled Pex5p Proteins**—The cDNA encoding a C-terminal truncated version comprising amino acid residues 1–324 of the human large isofrom of Pex5p (43, 44) (∆C1-Pex5p) was amplified using the pGEM4-Xpex5p plasmid (39) as template and the primer pair 5′-GGCAACTCTCATATTGGCATAGGTCTCTAGTTTAATGG-3′ and 5′-CCGCGGATCCCTCTATTAGGGTAATGG-3′. The resulting PCR product was digested with Ndel and BamHI and cloned into pET-28a (Novagen), originating pET-28a/GST (39).

**Import Experiments**—Import reactions using 400 μg of PNS from rat liver or human skin fibroblasts were performed as described before (39). *In vitro* import experiments containing 50 μg of purified peroxisomes were performed in import buffer comprising 0.25 M sucrose, 50 mM KCl, 5 mM MOPS-KOH, pH 7.2, 3 mM MgCl₂, 0.1 mM dithiothreitol, and 2 μg/ml E-64. ATP or ATPγS was used at 5 mM final concentration. Where indicated, 25 μg of GST-ubiquitin (GST- Ub (45)) or 4 μg of bovine ubiquitin (Sigma) were used per import reaction. TPRs-Pex5p (46), a PTS1-containing peptide (CRYHLKPLQSKL), and a control peptide (CRYHLKPLQLKSK) were used exactly as described before (37). Where specified, the reactions were supplemented with 250 ng of recombinant human E1 and 10 ng of the relevant E2. The reactions were incubated for 20 min at 37 °C, and the organelles were isolated by centrifugation and analyzed by SDS-PAGE and autoradiography.

**Protein Identification by Mass Spectrometry**—Following staining with colloidal Coomassie Brilliant Blue G-250, gel bands of interest were cut out and destained by alternately incubating them with 20 μl of 10 mM NH₄HCO₃ and 20 μl of 5 mM NH₄HCO₃/50% acetonitrile (v/v) for 10 min each. In-gel digestion was performed overnight at 37 °C using trypsin dissolved in 10 mM NH₄HCO₃ buffer (pH 7.8). The resulting peptides were extracted twice with 10 μl of acetonitrile/5% formic acid (50:50, v/v). Subsequently, acetonitrile was removed in vacuo, and samples were acidified by the addition of 5% formic acid to a final volume of 20 μl. Online reversed-phase nano-HPLC separations were performed using the Dionex LC Packings HPLC systems (Dionex LC Packings, Idstein, Germany) as described (47), with slight modifications. Peptides were separated on a C18 nano column (C18 PepMap; Dionex) using solvent A (0.1% formic acid) and solvent B (0.1% formic acid in 84% acetonitrile, v/v) to perform a binary linear gradient of 5–30% solvent B in 34 min. Electrospray ionization tandem mass spectrometry was performed on a Bruker Daltonics HCT ultra ion trap instrument (Bremen, Germany) equipped with a nanoelectrospray ion source (Bruker Daltonics) as described (36). Peak lists of tandem mass spectrometry spectra acquired were generated using DataAnalysis 3.4.179 with default parameters. For peptide identifications via MASCOT (release version 2.2) (48) and SEQUEST (Version TurboSEQUEST v. 27) (49), peak lists were correlated with a composite data base consisting of the Rat International Protein Index (Rat IPI V3.30) data base (at the European Bioinformatics Institute) containing 41,269 protein entries and a duplicate of the same data base, in which the amino acid sequences were randomly shuffled. Data base searches were performed with trypptic specificity, allowing two missed cleavages and oxidation of methionine. Proteins were assembled on the basis of peptide identifications using the ProteinExtractor tool (version 1.0) in ProteinScape (version 1.3 S.R. 2, Bruker Daltonics) as described (36). The presence of each protein isoform was confirmed by the identification of at least one unique peptide. Only protein identifications up to an accumulated false discovery rate of 0% are reported.

**Cell Culture**—Human primary skin fibroblast were grown in a standard Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum. The cells were grown at 37 °C in the presence of 5% CO₂. Cells at 90% confluence were harvested with a cell scraper, washed three times with phosphate-buffered saline, and centrifuged at 2,000 × g for 10 min at 4 °C. The resulting pellet was resuspended in SEM buffer supplemented with E-64. The sample was homogenized using a tight-fit glass-Teflon homogenizer, and the homogenate was centrifuged at 600 × g for 10 min at 4 °C. Aliquots of the PNS fraction were frozen in liquid N₂ and stored at −70 °C.

**FIGURE 1. Ubiquitination of ∆C1-Pex5p requires both cytosolic and peroxisomal components.** A, ³⁵S-labeled ∆C1-Pex5p was incubated in import medium containing ATP and GST-Ub with cytosol alone (Cyt; 500 μg of protein), cytosol and peroxisomes (Per + Cyt), or peroxisomes in the absence (Per) or presence of recombinant E1 (Per + E1). Organellar pellets and 1/5 of the Cyt sample were analyzed by SDS-PAGE under non-reducing (−DTT), and reducing conditions (+ DTT). Lane 1, 10% of the *E. coli* lysate containing ³⁵S-labeled ∆C1-Pex5p used in each lane. α, GST-Ub; ∆C1-Pex5p; numbers to the left indicate the molecular masses of the protein standards in kDa. B, ³⁵S-labeled ∆C1-Pex5p was incubated in import buffer containing cytosol, peroxisomes, GST-Ub and ATP. The organelles were isolated by centrifugation, solubilized with detergent, and subjected to a pull-down assay using glutathione-Sepharose beads. The total (T), unbound (Unb), and bound fractions (in this case, treated (B+) or not (B−) with DTT) were subjected to SDS-PAGE. Three equivalents of the bound fraction in relation to the total and unbound samples were loaded onto the gel.
E2D-mediated Ubiquitination of Mammalian Pex5p

To identify the mammalian counterpart of yeast Pex4p, we first asked whether or not we could detect its activity in purified rat liver peroxisomes by subjecting these organelles to in vitro import reactions supplemented with GST-UB and recombinant E1. 35S-labeled ΔC1-Pex5p synthesized in vitro in an E. coli-based transcription/translation system was used in most of these experiments as the substrate for ubiquitination. As shown before, this truncated version of Pex5p (which comprises amino acid residues 1–324 of human Pex5p) is completely competent in all the steps occurring at the peroxisomal protein translocation machinery (i.e. insertion, ubiquitination, and export) but, unlike the full-length peroxin, contains only one cysteine, the conserved Cys-11 (14, 37, 40). Thus, the existence of thiol-sensitive ubiquitinated ΔC1-Pex5p in these assays reflects ubiquitination at this cysteine (thiol ester bonds, unlike isopeptidic bonds, are thiol-sensitive; (42)).

It should also be noted that GST-UB (and not ubiquitin) was used in these experiments because it is used efficiently by the UCC acting on Cys-11 of Pex5p or ΔC1-Pex5p when the complete version of the in vitro import system is employed. However, in contrast to ubiquitin, conjugation of GST-UB to Pex5p or ΔC1-Pex5p gives rise to peroxisomal species that are not substrates for the peroxisomal export machinery (14). This choice simplifies the ubiquitination assays because only organelle pellets need to be analyzed.

As shown in Fig. 1A, purified rat liver peroxisomes are not competent in promoting ubiquitination of ΔC1-Pex5p when GST-UB and recombinant E1 are included in the import buffer (lane Per + E1). A similar result is obtained when ΔC1-Pex5p is incubated with a cytosolic fraction lacking organelles (lane Cyt). In contrast, thiol-sensitive GST-UB-ΔC1-Pex5p (Fig. 1A, arrow a; see also Fig. 1B) is easily observed when 35S-labeled ΔC1-Pex5p is subjected to import reactions containing both purified peroxisomes and the cytosolic fraction (lane Per + Cyt). Apparently, the cytosolic fraction contains at least one component (besides the E1 enzyme) required for the UCC acting on Cys-11 of Pex5p, a conclusion further corroborated by experiments performed with PNS fractions from rat liver and human skin fibroblasts (see below). Many different hypotheses could be forwarded to explain these observations. A plausible one is to assume that the required cytosolic component is an E2 enzyme. This possibility was addressed in the experiments described below.

To purify E2 enzymes from rat liver cytosol, we tried to use the two-step procedure developed by Ciechanover et al. (42). In the first step, cytosolic proteins are separated into two fractions by anion-exchange chromatography using DE52. The so-called Fraction I contains proteins (ubiquitin included) that do not bind to DE52. Fraction II is obtained by eluting the column with a high salt buffer and contains E1, several E2s, and many other proteins. In the second step, the ubiquitin-depleted Fraction II is subjected to covalent affinity chromatography using an ubiquitin-derivatized matrix. Unexpectedly, no ubiquitinating activity could be detected when using the DE52 Fraction II in our assays. Fraction I alone also produced a negative result. However, the addition of recombinant E1 to this fraction (but not to Fraction II) resulted in a full recovery of the ubiquitinating activity (see supplemental Fig. S1), an observation that sup-

FIGURE 2. Partial purification of the E2(s) mediating the ubiquitination of Cys-11 of ΔC1-Pex5p. A, protein fractions obtained during the purification procedure were analyzed by SDS-PAGE/Coomassie Blue staining (upper panel) and assayed for peroxisome-dependent ubiquitination activity on ΔC1-Pex5p (lower panel). In the upper panel, 100 μg of cytosolic protein (Cyt) were loaded onto the gel; the polyethylene glycol-precipitated fraction (PEG) and the affinity chromatography flow-through (FT), wash (W), and eluate (E) fractions shown were derived from 0.2, 0.2, 15, and 15 mg of cytosolic protein, respectively. In the ubiquitination assay, cytosolic protein (500 μg) and polyethylene glycol-precipitated, flow-through, wash, and eluate fractions derived from 500 μg of cytosolic protein were used. a, GST-UB-ΔC1-Pex5p; lanes 1, 10% of the E. coli lysate containing 35S-labeled ΔC1-Pex5p used in each lane; numbers to the left indicate the molecular masses of the protein standards in kDa (lanes MW); Ub, 2 μg of bovine ubiquitin. B, the affinity chromatography eluate and the DE52-bound (B) and unbound (Unb) fractions derived from 8, 25, and 25 mg of cytosolic protein, respectively, were analyzed by SDS-PAGE and stained with colloidal Coomassie Brilliant Blue (upper panel). Protein bands 1 and 2 were subjected to mass spectrometry. The activity assays (lower panel) were performed as described above.

Miscellaneous—The recombinant human E1 and E2s used in the in vitro import experiments were purchased from BIOMOL and were diluted in SEM buffer with 0.1% bovine serum albumin immediately before use. The anti-PMP70 antibody (50) was kindly provided by Dr. Wilhelm W. Just (University of Heidelberg, Heidelberg, Germany). The antibody directed to human Pex5p was described before (51). NEM and DTT treatment of protein samples, GST pull-down assays, SDS-PAGE, and Western blotting were performed as described recently (14).

RESULTS

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ported the idea that the cytosolic fraction contains the required E2.

The absence of E1 in Fraction I together with the more complicated problem that this fraction contains high amounts of ubiquitin precludes its utilization in the covalent affinity chromatography step. We found that by subjecting a cytosolic fraction to polyethylene glycol precipitation, we could recover the majority of the ubiquitinating activity in the pellet, whereas most of rat liver ubiquitin remained in the supernatant (Fig. 2A and data not shown). This ubiquitin-depleted fraction was then subjected to the covalent affinity chromatography step. As shown in Fig. 2A, virtually all the ubiquitinactivating activity present in the polyethylene glycol-precipitated fraction was recovered in the high pH/DTT eluate of the ubiquitin-agarose column (lane E, lower panel). As expected, the complexity of this protein fraction was still relatively high (Fig. 2A, upper panel, lane E). To further purify the component of interest, we explored the fact that it does not bind to DE52 (see supplemental Fig. S1). As shown in Fig. 2B, lower panel, almost no ubiquitinating activity could be detected when using the DE52-bound protein fraction in our assay. In contrast, a significant amount of GST-Ub-ΔC1-Pex5p was generated in the presence of the unbound protein fraction. SDS-PAGE analysis of this protein fraction revealed the presence of two protein bands (Fig. 2B, upper panel, lane Unb). Mass spectrometry analysis identified E2L3 (UbcH7) as the main protein present in the slower migrating band 1. This same E2 (or a fragment of it) together with E2N (UbcH13) and the three closely related E2 enzymes E2D3 (UbcH5c), E2D2 (UbcH5b), and E2D1 (UbcH5a) were identified in the faster migrating protein band 2 (see supplemental Table I).

To determine which of the five E2s identified (if any) is capable of mediating ubiquitination of Pex5p, a set of 13 human recombinant E2s comprising the homologues of the 5 rat E2s described above was tested in our ubiquitination assay. Particular care was given to the concentration of these enzymes in the assay. In the experiment presented in Fig. 2B, the amount of the DE52-unbound protein fraction used in the ubiquitination reaction (lower panel) was 2% of the amount loaded in the gel shown in the upper panel. Assuming that the strongest protein band visible in the Coomassie Blue-stained gel (containing E2L3/UbcH7) contains ~0.5 μg of protein, then 10 ng of the correct E2 per import reaction should be sufficient to observe an ubiquitinating activity. As shown in Fig. 3A, from all the E2s tested at this concentration, only UbcH5a (E2D1), UbcH5b (E2D2), and UbcH5c (E2D3) are capable of ubiquitinating ΔC1-Pex5p. The ubiquitinated Pex5p species are thiol-sensitive (Fig. 3B), and their production requires catalytically active versions of these E2s (Fig. 3A; lower panel, last three lanes). Finally, no ubiquitination of Pex5p could be detected in these assays in the absence of peroxisomes (data not shown).

The results presented above indicate that any of the three UbcH5 family members are capable of ubiquitinating ΔC1-Pex5p at the conserved cysteine residue. However, it remained unclear whether or not this event is linked to the normal transit of Pex5p through the peroxisomal protein translocation machinery. To clarify this issue, we explored the fact that insertion of full-length Pex5p into the docking/translocation machinery is cargo protein-dependent (37). As explained in detail elsewhere (12), the soluble phase of these import reactions contains high amounts of peroxisomal proteins that have leaked from the organelles during their preparation and manipulation. Under standard in vitro import conditions, these proteins are sufficient to allow efficient insertion of the peroxin into the peroxisomal translocation machinery. If, however,
and control peptide or a PTS1-containing peptide were added to samples in the presence of Pex5p (0.2 μM). These proteins are sequestered by adding to the import reaction at 37 °C was for 2.5 min (lanes 1–3) or 20 min (lanes 1 and 3). ATP was used in the reactions in lanes 1–3, whereas the reaction in lanes 4 received ATP instead of NEM. The addition of NEM, the reactions were subjected to centrifugation to separate organelles (lanes P) from soluble proteins (lanes S). Samples were then subjected to SDS-PAGE under non-reducing conditions and analyzed by Western blotting using an antibody against PMP70 to assess the separation into an organelle and a soluble phase. Exactly the same results were obtained when UbH5c isoforms were used in these experiments (data not shown). a, GST–Ub–ΔC1-Pex5p/Pex5p species; b, Ub–ΔC1-Pex5p/Pex5p species; lane I, 10% of the 35S-labeled proteins used in each lane. C, purified rat liver peroxisomes were incubated in import buffer supplemented with E1 and ubiquitin in the presence (lanes 2–4) or absence of UbH5c (lanes 1). ATP was used in reactions in lanes 1–3, whereas the reaction in lanes 4 received ATP instead of NEM. The addition of NEM, the reactions were subjected to centrifugation to separate organelles (lanes P) from soluble proteins (lanes S). Samples were then subjected to SDS-PAGE under non-reducing conditions and analyzed by Western blotting using an antibody directed to human Pex5p. Rat Pex5p and its ubiquitinated form (arrow b) are indicated. The asterisk indicates a nonspecific protein band occasionally detected by the antibody. 1, untreated rat liver peroxisomes (50 μg of protein). Numbers to the left indicate the molecular masses of protein standards in kDa.

**DISCUSSION**

The aim of this work was to identify the mammalian ubiquitin-conjugating enzyme(s) mediating the ubiquitination of the conserved cysteine of Pex5p at the peroxisomal membrane. For this purpose, we first developed a peroxisome-dependent Pex5p-ubiquitination assay, which was then used to monitor the distribution of the relevant E2 during the fractionation procedure of rat liver cytosol. Using the method described by Ciechanover et al. (42) (with some modifications), it was possible to obtain a low complexity protein fraction in which five E2s were identified. Subsequent ubiquitination assays using recombinant human versions of these E2s revealed that three of them, all members of the E2D family, were indeed capable of mediating ubiquitination of Pex5p. Importantly, the E2D-mediated Pex5p ubiquitination was shown to occur at the right residue of the receptor (i.e. Cys-11) and at the right moment/location (i.e. after the docking/translocation step). Finally, E2D-mediated ubiquitination of Pex5p resulted in export-competent species.
E2D-mediated Ubiquitination of Mammalian Pex5p

![Image](image_url)

**FIGURE 5.** Addition of recombinant E1 and UbcH5c to human and rat total organelles restores the UCC acting on Pex5p. A, five tubes containing a PNS from human fibroblasts in import buffer (100 µl final volume) were prepared. One of the tubes was kept on ice (lane C), whereas the others were subjected to a centrifugation to sediment the organelles. In one of these tubes, the supernatant was not removed but used instead to resuspend the organelles (lane S). The supernatants of the remaining tubes were discarded, and the organelles were carefully resuspended in 100 µl of import buffer. After the addition of ATP and GST-Ub to all the tubes, the following components were then added: recombinant E1 (samples in lane A, UbcH5c, and UbcH7); recombinant UbcH5c (lane UbcH5c); and recombinant UbcH7 (lane UbcH7). Import/ubiquitination reactions were initiated by adding 35S-labeled ΔC1-Pex5p and placing the tubes at 37 °C. After 20 min, NEM was added to all the samples, and the organelles were isolated by centrifugation and subjected to SDS-PAGE under reducing (+DTT) or non-reducing (−DTT) conditions. An autoradiograph is shown. , GST-Ub-ΔC1-Pex5p; lane I, 10% of the E. coli lysate containing 35S-labeled ΔC1-Pex5p used in each lane; numbers to the left indicate the molecular masses of protein standards in kDa. B, the same as in A with the exception that a rat liver PNS fraction was used.

Taken together, these findings strongly suggest that we have identified the rat liver E2s that participate in the UCC required for the Pex5p-mediated import pathway. Considering that members of the E2D family are ubiquitously expressed in human tissues (52, 53) and the results obtained with human organelles (Fig. 5), it is likely that this conclusion is also valid for humans.

The data presented here provide explanations for many of the observations reported before and reveal interesting properties of the mammalian Pex5p-mediated import pathway. For instance, it is now easier to understand why a peroxisome-associated E2 was never found in the several studies aiming at defining the mammalian peroxisomal protein repertoire. As shown in Fig. 5, the simple procedure of subjecting a PNS to a short centrifugation results in the complete depletion of the Pex4p-like activity from the sedimented organelles. Thus, besides lacking a specialized E2, as yeast/plant Pex4p may be regarded, mammalian peroxisomes also appear to lack their own pool of the E2 component required for ubiquitination of Pex5p. Furthermore, this observation also provides a biochemical ground to the in silico data, suggesting that mammals, as well as many other organisms, lack functional counterparts of yeast/plant Pex22p and is in agreement with the fact that E2-E3 interactions are generally very weak and transient with large dissociation constants (54, 55).

Finally, our data may also have implications to the field of human peroxisomal biogenesis disorders. Indeed, considering that any of the three enzymes identified here, each encoded by a different gene, is capable of supporting the UCC acting on Pex5p and the fact that several important biological roles have been ascribed to these E2s (e.g. in controlling the cellular levels of p53 or IkBα (56, 57)), it now seems unlikely that defects in an E2 enzyme in humans will ever be linked to an isolated defect in peroxisomal biogenesis, as observed in yeasts and plants.

The finding that members of the E2D family participate in a novel, previously uncharacterized, ubiquitin-conjugating cascade is not surprising. Indeed, mammalian E2D enzymes are known to be highly active with a variety of E3s (58) and, at least in rat liver, they probably mediate a large fraction of the total ubiquitinating activity (59). However, by showing that ubiquitination of a cysteine residue in a particular protein does not require a specialized E2, our results do raise the possibility that this unusual type of ubiquitination is more common than presently realized.

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