The Sorting Signals for Peroxisomal Membrane-bound Ascorbate Peroxidase Are within Its C-terminal Tail

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Peroxisomal ascorbate peroxidase (APX) is a carboxyl tail-anchored, type II (Ncytosol-Cmatrix) integral membrane protein that functions in the regeneration of NADH in glyoxysomes of germinated oilseeds and protection of peroxisomes in other organisms from toxic H2O2. Recently we showed that cotranssed peroxisomal APX was sorted post-translationally from the cytosol to peroxisomes via a novel reticular/circular membranous network that was interpreted to be a subdomain of the endoplasmic reticulum (ER), named peroxisomal ER (pER). Here we report on the molecular signals responsible for sorting peroxisomal APX. Deletions or site-specific substitutions of certain amino acid residues within the hydrophilic C-terminal-most eight-amino acid residues (includes a positively charged domain found in most peroxisomal integral membrane-destined proteins) abolished sorting of peroxisomal APX to peroxisomes via pER. However, the C-terminal tail was not sufficient for sorting chloramphenicol acetyltransferase to peroxisomes via pER, whereas the peptide plus most of the immediately adjacent 21-amino acid transmembrane domain (TMD) of peroxisomal APX was sufficient for sorting. Replacement of the peroxisomal APX TMD with an artificial TMD (devoid of putative sorting sequences) plus the peroxisomal APX C-terminal tail also sorted chloramphenicol acetyltransferase to peroxisomes via pER, indicating that the peroxisomal APX TMD does not possess essential sorting information. Instead, the TMD appears to confer the proper context required for the conserved positively charged domain to function within peroxisomal APX as an overlapping pER sorting signal and a membrane peroxisome targeting signal type 2.

Peroxisomes are single membrane-bound organelles that do not contain DNA nor protein-synthesizing machinery. Data published thus far indicate that they acquire their nuclear-encoded matrix and boundary membrane protein constituents from the cytosol in a regulated, post-translational manner. For instance, proteins destined for the peroxisomal matrix are sorted by at least two evolutionarily conserved targeting signals, the type 1 and type 2 peroxisomal targeting signal (PTS).¹ Most of our understanding of the molecular mechanisms responsible for sorting and import of PTS1- and PTS2-bearing proteins has come from studies of yeast and cultured mammalian cells with defects in peroxisomal biogenesis (pex mutants) (1). Characterization of these mutants has led to the isolation of a number of genes (PEX) and gene products (peroxins) (2–4). More recently, information related to the targeting signals and sorting/insertion/assembly of peroxisomal membrane proteins (PMPs) has become available. PMPs also seem to be synthesized in the cytosol and sorted post-translationally to the organelle. However, with one exception (5), PMPs do not possess a PTS1 or PTS2, and insertion/assembly of PMPs requires several protein components (peroxins) distinct from those required for the import of matrix proteins (2, 6).

Newly synthesized PMPs seem to be sorted either directly to peroxisomes from the cytosol or indirectly to peroxisomes via the endoplasmic reticulum (ER). Data on the routing of PMPs directly to the peroxisomal boundary membrane has been derived mostly from in vitro studies (7–10). Direct sorting of PMPs appears to be mediated by molecular chaperones and a two-step binding and insertion process that is temperature-dependent but does not require ATP hydrolysis. Targeting information also has been obtained for direct in vivo sorting of PMPs to peroxisomes (11–14). For example, the Candida boidinii PMP47 sorting signal, referred to as a membrane PTS (mPTS), was shown to be a cluster of basic residues within a 20-amino acid hydrophilic loop between transmembrane domains (TMDs) four and five of the protein (11).

Another subset of PMPs seems to be sorted initially from the cytosol to the ER, then to peroxisomes. For example, Saccharomyces cerevisiae Pex15p, a tail-anchored type II (Ncytosol-Cmatrix) PMP, and Yarrowia lipolytica Pex2p and Pex16p are glycosylated within the ER lumen while en route to peroxisomes (15, 16). Overexpression of human Pex3p or a portion of the Hansenula polymorpha counterpart fused to a reporter protein resulted in a profound proliferation of ER membranes, consistent with the premise that these proteins are sorted initially to the ER and then to peroxisomes (17, 18). Targeting information, albeit somewhat limited when compared with that for C. boidinii PMP47, also has been reported for certain PMPs (H. polymorpha Pex3p and S. cerevisiae Pex15p) that are sorted indirectly to peroxisomes via the ER (15, 17). Herein, a cluster of basic residues similar to sequences identified in the C. boidinii PMP47 targeting signal were postulated to be part of the sorting signal.

To date, only one plant PMP has been reported to be sorted to peroxisomes via the ER. Cottonseed peroxisomal ascorbate

low-2; CAT, chloramphenicol acetyltransferase; Cy2, cyanine 2; ER, endoplasmic reticulum; pER, peroxisomal ER; HA, hemagglutinin; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMP, peroxisome membrane protein; TMD, transmembrane domain; VAMP, vesicle-associated membrane protein.
peroxidase (APX), a tail-anchor type II (N\textsubscript{cys}-C-matrix) PMP, was shown in vitro to insert specifically into ER membranes and not into peroxisome membranes (19). This insertion occurred in a post-translational (signal recognition particle-independent) manner, was dependent upon the presence of ATP and the Hsp70 chaperone machine, and seemed to involve a membrane-bound receptor. In the same study, transiently expressed peroxisomal APX was found to sort in vivo to a distinct subdomain of the ER, which was designated as peroxisomal ER (pER). Suggestive evidence that the peroxisomal APX was transported by vesicles from pER to peroxisomes was obtained from results with brefeldin A, a fungal toxin that disrupts protein export from the ER (19). Vesicular transport of PMPs from the ER to peroxisomes has been proposed for sorting of other PMPs in yeast (15, 16, 20–22) and mammals (23); however, in only one system hitherto (i.e. Y. lipolytica) has an ER-to-peroxisome connection been demonstrated experimentally (for review, see Ref. 24). Nevertheless, important implications of this indirect PMP sorting pathway are that the ER serves as the initial targeting site of the nascent PMP and as the source of new or differentiating peroxisomes (2, 4, 24, 25).

Here, we describe results of in vivo immunofluorescence experiments designed to delineate the molecular signal(s) responsible for sorting peroxisomal APX in plant cells. We demonstrate that a conserved positively charged domain at the C terminus of peroxisomal APX (KKRRMK) is required for sorting this protein to peroxisomes via pER. Furthermore, we show also that an adjacent TMD is critical for sorting peroxisomal APX; the TMD apparently confers the proper context necessary for the C-terminal sorting signals to function.

EXPERIMENTAL PROCEDURES

Reagents and Biochemicals—Restriction enzymes and other DNA-modifying enzymes were purchased from Promega Corp. (Madison, WI) or New England Biolabs (Beverly, MA). The TA cloning vector, pCR2.1, was obtained from Invitrogen Corp. (San Diego, CA). Purification of polymerase chain reaction (PCR) products, DNA fragment isolation, and plasmid DNA isolations were carried out using commercially available kits (Qiagen, Chatsworth, CA). Triton X-100 was obtained from Sigma, pectolyase Y-23 was purchased from Seishin Pharmaceutical Co., (Tokyo, Japan), and paraformaldehyde was from Ted Pella Inc. (Redding, CA). IgGs in rabbit sera raised to purified cottonseed catalase (26) were affinity-purified with protein A-Sepharose (Amersham Pharmacia Biotech). Monoclonal antibodies to chlorophenolic acetyltansferase (CAT) in mouse hybridoma medium were a gift from S. Subramani (University of California, San Diego, CA). Mouse anti-hemagglutinin (HA) epitope monocalon antibodies (12CA5) were purchased from Sigma, and fluorescent dye-hemagglutinin (HA) epitope monoclonal antibodies (12CA5) were purchased from Roche Molecular Biochemicals. All HA-pAPX derivatives and CAT-pAPX fusion constructs examined in this study (see Table I) were inserted into pRTL2/CAT-pAPX (19), pRTL2/CAT-XbaI, and pRTL2/CAT-XbaI is a general purpose CAT fusion cassette vector whereby an in-frame XbaI site was substituted for sequences encoding the CAT stop codon (30).

Table I) were inserted into pRTL2/CAT-pAPX (19), pRTL2/CAT-pAPX (36-TMDATMD\textsuperscript{\textdagger}), and pRTL2/CAT-pAPX (36-TMDATMD\textsuperscript{\textdagger\textdagger}). Two sets of appropriate synthetic double-stranded oligonucleotides possessing HpaI and BspEI complementary ends coding for amino acid residues in an order reverse to those in the TMD of wild-type peroxisomal APX were ligated into HpaI-BspEI digested pCR2.1/pAPX (36-TMDATMD\textsuperscript{\textdagger}), then ligated into XbaI-digested pRTL2/CAT-pAPX to yield pRTL2/CAT-pAPX (36-TMDATMD\textsuperscript{\textdagger\textdagger}). Next, two sets of appropriate synthetic double-stranded oligonucleotides possessing HpaI and BspEI complementary ends coding for amino acid residues in an order reverse to those in the TMD of wild-type peroxisomal APX were ligated into HpaI-BspEI digested pCR2.1/pAPX (36-TMDATMD\textsuperscript{\textdagger}), then ligated into XbaI-digested pRTL2/CAT-pAPX (36-TMDATMD\textsuperscript{\textdagger\textdagger}) to construct pRTL2/CAT-pAPX (36-TMDATMD\textsuperscript{\textdagger\textdagger\textdagger}). Two sets of appropriate synthetic double-stranded oligonucleotides possessing HpaI and BspEI complementary ends coding for amino acid residues in an order reverse to those in the TMD of wild-type peroxisomal APX were ligated into HpaI-BspEI digested pCR2.1/pAPX (36-TMDATMD\textsuperscript{\textdagger\textdagger\textdagger}), then ligated into XbaI-digested pRTL2/CAT-pAPX (36-TMDATMD\textsuperscript{\textdagger\textdagger\textdagger\textdagger}) to yield pRTL2/CAT-pAPX (36-TMDATMD\textsuperscript{\textdagger\textdagger\textdagger\textdagger}).

Cell Culture, Microprojectile Bombardment, and Immunofluorescence Microscopy—Nicotiana tabacum L. cv. Bright Yellow 2 (BY-2) suspension cells were maintained in cultures as described previously (32). Cells were transformed transiently with 10 μg of plasmid DNA by means of a microprojectile particle delivery system (Bio-Rad) (32, 33). Approximately 20 to 24 h after bombardment (to allow transient gene expression and protein sorting) cells were processed for immunofluorescence microscopy (29). Briefly, fixed cells were immersed in phosphate-buffered saline (PBS), then incubated in perctolyase Y-23 (0.1% v/v) in PBS for 2 h at 30 °C. After several washes in PBS, plasma and organellar membranes were permeabilized by incubating cells in Triton X-100 (0.3% v/v) in PBS for 15 min at room temperature (29). Fixed, perctolyase-treated, and detergent-permeabilized cells were incubated with primary antibodies and then fluorescent dye-conjugated secondary antibodies. Application of antibodies was varied: concentrations and staining of ER (pER) with 3,3′-dihexyloxacarbocyanine iodide (Molecular Probes Inc., Eugene, OR) were done as described previously (19). A portion of immunostained cells were counterstained with glass slides and viewed with a Zeiss Axiovert 100 epifluorescence microscope (Carl Zeiss Inc., Thornwood, NY). Photographs were taken using Kodak Tmax400 ASA black and white print film (Eastman Kodak Co.,) and printed images or photographic negatives were computer-scanned and subsequently composed into figures using Adobe Photoshop 5.0.2 (Adobe Systems, Mountain View, CA).
Cytosol

Experimental results supporting this premise were that a single TMD domain (that is not found in cytosolic isoforms of a unique 41-amino acid C-terminal extension (including the sorting signal for cottonseed peroxisomal APX is located within BY-2 cells (19). Results of a replicate experiment are presented terminal HA epitope tag) sorted to pER and peroxisomes in HA-pAPX (full-length cottonseed peroxisomal APX with a N-

truncations were constructed via PCR-based mutagenesis to identify regions within the C-terminal extension that possess the necessary sorting information, a series of HA epitope-tagged pAPX mutants with different C-terminal polypeptide sequences were used in the current study for presenting results of our experiments.

RESULTS

The C Terminus of Peroxisomal APX Is Necessary for Sorting to Peroxisomes via pER—We speculated earlier (34) that the sorting signal for cottonseed peroxisomal APX is located within a unique 41-amino acid C-terminal extension (including the single TMD domain) that is not found in cytosolic isoforms of APX. Experimental results supporting this premise were that a portion (36 C-terminal residues) of the peroxisomal APX extension was sufficient for sorting CAT (CAT-pAPX+36) from the cytosome to peroxisomes via pER in tobacco BY-2 cells (19). To identify regions within the C-terminal extension that possess the necessary sorting information, a series of HA epitope-tagged pAPX mutants with different C-terminal polypeptide truncations were constructed via PCR-based mutagenesis (Table I).

In previous studies we showed that transiently expressed HA-pAPX (full-length cottonseed peroxisomal APX with a N-terminal HA epitope tag) sorted to pER and peroxisomes in BY-2 cells (19). Results of a replicate experiment are presented as a control for the present study. Fig. IA shows in a representative single cell a reticular/circular (open arrows) and punctate (solid arrows) Cy2 immunofluorescence image attributable to the subcellular localizations of HA-pAPX to membranes of pER and individual peroxisomes, respectively. Evidence that the punctate image is peroxisomal is given in the double-labeling comparison of Fig. IA compared with B. The punctate portion of HA-pAPX in the transformed cell (Fig. IA) is superimposable upon the punctate rhodamine pattern (solid arrows) attributable to endogenous catalase immunofluorescence in the same cell (Fig. IB). The non-colocalized HA-pAPX staining (Fig. IA; open arrows) is the pER compartment. Immunofluorescence evidence that this HA-pAPX-stained compartment is pER is the superimposable confocal images of HA-pAPX immunofluorescence and 3,3′-dihexyloxacarbocyanine iodide-stained ER that is illustrated in a color overlay (Fig. 3, A–C) in a previous study (19). This ER compartment (pER) is readily recognized in the epifluorescence images that are used in the current study for presenting results of our experiments.

Sorting of HA-pAPX to pER and/or peroxisomes was abolished when various C-terminal polypeptides were removed from the protein. HA-pAPX lacking its C-terminal 38 amino acid residues (HA-pAPX-38) accumulated throughout the non-organellar portion of the cytoplasm (cytosol) (Fig. 1C); HA-pAPX-38 (C), HA-pAPX-5 (D), or pRTL2 vector alone (mock-transformed) (F). Cells were fixed in formaldehyde (20–24 h after biolistic bombardment), permeabilized with pectolyase and Triton X-100, and incubated with anti-HA IgGs (except in E) and anti-mouse Cy2-conjugated IgGs as described under “Experimental Procedures.” B, shows the corresponding endogenous catalase immunofluorescence (anti-catalase IgGs and anti-rabbit rhodamine-conjugated IgGs in individual peroxisomes in the same cells as A. Solid arrows (A and B) indicate obvious colocalizations, whereas open arrows (A) indicate obvious non-colocalizations of HA-pAPX in membranous/reticular pER and peroxisomal catale. The bar in A indicates 10 μm.

from the protein. HA-pAPX lacking its C-terminal 38 amino acid residues (HA-pAPX-38) accumulated throughout the non-organellar portion of the cytoplasm (cytosol) (Fig. 1C); compare this image with the reticular/circular, punctate image of HA-pAPX localization (Fig. 1A). Similarly, a HA-pAPX construct that was truncated by either its 29 or 11 C-terminal residues (data not shown, see Table I) or lacking its C-terminal five amino acid residues (Fig. 1D) also did not sort to pER and/or peroxisomes, i.e. the protein remained in the cytosol. These results clearly indicate that C-terminal residues within the peroxisomal APX extension are necessary for sorting to pER and peroxisomes in BY-2 cells.

In one control experiment, authentication of the immunoflu-
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images of cells transformed transiently with either HA-pAPX/S-RRKΔGG (A), HA-pAPX-SΔG (B), HA-pAPX-RRKΔG (C), HA-pAPX-KRRMKΔKMKRR (E), or HA-pAPX-YEVΔGGG (F). Cells were fixed 20–24 h after biologic bombardment, then permeabilized with pepsylase and Triton X-100, and incubated with anti-HA IgGs and anti-mouse Cy2-conjugated IgGs as described under “Experimental Procedures.” The bar in A indicates 10 μm.

**Fig. 2.** Basic amino acid residues at the C terminus of HA epitope-tagged peroxisomal APX are necessary for sorting to peroxisomes via pER—That the C-terminal five amino acid residues (RRKΔG) of peroxisomal APX were minimally necessary for sorting (Fig. 1D) prompted us to examine this positively charged region in more detail. As stated in the Introduction, positively charged domains have been identified as part of the sorting signal of several PMPs. For example, the mPTS identified in C. boidinii PMP47 includes a cluster of positively charged residues (KIKKR) flanked by, among other residues, a conserved serine or threonine (11). An alignment of sequences within the PMP47 mPTS and the peroxisomal APX C terminus revealed that at least two basic residues (arginine and lysine) within the positively charged domain of peroxisomal APX as well as an upstream serine residue (underlined, ILGVYEVKKRM- COOH) were highly conserved (comparison not shown). To determine whether these residues as well as other basic residues within the C-terminal positively charged domain of pAPX were necessary for sorting, several HA epitope-tagged pAPX mutants containing various site-specific glycine substitutions were constructed (Table I). Glycine residues were chosen for substitution experiments because they were predicted to least likely adversely influence proper sorting of pAPX by destabilization of three-dimensional protein structure.

HA-pAPX initially was modified so that the conserved serine (position 11), arginine (position 5), and lysine (position 4) together were replaced with glycines (underlined, ILGVYEVGYKKRM-COOH). This construct, designated HA-pAPX-S/RKΔG/ GG, accumulated in the cytosol (Fig. 2A), indicating that these residues are critical for proper sorting of peroxisomal APX to pER and peroxisomes. HA-pAPX was modified next so that only the serine was replaced with glycine (underlined, ILGVYEVKKRM-COOH), leaving the positively charged domain unaltered. HA-pAPX-SΔG, unlike HA-pAPX-S/RKΔG/GG, was not mislocalized within the cytosol (Fig. 2B) but instead was efficiently sorted to pER and peroxisomes. Thus, the conserved serine residue located within the TMD and upstream of the positively charged domain of peroxisomal APX does not appear to be essential for sorting.

Next, experiments were done to test the sorting function of conserved basic residues within the C-terminal positively charged domain. HA-pAPX was modified so that arginine (position 5) and lysine (position 4) each were replaced with a glycine (underlined, YEVGGRMK-COOH). HA-pAPX-RKΔG was not localized to pER and/or peroxisomes but instead accumulated throughout the cytosol (Fig. 2C), indicating that these conserved basic residues are essential for sorting function. However, two other HA-pAPX mutants with glycine substitutions of similar basic residues within the positively charged domain did not abolish sorting. When the lysine at position 4 and the arginine at position 3 were replaced with glycines (underlined, YEVRGGMK-COOH), the resulting mutant (HA-pAPX-RKΔGG) was localized to pER and peroxisomes (Fig. 2D). Sorting of HA-pAPX to organelles also was preserved when a glycine was substituted for the arginine at position 5 (underlined, YEVKRMK-COOH, HA-pAPX-RΔG) (data not shown, see Table I). Interestingly, HA-pAPX still localized to pER and peroxisomes (Fig. 2E) when the sequence of amino acid residues within the C-terminal positively charged domain was reversed (underlined, GGRKKRMK-COOH; HA-pAPX- RKKMKΔKMKRR). Taken together, these results indicate that there does not appear to be a strict requirement for a single basic amino acid residue or a specific sequence (polarity) of residues within the positively charged domain. Instead, the sorting function of the domain appears to be provided by some more general property conferred at least in part by its overall basic amino acid composition. Fig. 2F shows that glycine substitutions of amino acid residues immediately upstream of the positively charged domain (underlined, GGRKRKKMK-COOH) and not within the TMD does not affect sorting, i.e. transiently expressed HA-pAPX-YEVΔGGG localized to pER and peroxisomes.

The C Terminus of Peroxisomal APX Including the TMD and the Positively Charged Domain Is Sufficient for Sorting CAT to Peroxisomes via pER—To test whether the C-terminal five amino acid residues of peroxisomal APX were sufficient for subcellular sorting, sequences encoding this region were appended in-frame (with a serine linker) to the 3’ end of the DNA coding for CAT (CAT-pAPX+RKΔG/KMK) (Table I). Both CAT (Fig. 3A) and CAT-pAPX+RKΔG (Fig. 3B) accumulate in the cytosol of transformed cells, indicating that the C-terminal positively charged domain alone is not sufficient for rerouting CAT from the cytosol to pER and/or peroxisomes. Similar results were observed when either the C-terminal 11 or 20 amino acid residues of peroxisomal APX were fused (with a serine linker) to CAT. That is, both CAT-pAPX+11 (CAT plus the C-terminal 3 amino acid residues of the peroxisomal APX TMD and the 8 C-terminal-most amino acid tail) (Fig. 3C) and CAT-pAPX+20 (CAT plus the C-terminal 12 residues of the peroxisomal APX TMD and the C-terminal tail) (Fig. 3D) accumulated in the cytosol of transformed cells.

CAT was sorted to peroxisomes and pER, however, when the C-terminal 29 amino acids of peroxisomal APX containing the entire 21 amino acid TMD (underlined, VLAAGAVGVYAVAAA-VVILSYFEVYKRKMK-COOH) and C-terminal tail were appended to CAT (CAT-pAPX+29) (Fig. 3E). This shows a reticular/ circular (open arrows) and globular (solid arrows) fluorescence pattern attributable to the localization of CAT-pAPX+29 to pER and peroxisomal aggregates, respectively. Evidence for the localization of CAT-pAPX+29 to peroxisomes (aggregated) is shown in comparisons of Fig. 3, E and F. Endogenous catalase in the CAT construct-transformed cell is localized in globular structures (Fig. 3F, solid arrows) and is colocalized with...
pAPX+36-TMDΔTMDrev (Fig. 4B) and CAT-pAPX+36 (Fig. 4A) (and CAT-pAPX+29; Fig. 3E) have exactly the same amino acid composition, we conclude that a global property(s) and not a strict (polar) amino acid sequence within the hydrophobic region confers in part the proper sorting of peroxisomal APX.

Next, the possibility was tested that the apparent sorting property(s) possessed by the peroxisomal APX TMD is shared among TMDs within other PMPs. Toward this end, sequences coding for the TMD within CAT-pAPX+29 and CAT-pAPX+36 were replaced with sequences coding for either the single TMD of S. cerevisiae Pex15p (15) or the fourth (of six) TMD of C. boidinii PMP47 (35), respectively (see Table I). The fourth TMD (TMD4) of PMP47 was chosen because it is immediately upstream of the mPTS (11). Both CAT-pAPX+29-TMDΔPex15pTMD (Fig. 4C) and CAT-pAPX+36-TMDΔPMP47TMD4 (Fig. 4D) were sorted to pER and aggregated peroxisomes, indicating that these TMDs convey the same global property(s) sufficient in part for sorting CAT to pER and peroxisomes in BY-2 cells.

An examination of the TMD sequences within peroxisomal APX, Pex15p, and PMP47TMD4 did not reveal any noticeable conserved features, i.e. the TMDs range in length (18–22 residues long) and in overall amino acid composition (0–29% alanine and 10–56% leucine) (see Table I). Therefore the possibility was tested that only an overall hydrophobicity of a TMD (along with the positively charged domain) was functional in sorting peroxisomal APX. CAT-pAPX+29-TMDΔAsnTMD (Fig. 4A and E) and CAT-pAPX+29 (Fig. 3E) shows an absence of any distinguishable immunofluorescence staining when cells were mock-transformed with pRTL2 alone.

Taken together, results presented in Fig. 4 indicate that the role of the peroxisomal APX TMD in subcellular sorting is primarily to convey via its hydrophobic α-helical structure and apparently not a more subtle feature of sequence, the proper

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**Fig. 3.** The C-terminal 29 amino acid residues of peroxisomal APX are sufficient for sorting CAT to peroxisomes via pER. Representative immunofluorescence images of cells transformed transiently with either CAT (A), CAT-pAPX+RKRMK (B), CAT-pAPX+11 (C), CAT-pAPX+20 (D), or CAT-pAPX+29 (E and F). Cells were fixed 20–24 h after biolistic bombardment, then permeabilized with pectolyase and Triton X-100, and incubated with anti-CAT IgGs and anti-mouse Cy2-conjugated IgGs as described under “Experimental Procedures.” F, shows the corresponding endogenous catalase immunofluorescence (anti-catalase IgGs and anti-rabbit rhodamine-conjugated IgGs) in globular (aggregated) peroxisomes in the same cells as E. Solid arrows (E and F) indicate obvious colocalizations, whereas open arrows (E) indicate obvious non-colocalizations of HA-pAPX in membranous/reticular pER and peroxisomal catalase. The bar in A indicates 10 μm.

CAT-pAPX+29 within the same transformed cell (Fig. 3E). In neighboring non-transformed cells, catalase is localized within individual peroxisomes (punctate fluorescence). The aggregation of peroxisomes observed in all BY-2 cells transformed with sorting-competent CAT-pAPX fusion proteins (Table I) already has been reported (19) and was shown by us to be due to the “zipping together of individual peroxisomes as a consequence of the oligomerization of cytosolic-facing membrane-bound CAT (CAT-pAPX) subunits.” Peroxisomal aggregation, therefore, provides convincing evidence that a CAT-pAPX fusion protein localizes (via pER) to pre-existing peroxisomes.

**Varied TMDs within the Peroxisomal APX C-Terminal Extension Are Sufficient for Sorting CAT to Peroxisomes via pER**—The above results (Fig. 3) indicate that sorting of peroxisomal APX to pER and peroxisomes is in part dependent upon the TMD, i.e. the 21-amino acid TMD along with the C-terminal 8-amino acid tail (CAT-pAPX+29) are sufficient for sorting CAT to pER and peroxisomes (Fig. 3E). Moreover, data not shown indicated that the 26 C-terminal residues of peroxisomal APX, including the majority of the TMD (C-terminal 18 amino acid residues) and the C-terminal tail, also were sufficient for sorting CAT to pER and peroxisomes (Table I). To test whether sorting is mediated either by specific amino acid sequences within the peroxisomal APX TMD or by a more global property, e.g. overall hydrophobicity and/or TMD length, several different CAT-pAPX fusion proteins containing altered TMDs were generated (Table I). The results from this set of experiments are presented in Fig. 4.

Fig. 4A shows as a control a replicate image of that shown previously (19) of the localization of CAT-pAPX+36 (CAT plus the 36 C-terminal residues of peroxisomal APX) to pER (open arrows) and aggregated peroxisomes (solid arrows). Fig. 4B shows that when the 21-amino acid sequence within the peroxisomal APX TMD was reversed, the resulting CAT-pAPX fusion protein (CAT-pAPX+36-TMDΔTMDrev) also was sorted to aggregated peroxisomes and pER. Because the TMD in CAT-
It is well established that targeting and membrane insertion of several ER proteins occurs in a post-translational manner and, therefore, are not dependent upon an N-terminal hydrophobic signal sequence and the cotranslational signal recognition particle/Sec61p pathway. This group of proteins is known collectively as “tail-anchored” membrane proteins because they (i) possess a single hydrophobic sequence (TMD) located close to or at the C terminus of the protein and (ii) are orientated such that the bulk of protein resides on the cytosolic side of the membrane (37). In addition to the ER, members of this unique class of membrane proteins are found in several other organelles including various post-ER compartments within the secretory pathway (e.g. Golgi, vacuoles, and synaptic vesicles) (38–42), mitochondria (43–46), and peroxisomes (15, 19). For all tail-anchored proteins examined to date, the initial membrane-targeting event is mediated by sequences within the C-terminal region of the protein. The varied subcellular locations of these proteins suggest, however, that the C-terminal topogenic sequences and the mechanisms that regulate membrane selectivity are complex.

Immunofluorescence data presented in this paper (Table I) clearly show that a C-terminal-mediated sorting paradigm pertains to tail-anchored peroxisomal APX. For instance, the sorting of HA-pAPX to pER and peroxisomes was abolished, i.e. mislocalized within the cytosol, when C-terminal sequences including the positively charged domain (RKRKM-CCOHH) either were deleted (Fig. 1) or modified by specific amino acid substitutions (Fig. 2). In addition, the C-terminal tail of peroxisomal APX, including the positively charged domain and the immediately adjacent TMD, was sufficient for redirecting CAT from the cytosol to peroxisomes via pER (Figs. 3E and 4). Taken together, these data suggest that the sorting of peroxisomal APX to pER and peroxisomes is mediated primarily by amino acid residues within the C-terminal positively charged domain. It is important to point out that all of the modified HA-pAPX proteins and CAT-pAPX fusion proteins examined in this study either sorted to both pER and peroxisomes or remained in the cytosol (Table I); no evidence was ever found for one of these peroxisomal APX derivatives to be localized exclusively to either pER or peroxisomes. In a previous study, however, we found that a CAT-pAPX fusion protein (CAT-pAPX+36) localized only to pER when export from the pER was blocked by incubating cells in brefeldin A (19). After removal of brefeldin A from these cells, the CAT-pAPX sorted to peroxisomes. A plausible explanation for these collective results is that residues within the positively charged domain are part of overlapping pER and peroxisome sorting signals. Given this possibility and the sorting pathway of peroxisomal APX from the cytosol to pER and then to peroxisomes (19), it follows that certain deletions or modifications of the positively charged domain would inhibit initial sorting to pER and subsequent sorting to peroxisomes. This scenario is consistent with a model (2, 15) that suggests that the indirect sorting of a subset of PMPs to peroxisomes via ER is mediated by an ER sorting signal and a distinct mPTP that can only function from within the ER (discussed below).

The C-terminal TMD also appears to play an important role in sorting peroxisomal APX to pER and peroxisomes. Our results suggest that the TMD, i.e. a hydrophobic stretch of amino acid residues that has the propensity to form an α-helical structure, functions in conveying the proper context for the positively charged domain to function as a pER and peroxisome sorting signal. However, there does not appear to be a specific amino acid sequence requirement for sorting signal functioning within the peroxisomal APX TMD. One of the more convincing evidences for this conclusion was the result that replacement of the peroxisomal APX TMD with an artificial TMD was sufficient for sorting CAT to peroxisomes via pER (CAT-pAPX+29-TMDΔsynTMD, Fig. 4E). The overall hydrophobicity and length of the TMD apparently are the primary determinants for proper sorting of peroxisomal APX to peroxisomes via pER. CAT-pAPX fusion proteins containing a TMD with a hydrophobicity of at least 32, such as CAT-pAPX-Pex15p (Fig. 4C) (using the Kyte-Doolittle hydrophathy indices (47) and 18 or more amino acid residues) were sorted to pER and peroxisomes, whereas those constructs containing a TMD with a hydrophobicity of 28 or less and 12 or fewer residues, e.g. CAT-pAPX+20 (Fig. 3D), were not sorted. These results are consistent with a previous conclusion (48) that both a “minimum” length and overall hydrophobicity of the TMD are necessary to provide at least the thermodynamic driving force for membrane integration of tail-anchored proteins. Of course, we cannot exclude the possibility that sequences within the peroxisomal APX TMD also could mediate specific protein-protein and/or protein-lipid interactions that are important in, among other events, the proper (efficient) assembly and organization of the protein in its functional site within the peroxisomal boundary membrane. Similar roles have been postulated for the TMDs within other tail-anchored proteins (38, 49–51); additional experiments need to be done to test whether this possibility exists for peroxisomal APX.

Several striking similarities are revealed in a comparison of the mechanisms by which C-terminal sequences within peroxisomal APX and at least one other tail-anchored membrane protein mediate sorting and insertion into ER (pER) membranes. Synaptobrevin/vesicle-associated membrane proteins (VAMP) are tail-anchored soluble N-ethylmaleimide-sensitive factor attachment receptor proteins (SNAREs) that function in intracelluar vesicle targeting and fusion (52). With the exception of a novel VAMP isoform localized to mitochondria (44, 53), all VAMPs examined to date are sorted initially to the ER and then are transported to their functional sites throughout the secretory pathway (39, 49). Both VAMP and peroxisomal APX require ATP and a proteinaceous membrane-bound component for membrane integration in vitro (19, 54, 55). Furthermore, as we have shown here for peroxisomal APX the ER sorting sequence of VAMP includes a cluster of basic amino acid residues (lysines) immediately adjacent, albeit N-terminal, to the TMD (56). Replacement of the lysine residues within the VAMP signal with hydrophilic serines diminished binding to ER membranes in vitro (56), consistent with the mislocalization of HA-pAPX-RKΔGG within the cytosol (Fig. 2C). These results indicate that at least these positively charged residues tested are important for proper sorting of peroxisomal APX and VAMP to the ER (pER). Some notable differences exist, however, between sequences within the VAMP and peroxisomal APX ER sorting signals. For instance, sequence alignments of positively charged domains revealed almost no conservation (data not shown). Several secondary prediction programs indicate that whereas the C terminus of peroxisomal APX has a tendency to form an α-helical structure throughout the TMD that terminates prior to the positively charged domain (data not shown), residues within the VAMP positively charged domain are predicted to form part of an amphipathic helix (56). It now remains to be determined whether these differences between VAMP and peroxisomal APX sorting sequences imply that distinct protein components are involved in recognition and membrane integration into ER membranes or whether shared protein components participate in the initial sorting events of these two...
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FIG. 5. Sequence comparison of the cottonseed peroxisomal APX C terminus with other PMPs from plants, yeast, and mammals. Conserved positively charged domains are in bold, and predicted TMD regions are underlined. Sequence data were obtained using GenBank™ and aligned using ClustalW and by visual inspection. At, Arabidopsis thaliana; Ch, C. boidinii; Gh, Gossypium hirsutum; Hp, H. polymorpha; Hs, H. sapiens; Pa, Podospora anserina; Pp, P. pastoris; Sc, S. cerevisiae; So, Spinacia oleracea; Rn, Rattus norvegicus; Yl, Yarrowia lipolytica.

tail-anchored proteins to ER.

Other sequence comparisons between peroxisomal APX and PMPs from various organisms reveal that the positively charged domain is a remarkably conserved feature (Fig. 5). Most integral PMPs examined possess a cluster of 3–5 basic residues (arginines and lysines) and a single hydrophilic or small polar residue, all adjacent to at least one membrane-spanning domain (Fig. 5). PMPs predicted to be peripherally associated with the peroxisome boundary membrane and some other integral PMPs do not appear to possess the motif described above, suggesting that they may be sorted to peroxisomes by a different pathway.

For several of the PMPs listed in Fig. 5 (C. boidinii PMP47, Pichia pastoris and Homo sapiens Pex3p, and P. pastoris Pex22p) sequences including the positively charged motif have been identified as the targeting signal (mPTS) responsible for sorting the protein directly from the cytosol to the peroxisome boundary membrane (11–14). Interestingly, the same positively charged motif has been implicated in the sorting of another group of PMPs (H. polymorpha Pex3p and S. cerevisiae Pex15p) initially to the ER (15, 17). How can the same positively charged motif be responsible for sorting some PMPs directly to peroxisomes and others to peroxisomes via the ER? One possibility (mentioned earlier) put forth by Subramani and coworkers (2, 15) is that PMPs are sorted to the peroxisomal boundary membrane either directly or indirectly by two individual but related mPTSs. One mPTS, designated as mPTS1, would be responsible for targeting membrane proteins to peroxisomes directly from the cytosol, whereas the other mPTS, designated mPTS2, would require that the PMP be sorted first to the ER (via an ER sorting signal) before it could direct the PMP to the peroxisomal boundary membrane. The relatedness of the mPTS1 and mPTS2 would be that they individually include the conserved positively charged motif, whereas the context conferred by adjacent residues (e.g., residues within the TMD) likely contributes to the overall topogenic signal and thereby defines a mPTS1 and mPTS2.

Although there are several studies that support the existence of an mPTS1-mediated pathway (7–14), evidence for a two-mPTS model comes primarily from studies of another tail-anchored PMP, namely S. cerevisiae Pex15p. Elgersma et al. (15) demonstrate that the C-terminal 82-amino acid residues including the positively charged domain (KKYK) and immediately adjacent single TMD of Pex15p were sufficient for sorting a reporter protein to both peroxisomes and ER. However, removal of the C-terminal 30-amino acid residues from wild-type Pex15p, thereby leaving the positively charged domain exposed at the C terminus (similar to the context of the positively charged domain in peroxisomal APX), resulted in accumulation of the protein in the ER. The C-terminal 34 amino acid residues were not sufficient to target a passenger protein to peroxisomes. These findings were interpreted by Elgersma et al. (15) to mean that a mPTS2 lies somewhere within the entire C-terminal 82 amino acids and functions in targeting Pex15p to peroxisomes only after the ER targeting signal (upstream of the C-terminal 30 amino acids and including the positively charged domain) directed the protein to the ER. Elgersma et al. (15) also point out that since several of the critical Pex15p constructs were expressed on low copy plasmids in yeast, aberrant targeting should have been minimized.

Our results suggest that like Pex15p, the C terminus of peroxisomal APX appears to contain an overlapping ER (pER) targeting signal and a mPTS2. Convincing evidence that peroxisomal APX contains a mPTS2 and not a mPTS1 comes from observations that the protein inserts into ER membranes but not into peroxisome membranes in vitro (whereas C. boidinii PMP47 with a mPTS1 inserts exclusively into peroxisome membranes in vitro) (19). One notable difference between Pex15p and peroxisomal APX is that the ER (pER) sorting signal and mPTS2 of peroxisomal APX could not be dissected by loss or gain of function experiments; site-specific modifications or deletions of the mPTS2 of peroxisomal APX constructs resulted in mislocalization within the cytosol, whereas deletion of the mPTS2 of Pex15p results in accumulation in the ER (15). Apparently, the ER sorting signal and mPTS2 are not as tightly overlapped in Pex15p as they are in peroxisomal APX. Further studies of the Pex15p sorting signals will be required to resolve this issue. The observed mislocalization within the cytosol of certain HA-pAPX proteins also suggests that pER localization of sorting-competent peroxisomal APX proteins is not due to overexpression alone. For instance, HA-pAPX-RK (Fig. 3C) accumulated in the cytosol, although replacement of positively charged residues with non-charged glycines should have facilitated localization to pER if this compartment represented simply a non-peroxisomal membrane system.

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REFERENCES

1. Distel, B., Erdmann, R., Gould, S. J., Blobel, G., Crane, D. I., Cregg, J. M., Dudo, G., Fujiki, Y., Goodman, J. M., Just, W. W., Kiel, J. A. K. W., Kuman, 16343
13. Koller, A., Snyder, W. B., Faber, K. N., Wenzel, T. J., Rangell, L., Keller, G. A., Wiemer, E. A. C., Luers, G. H., Faber, K. N., Wenzel, T., Veenhuis, M., and Tugal, H. B., Pool, M., and Baker, A. (1999) Plant Physiol.

12. Dyer, J. M., McNew, J. A., and Goodman, J. M. (1996) J. Cell Biol.

11. Mullen, R. T., Lisenbee, C. S., Miernyk, J. A., and Trelease, R. (1999) Mol. Cell. Biol.

10. Tugal, H. B., Pool, M., and Baker, A. (1999) J. Biol. Chem.

9. Pause, B., Diestelko¨tter, P., Heid, H., and Just, W. W. (1997) FEBS Lett.

8. Just, W. W., and Diestelko¨tter, P. (1996) FEBS Lett.

7. Imanaka, T., Shiina, Y., Takano, T., Hashimoto, T., and Osumi, T. (1996) Biochim. Biophys. Acta

6. Hettema, K. H., Distel, B., and Tabak, H. F. (1999) Biochim. Biophys. Acta 1451, 17–34

5. Yamashita, H., Avraham, S., Jiang, S., London, R., Van Veldhoven, P. P., and Subramani, S. (1996) Mol. Cell. Biol.

4. Tocanne, J. -F. (1999) FEBS Lett.

3. Olsen, L. J. (1998) J. Biol. Chem.

2. Subramani, S. (1998) J. Biol. Chem.

1. Subramani, S. (1996) J. Biol. Chem.