A novel method to determine the topology of peroxisomal membrane proteins in vivo using the tobacco etch virus protease
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Published in:
The Journal of Biological Chemistry

DOI:
10.1074/jbc.M105828200

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2001

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Most proteins essential for the biogenesis of peroxisomes (peroxins) that are identified to date are associated with or are integral components of the peroxisomal membrane. A prerequisite in elucidating their function is to determine their topology in the membrane. We have developed a novel tool to analyze the topology of peroxisomal membrane proteins in the yeast *Hansenula polymorpha in vivo* using the 27-kDa Nia protease subunit from the tobacco etch virus (TEVp). TEVp specifically cleaves peptides containing the consensus sequence, EKKYQX/S (tev). We show that cytosolic TEVp and peroxisomal TEVp.SKL are selectively active on soluble cytosolic and peroxisomal tev-containing proteins in *vivo*, respectively, without affecting the viability of the yeast cells. The tev sequence was introduced in between the primary sequence of the peroxisomal membrane proteins Pex3p or Pex10p and the reporter protein enhanced green fluorescent protein (eGFP). Co-synthesis of these functional tev-GFP tagged proteins with either cytosolic TEVp or peroxisomal TEVp.SKL revealed that the C termini of Pex3p and Pex10p are exposed to the cytosol. Additional applications of the TEV protease to study peroxisome biogenesis are discussed.

Peroxisomes are organelles present in all eukaryotic organisms studied so far. Unlike other cellular organelles, their function may be highly diverse, dependent on cell type and the external stimuli the cell encounters. In plants they are involved in photorespiration, in trypanosomes they are involved in glycolysis, and in fungi they are involved in the synthesis of purine, and cholesterol biosynthesis as well as fatty acid and purine degradation. Peroxisome malfunctioning is the cause of severe inherited human disorders, such as Zellweger syndrome (5, 6).

Peroxisomes do not contain DNA or a protein-synthesizing machinery. Consequently, peroxisomal proteins are synthesized on cytosolic polysomes and sorted post-translationally to their target organelle (7). Two distinct signal sequences for peroxisomal matrix proteins have been defined (designated as PTSs)

Most peroxins (18 out of 23) are membrane-associated proteins, either peripheral or integral. To study the specific functions of these peroxisomal membrane (PM)-bound peroxins, information about the topology of these proteins is required. In particular, the location of functional domains, either catalytic or domains involved in physical interactions with other proteins, needs to be established to get insight into the roles of the PM peroxins.

Biochemical techniques to discriminate between peripheral versus integral membrane protein (differential extraction by low salt, high salt, and sodium carbonate) and to determine the topology of PM peroxins (protease protection assays on purified organelles or differential permeabilization of cellular membranes) have been performed on most of the PM peroxins reported. In many cases, however, these procedures have not led to unequivocal information on the topology of these PM peroxins. Typical examples of this include the analyses on Pex8p (11–13), Pex10p (14–17), Pex11p (18–24), Pex14p (25–28), Pex16p (29, 30), and Pex17p (31, 32). Therefore, we set out to develop an alternative method to establish the topology of PM peroxins. The basic idea was to introduce a specific, heterologous

* The abbreviations used are: PTS, peroxisomal-targeting signal; TEV, tobacco etch virus; TEVp, TEV protease; GFP, green fluorescent protein; eGFP, enhanced green fluorescent protein; PM, peroxisomal membrane; PCR, polymerase chain reaction; kDa, kilodalton; P*_{AUX}, alcohol oxidase promoter; P*_{AMO}, amine oxidase promoter.
gous protease in the yeast *Hansenula polymorpha*. Prerequi-
site is that the protease should be active on a defined amino
acid sequence not present in essential proteins of the yeast,
which can be introduced in substrate PM peroxins. Co-expres-
sion of a PM peroxin containing a protease-processing site with
a cytosolic protease in one strain or a peroxisomal protease in
another strain should reveal whether the processing site is
accessible in the cytosol or in the peroxisomal matrix. The
major advantage of such a system would be that no other
proteins need to be analyzed to determine the accessibility of
the peroxisomal matrix as it is the strain expressing the cyto-
solic variant of the protease that is the control for the strain
expressing the peroxisomal variant and vice versa.

We selected a 239-amino acid fragment of the 346-kDa to-
bacco etch virus (TEV) polyprotein containing a proteinase
activity specifically processing the consensus sequence (heptapeptide) EXXXQX(S/G) in cis and in trans (33). Cleavage
occurs between glutamine and serine or glycine. This protease
has been used for site-specific proteolysis both in vitro and in
vivo in *Escherichia coli* and *Saccharomyces cerevisiae* of
stratate proteins containing the consensus sequence for process-
ing (34–36).

Here, we describe the synthesis and sorting of the TEV
protease to *H. polymorpha* peroxisomes or the cytosol. The
protease was shown to be active in both subcellular compart-
ments without the loss of cell viability. In an *in vivo* applica-
tion, we show that the C termini of both Pex3p (containing a
Pex19p-interaction domain) (37–39) and Pex10p (containing a
zinc-binding domain) (14) face the cytosol. Additional appli-
cations of the TEV protease to study peroxisome biogenesis are
discussed.

### EXPERIMENTAL PROCEDURES

**Strains and Cultivation—** *H. polymorpha*NCYC495 (les1.1) and
derivatives (Table I) were grown at 37 °C in batch cultures in YPD (1% yeast extract, 2% Bacto peptone, 2% glucose) or in mineral medium (40) containing either 0.5% (w/v) glucose or 0.5% (v/v) methanol as carbon and energy sources in combination with 0.25% (w/v) ammonium sulfate or 0.25% (w/v) ethylamine or 0.25% (w/v) methylvamine as sole nitrogen sources. For growth on solid medium, a 0.67% (w/v) yeast nitrogen base
was used, supplemented with 1% (w/v) glucose and 2% (w/v) agar. When
required, leucine was added to the medium to a final concentration of 30
mg/liter.

**Molecular Biological Techniques—** *E. coli* DH5α and XLIblue were
used for the propagation and amplification of plasmid DNA. Recombi-
nant DNA procedures (enzyme digestion, cloning, plaque isolation, PCR, and Southern blotting) were performed as described (41). Transfor-
mation of *H. polymorpha* strains and site-specific integration of
single and multiple copies of plasmid DNA in the genomic AOX or AMO
locus were performed as described (37, 42, 43).

**Plasmid Constructions—** The oligonucleotides and plasmids used in
this study are listed in Table II. For co-synthesis of tev-containing
substrate proteins and TEV protease (TEVP) derivatives, novel *H. poly-
morpha* expression vectors were constructed based on the dominant
zeocin resistance gene. Vector pHIPZ4, which contains the *H. polymor-
pha* alcohol oxidase promoter (P*AOX*) for heterologous expression, has
recently been described (44). pHIPZ5, which contains the *H. polymor-
pha* amine oxidase promoter (P*AMO*), was constructed by replacing the
P*AOX* locus in pHIPZ4 by a 1.0-kilobase pair NotI-BamHI DNA frag-
ment from pHIPX5 (45) containing the P*AMO*. A DNA fragment encoding
the 27-kDa Nla protease subunit from TEVP was obtained by PCR using
primers KN11 and KN12 introducing a HindIII, BamHI, and
start codon upstream amino acid sequence SLFKG at amino acid posi-
tion 2040 in the full-length TEV and a stop codon followed by a SaI site
downstream amino acid sequence NELVIS at amino acid position
2278 in full-length TEV. A PTS1-type signal (SKL) and a SaI site were
introduced downstream the TEVP coding region using PCR and primer
KN13. The genes encoding the TEV- substrate molecules were con-
structed as follows. By sequential cloning steps, a DNA fragment en-
coding the Msc epitope (MEKQKLISEEDL), preceded by a
GFP was performed in a similar way by using the 27-kDa NIa protease subunit from TEVP as a template for PCR
using primers KN2 and KN14, followed by a SaI site and
eGFP with a PTS1 sequence (primer combination KN2-KN14) or with-
out a PTS1 (primer combination eGFP-SaI1 (46)-KN14). The *HindIII*
site upstream and the SaI site downstream the hybrid genes (TEVp
derivatives and TEVp substrate molecules) were used for insertion into
pHIPX4 and pHIPZ4. For insertion of TEVp derivatives into pHIPZ5,
*BamHI and SaI digestion were used. For constructing the
P*AMO* expression construct to introduce
the C-terminal codon R295 of *H. polymorpha* PEX10 by PCR and primer
KN20 and fused to the XhoI site preceding tev-GFP. Similarly, using
PCR and primer KN15, a SaI site was introduced downstream C-
terminal codon A457 of *H. polymorpha* PEX3 and fused to the XhoI site
preceding tev-GFP to construct the Pex3p-TEVP hybrid gene. The
PE3x-tev.GFP and PEX10-tev.GFP hybrid genes were inserted as
BamHI-SaI fragments into *pHPX4*

**Biochemical Methods—** Preparation of crude extracts of *H. polymor-
pa* (46), SDS-polyacrylamide gel electrophoresis (47), and Western blot
analysis (48) was performed as described; blots were probed using
specific antibodies against various *H. polymorpha* proteins. Polyclonal
antibodies were generated in rabbits against the 27-kDa fragment of the
TEVP protease used in this study. The antibodies against GFP were a
gift from Dr. W.-H. Kunau, Bochum, Germany. Goat anti-rabbit alkaline
phosphatase and goat anti-rabbit horse radish peroxidase (Roche
Molecular Biochemicals) were used as secondary antibodies that were
detected by bromochloroindolyl phosphate/nitro blue tetrazolium
(Roche Molecular Biochemicals) or ECL (Amersham Pharmacia Bio-
tech) according to the manufacturers’ protocols.

**Microscopical Procedures—** Fluorescent microscopy to localize hybrid
proteins containing GFP was performed as described (46) using an
Axioskop 2 fluorescence microscope (Zeiss, Germany). The Nether-
lands) equipped with a Princeton Instruments CCD camera (RTE/
CCD-1300 Y; Princeton Instruments b.v., The Netherlands). Whole
cells were fixed and prepared for electron microscopy and immunocy-
tochemistry as described previously (11). Immunolabeling was
performed on ultrathin sections of unincryl-embedded cells using specific
antibodies against various *H. polymorpha* proteins and GFP and gold-
conjugated goat anti-rabbit (GAR-gold) antibodies according to the
instructions of the manufacturer (Amersham Pharmacia Biotech).

<table>
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<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Reference</th>
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<tr>
<td>NCYC 495</td>
<td><em>H. polymorpha</em> WT, leu1.1 derivative</td>
<td>(60)</td>
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<td>HF34</td>
<td>NCYC495::P<em>AOX</em>-Nmyc.tev.GFP::P<em>AOX</em>-TEVP</td>
<td>This study</td>
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<td>HF35</td>
<td>NCYC495::P<em>AOX</em>-Nmyc.tev.GFP::P<em>AOX</em>-TEVP</td>
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<td>HF36</td>
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<td>HF38</td>
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<td>HF39</td>
<td>NCYC495::P<em>AMO</em>-Nmyc.tev.GFP::P<em>AMO</em>-TEVP</td>
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<td>HF40</td>
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<td>HF42</td>
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<td>HF43</td>
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<td>HF44</td>
<td>NCYC495::P<em>AMO</em>-Nmyc.tev.GFP::P<em>AMO</em>-TEVP</td>
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<td>HF193</td>
<td>NCYC495::P<em>AMO</em>-TEVP::P<em>AMO</em>-PEX10.tev.GFP</td>
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RESULTS

The use of the TEV protease to study the principles of peroxisome biogenesis in *H. polymorpha* is critically dependent on the following prerequisites. 1) The protein should be synthesized and become active in this yeast without loss of cell viability, 2) it should be active on substrate proteins in the cytosol and peroxisomal substrate molecules. The experiments to analyze peroxisomal TEVp should be selective toward cytosolic and peroxisomal substrate proteins. To establish the activity and subcellular localization of TEVp and TEVp.SKL in *H. polymorpha*—in *vivo*, we constructed two hybrid genes encoding either a cytosolic or a peroxisomal tev-containing substrate protein molecule (Fig. 1A). These proteins consist of an N-terminal Myc molecule (abbreviated as tev in text and figures) and the reporter protein eGFP (Nmyc.tev.GFP, cytosolic protein). In the case of the peroxisomal substrate, a PTS1 sequence, SKL, is added at the C terminus (Nmyc.tev.GFP.SKL). Synthesis of the TEVp substrate molecules was determined by Western blot analysis using antibodies directed against GFP or the Myc epitope. Both antisera specifically

### TABLE II

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<th>Primers</th>
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<tr>
<td>KN3</td>
<td>CCCAGGCTATGGGACGAAATTGGTACGTAGAGGTAACATCGAGCTTCTCAAGAAAGCGG</td>
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<td>CCCAGGCTATGGGACGAAATTGGTACGTAGAGGTAACATCGAGCTTCTCAAGAAAGCGG</td>
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<td>CCCAGGCTATGGGACGAAATTGGTACGTAGAGGTAACATCGAGCTTCTCAAGAAAGCGG</td>
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<td>KN15</td>
<td>AACTGAGGAAAGTACAGGTTCTCTCTGAGCATCGAAATTGAGTAG</td>
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<td>KN20</td>
<td>GGGCTGACTTACGAGTACAAATTGGTACGTAGAGGTAACATCGAGCTTCTCAAGAAAGCGG</td>
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<th>Plasmids</th>
<th>Relevant characteristics</th>
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<tr>
<td>pHIPZ4*</td>
<td>Kan&lt;sup&gt;a&lt;/sup&gt;, ScLEU2, P&lt;sub&gt;AOX&lt;/sub&gt;-driven expression</td>
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<tr>
<td>pHIPZ4*</td>
<td>Amp&lt;sup&gt;a&lt;/sup&gt;, Zeo&lt;sup&gt;a&lt;/sup&gt;, P&lt;sub&gt;AOX&lt;/sub&gt;-driven expression</td>
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<tr>
<td>pHIPZ5*</td>
<td>Amp&lt;sup&gt;a&lt;/sup&gt;, Zeo&lt;sup&gt;a&lt;/sup&gt;, P&lt;sub&gt;AOX&lt;/sub&gt;-driven expression</td>
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<tr>
<td>pFEM45</td>
<td>pHIPZ4-TEV</td>
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<td>pFEM46</td>
<td>pHIPZ4-TEV.SKL</td>
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<td>pFEM68</td>
<td>pHIP5-TEV</td>
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<td>pFEM69</td>
<td>pHIP5-TEV.SKL</td>
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<td>pFEM72</td>
<td>pHIPX4-Nmyc.tev.GFP</td>
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<td>pFEM152</td>
<td>pHIPX4-PEX3.tev.GFP</td>
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<tr>
<td>pFEM147</td>
<td>pHIPX4-PEX10.tev.GFP</td>
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<sup>a</sup> Plasmids reported in Refs. 61 and 45, respectively.

<sup>b</sup> All other plasmids first reported in this study.
recognized a protein band of the expected size of ~30 kDa in crude extracts prepared from methanol-grown transformants (Fig. 2, lanes 1 and 2). These protein bands were undetectable in extracts prepared from the control host strain (data not shown). The subcellular localization of the TEVp-substrate proteins was determined by fluorescence microscopy and immunocytochemistry. As shown in Fig. 1, a diffuse fluorescence was observed in cells of the strain synthesizing Nmyc.tev.GFP (Fig. 1B), which is indicative of a cytosolic location of the protein. Immunolabeling experiments using antibodies against the Myc epitope confirmed that indeed this substrate molecule had accumulated in the cytosol (Fig. 1D). Also, significant labeling was localized on the nucleus of these cells, which is however not unexpected since this substrate molecule has a size that should allow free passage through the nuclear pore (49, 50). In contrast, fluorescence was observed as bright dots in the strain producing Nmyc.tev.GFP.SKL (Fig. 1C). The peroxisomal localization of this protein was corroborated by immunocytochemical experiments (Fig. 1E) in which Myc antibody-dependent labeling was exclusively located on these organelles. Unexpectedly, the protein did not diffuse into the crystalline AO matrix of the organelle as for instance observed for the endogenous enzyme dihydroxyacetone synthase (51). Instead, it accumulated in the narrow space between the crystalline matrix and the peroxisomal membrane (Fig. 1E), a location also observed for endogenous catalase protein (51).

Synthesis of Cytosolic or Peroxisomal TEV-Protease Does Not Affect H. polymorpha Viability—Two TEV protease expression plasmids were constructed, one designed to produce cytosolic TEVp and the other one designed to produce peroxisomal TEVp. To this end, the active 27-kDa domain of the TEV protease (52) was modified by introducing an initiation codon (ATG/Met) in front of amino acid 2040 and a termination codon (TAA) downstream amino acid 2278 of full-length TEV. For peroxisomal targeting, the typical C-terminal PTS1 signal, SKL, was introduced at the extreme C terminus (TEVp.SKL). In initial experiments, expression of the TEVp variants was controlled by the PAMO. Four strains were constructed in which the TEVp substrate proteins were co-synthesized with either TEVp or TEVp.SKL.

Since the TEV protease might act on any endogenous protein containing the consensus sequence EXXXQ|/SG, we first determined whether the TEV protease affected cell viability. As shown in Fig. 2, lanes 3-6, synthesis of the TEV protease destined for the cytosol or peroxisomes was readily demonstrated in cells grown for 16 h in methanol-containing medium. All four strains were viable and showed growth characteristics in methanol-containing medium akin to the host strain. Electron microscopical analysis did not reveal any significant morphological difference between cells producing either TEVp or TEVp.SKL as compared with wild type controls (data not shown).

Cytosolic and Peroxisomal TEV-Protease Are Active in Vivo in H. polymorpha—To determine whether the TEV protease is active in H. polymorpha in vivo, cell-free extracts of the four strains described above were analyzed for processing of the TEVp-substrate proteins (Fig. 2). Western blot analysis of these extracts probed with antibodies against GFP or the Myc epitope revealed that in all four strains, most of the substrate proteins were processed, reducing the size of and eliminating the Myc epitope from these molecules (Fig. 2, lanes 3-6). These results suggest that the TEV protease is active in H. polymorpha cells. However, processing of the substrate protein is also observed when the substrate and protease were supposed to be spatially separated. At least two possibilities may account for this phenomenon, namely (i) processing of the substrates occurs either in vitro during the preparation of the cell free extracts or (ii) processing occurs in vivo. Since both substrate and protease are sorted by the same pathway, the peroxisome-destined protease might already function en route to the organelle. Similarly, the peroxisome-destined substrate molecule might become processed by cytosolic TEVp before import into the organelle. The possibility of processing in vitro is less likely as it was prevented by trichloroacetic acid precipitation of whole cells immediately after harvesting. Therefore, we anticipated that the processing of the GFP substrate molecules occurred in vivo. Two alternative experiments were designed to limit the possible in vivo processing during sorting of GFP substrate proteins and TEVp to different subcellular locations. First, the expression levels of TEVp and TEVp.SKL were lowered through the control of weaker promoter elements while keeping the expression of the GFP substrate molecules under the control of the AOX promoter. As can be seen in Fig. 2, lanes 7-10, a reduced production level of the TEV protease by the amine oxidase promoter element (PAMO) resulted in a drastic increase in the level of unprocessed substrate protein when the two are spatially separated in the cell (Fig. 2, lanes 8 and 9). In contrast, when the protease and the substrate protein were destined for the same cellular compartment, most to all of the substrate proteins were processed (Fig. 2, lanes 7 and 10). These data show that by lowering the level of the protease, the processing of substrate molecules in the same subcellular location still proceeds efficiently, whereas unwanted in vivo processing of substrate molecules destined for a different subcellular location is reduced. In a second approach, we sought to prevent in vivo processing of spatially separated substrate proteins and TEVp by introducing a timely separation in the synthesis of the two proteins. To achieve this, cells producing TEVp.SKL under the control of the PAMO together with either
Nmyc.tev.GFP or Nmyc.tev.GFP.SKL under the control of the $P_{AMO}$ were first grown in glucose/ethylamine-containing medium. Under these conditions, the $P_{AMO}$ is induced, and thus TEVp.SKL is synthesized and sorted to peroxisomes. Concurrently, because of the presence of glucose, the $P_{AOX}$, and thus the synthesis of the GFP substrate molecule, is repressed. Subsequently, these cells were shifted to medium containing methanol and ammonium sulfate as the sole carbon source and nitrogen source, respectively. Now the $P_{AMO}$ is fully repressed (by NH$_4^+$), and thus production of TEVp.SKL is prevented, whereas the production of the GFP substrate proteins is now induced (by $P_{AOX}$). In a time course of 8 h of the shift of cells to methanol/ammonium sulfate-containing medium (Fig. 3), it can be seen that amine oxidase (an indicator for TEVp synthesis) is present at $t = 0$ and is still detectable after 8 h of growth in methanol/NH$_4^+$-containing medium. In contrast, alcohol oxidase is virtually absent at $t = 0$ but is readily detectable after 1 h in methanol medium. In the strain synthesizing the cytosolic substrate protein (Fig. 3, $\alpha$-GFP, top panel), no processing is observed at 8 h after the shift of these cells from glucose/ethylamine to methanol/ammonium sulfate medium. In contrast, significant amounts (>50%) of the peroxisomal GFP substrate molecule were found to be processed after the shift even after 6–8 h (Fig. 3, $\alpha$-GFP, bottom panel). Unprocessed peroxisomal substrate protein, however, remained detectable throughout the 8-h time interval. Most likely, this is attributable to the development of new peroxisomes in this period that imported Nmyc.tev.GFP.SKL under conditions that repress TEVp.SKL production and thus was not available to the peroxisomes to process. These data convincingly show that accumulation of TEVp.SKL in peroxisomes prior to the synthesis of the substrate protein in the cytosol prevents in vivo processing of a substrate molecule at a different subcellular location but will process substrate molecules targeted to the same location.

Taken together, these data show that heterologously synthesized TEVp and TEVp.SKL are active in H. polymorpha in the cytosol and peroxisomes, respectively, and can act specifically in these compartments. Under the experimental conditions, no vital H. polymorpha proteins are targets for the TEVp activity.

The C Termini of the Peroxisomal Membrane Proteins Hpx3p and Hpx10p Face the Cytosol—A potential application of the TEV protease is the determination of the topology of peroxisomal membrane proteins. For several of these proteins, the data are controversial to some extent and may vary with the method used. A typical example is Pex10p. In the yeast Pichia pastoris, the zinc finger containing the C terminus was reported to reside in the peroxisomal matrix (15), whereas it was reported to face the cytosol in human cells (16, 17). Pex3p on the other hand is a peroxisomal membrane protein of which the C terminus has consistently been reported to face the cytosol (53–55). Therefore, we decided to determine the localization of the C termini of these two proteins in H. polymorpha using the TEVp-based system. To this end, functional hybrid proteins, consisting of the peroxin tagged at its C terminus to GFP linked by a tev-processing sequence, were co-synthesized with either TEVp or TEVp.SKL. The GFP-tagged membrane proteins were expressed by the $P_{AOX}$, and the TEV proteases were expressed by the $P_{AMO}$. The cells were grown in methanol/methyamine-containing medium, similar to the strains expressing the soluble substrate proteins as shown in Fig. 2, lanes 7-10. As can be seen in Fig. 4, fluorescence microscopic analysis of these cells showed that when either Pex3p.tev.GFP or Pex10p.tev.GFP was co-expressed with TEVp.SKL, a clear peroxisomal fluorescent staining was observed (Fig. 4, B and D). In contrast, when these hybrid proteins were co-expressed with cytosolic TEVp, a predominantly cytosolic fluorescent staining was observed (Fig. 4, A and C). Western blot analyses using crude extracts prepared of these cells confirm that in cells producing the cytosolic TEVp, specific processing of the hybrid proteins was observed (Fig. 5, shown for Pex3p.tev.GFP). Since synthesis of both the hybrid protein and the TEV protease was induced simultaneously, unprocessed Pex3p.tev.GFP was expected to be detectable in these strains. In contrast, no signif-
icient processing of Pex3.tev.GFP was observed when it was co-synthesized with TEVp.SKL. This result implies that the tev site at the C terminus of Pex3p is not accessible from the peroxisomal matrix.

**DISCUSSION**

In this study we describe the synthesis of the 27-kDa Nla protease subdomain of the tobacco etch virus in the yeast *H. polymorpha*. The protease was shown to be selectively active in vivo on soluble TEVp-substrate molecules that were produced in the same compartment either in the cytosol (TEVp) or in the peroxisomal matrix (TEVp.SKL). This system was successfully used to show that the C termini of two peroxisomal membrane proteins, Pex3p and Pex10p, face the cytosol.

Besides several other obvious applications of the TEV protease to study peroxisome biogenesis, this procedure to determine membrane protein topology seems to be pre-eminently suited to establish the location of functional domains in PM peroxins. To understand the function of peroxisomal membrane proteins, knowledge of the topology of these proteins is crucial. However, the current procedures to determine protein topology have resulted in virtually controversial data for many (inter-) peroxisomal membrane proteins, including Pex8p (11–13), Pex10p (14–17), Pex11p (18–24), Pex14p (25–28), Pex16p (29, 30), and Pex17p (31, 32). For example for Pex10p, these experiments have resulted in two contradicting topologies. In *P. pastoris*, the C-terminal zinc-finger domain was proposed to face the peroxisomal matrix (15). In human cells, the C terminus was proposed to face the cytosol (16, 17). Since two other zinc-binding PM peroxins, Pex2p and Pex12p, are essential for peroxisome biogenesis, it is important to know whether their zinc-binding domains face the same side of the peroxisomal membrane.

The TEV-based system was effectively used to resolve the location of the C terminus of HpPex10p. Co-synthesis of Pex10.tev.GFP with cytosolic TEVp resulted in cleavage of the Pex10.tev.GFP hybrid. Cleavage was prevented when Pex10.tev.GFP was co-synthesized with peroxisomal TEVp.SKL. Identical results were obtained with a Pex3.tev.GFP hybrid protein, the C terminus of which is known to face the cytosol in both baker’s yeast and human cells (53–55). These data convincingly demonstrate that the C terminus of HpPex10p has the same location as Pex3p and thus protrudes into the cytosol.

**Advantages of the TEVp-processing System**—The advantage of the TEVp-based procedure over other available methods is that no (endogenous) control proteins need to be characterized that may display other susceptibility toward a protease or antibody. Rather, one protein is analyzed with either a protease acting in the peroxisome or one active in the cytosol, and probably other applications will further advance our knowledge about the specific function of the peroxins.

**REFERENCES**


**Acknowledgment**—We thank Dr. W.-H. Kunau (Ruhr University, Bochum, Germany) for the generous gift of antibodies against GFP.