University of Groningen

A novel method to determine the topology of peroxisomal membrane proteins in vivo using the tobacco etch virus protease

Faber, Klaas; Kram, Anita; Ehrmann, Michael; Veenhuis, Marten

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

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

Publication date:
2001

Link to publication in University of Groningen/UMCG research database

A Novel Method to Determine the Topology of Peroxisomal Membrane Proteins *in Vivo* Using the Tobacco Etch Virus Protease*

Klaas Nico Faber‡, Anita M. Kram§, Michael Ehrmann¶‖, and Marten Veenhuis**

From the Eukaryotic Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, P. O. Box 14, 9750 AA Haren, The Netherlands and ¶Biosi-2, Cardiff University, Museum Avenue, P. O. Box 911, Cardiff CF10 3US, United Kingdom

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, EXXYXQ↓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 secondary metabolites, such as β-lactam penicillins (1–3). In yeast they are essential for the metabolism of unusual carbon sources such as oleic acid, primary amines, purines, D-amino acids, and methanol (4). In humans they are involved in a variety of anabolic and catabolic pathways, including plasmalogen 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)[1], the C-terminal PTS1, SKL, and variants and the N-terminal PTS2 (8). In general, import of peroxisomal proteins does not involve any significant protein modification (9). Recently, several genes involved in peroxisome biogenesis, so-called *PEX* genes encoding peroxins, have been cloned by the functional complementation of yeast mutant strains lacking functional peroxisomes (10). Up to now, 23 *PEX* genes have been described, and more are likely to be identified in the near future. Data base screening has been shown to be a powerful tool to identify human homologues of the yeast peroxins. Thirteen human PEX homologues have been identified, so far of which 11 were shown to restore peroxisome biogenesis in cell lines of patients with peroxisomal disorders (6).

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, heterolo-

[1] 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_{AOX}, alcohol oxidase promoter; P_{AMO}, amine oxidase promoter.

TABLE I
H. polymorpha strains used in this study

Strain	Relevant genotype	Reference
NCYC 495	*H. polymorpha* WT, *leu1.1* derivative	(60)
HF34	NCYC495::P$_{AOX}$-N*myc.tev.GFP*::P$_{AOX}$-*TEVp*	This study
HF35	NCYC495::P$_{AOX}$-N*myc.tev.GFP.SKL*::P$_{AOX}$-*TEVp*	This study
HF36	NCYC495::P$_{AOX}$-N*myc.tev.GFP*::P$_{AOX}$-*TEVp.SKL*	This study
HF37	NCYC495::P$_{AOX}$-N*myc.tev.GFP.SKL*::P$_{AOX}$-*TEVp.SKL*	This study
HF38	NCYC495::P$_{AOX}$-N*myc.tev.GFP*::P$_{AMO}$-*TEVp*	This study
HF39	NCYC495::P$_{AOX}$-N*myc.tev.GFP.SKL*::P$_{AMO}$-*TEVp*	This study
HF40	NCYC495::P$_{AOX}$-N*myc.tev.GFP*::P$_{AMO}$-*TEVp.SKL*	This study
HF41	NCYC495::P$_{AOX}$-N*myc.tev.GFP.SKL*::P$_{AMO}$-*TEVp.SKL*	This study
HF42	NCYC495::P$_{AOX}$-N*myc.tev.GFP*	This study
HF45	NCYC495::P$_{AOX}$-N*myc.tev.GFP.SKL*	This study
HF151	NCYC495::P$_{AMO}$-*TEVp*::P$_{AOX}$-*PEX3.tev.GFP*	This study
HF153	NCYC495::P$_{AMO}$-*TEVp.SKL*::P$_{AOX}$-*PEX3.tev.GFP*	This study
HF191	NCYC495::P$_{AMO}$-*TEVp*::P$_{AOX}$-*PEX10.tev.GFP*	This study
HF193	NCYC495::P$_{AMO}$-*TEVp.SKL*::P$_{AOX}$-*PEX10.tev.GFP*	This study

gous protease in the yeast *Hansenula polymorpha*. Prerequisite 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-expression 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 cytosolic 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 tobacco etch virus (TEV) polyprotein containing a proteinase activity specifically processing the consensus sequence (heptapeptide) E*XXYX*Q(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 substrate proteins containing the consensus sequence for processing (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 compartments without the loss of cell viability. In an *in vivo* application, 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 applications of the TEV protease to study peroxisome biogenesis are discussed.

EXPERIMENTAL PROCEDURES

Strains and Cultivation—*H. polymorpha* NCYC495 (*leu1.1*) 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) methylamine 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 XL1blue were used for the propagation and amplification of plasmid DNA. Recombinant DNA procedures (enzyme digestion, cloning, plasmid isolation, PCR, and Southern blotting) were performed as described (41). Transformation 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. polymorpha* alcohol oxidase promoter (P$_{AOX}$) for heterologous expression, has recently been described (44). pHIPZ5, which contains the *H. polymorpha* amine oxidase promoter (P$_{AMO}$), was constructed by replacing the P$_{AOX}$ locus in pHIPZ4 by a 1.0-kilobase pair *Not*I-*Bam*HI DNA fragment from pHIPX5 (45) containing the P$_{AMO}$. A DNA fragment encoding the 27-kDa NIa protease subunit from TEVp was obtained by PCR using primers KN11 and KN12 introducing a *Hin*dIII, *Bam*HI, and start codon upstream amino acid sequence SLFKG at amino acid position 2040 in the full-length TEV and a stop codon followed by a *Sal*I site downstream amino acid sequence NELVIS at amino acid position 2278 in full-length TEV. A PTS1-type signal (SKL) and a *Sal*I site were introduced downstream the TEVp coding region using PCR and primer KN13. The genes encoding the TEVp-substrate molecules were constructed as follows. By sequential cloning steps, a DNA fragment encoding the Myc epitope (MEQKLISEEDL), preceded by a *Hin*dIII site, and followed by an *Xho*I site (primer KN3) was fused to an *Xho*I site upstream of a DNA fragment containing the TEV protease cleavage sequence (ENLYFQ↓S, abbreviated as tev) followed by a *Bgl*II site and eGFP with a PTS1 sequence (primer combination KN2-KN14) or without a PTS1 (primer combination eGFP-*Sal*I (46)-KN14). The *Hin*dIII site upstream and the *Sal*I 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, *Bam*HI and *Sal*I digestions were used. For constructing the *PEX10.tev.GFP* hybrid gene, a *Xho*I site was introduced downstream C-terminal codon R^{295} of *H. polymorpha* PEX10 by PCR and primer KN20 and fused to the *Xho*I site preceding tev-GFP. Similarly, using PCR and primer KN15, a *Sal*I site was introduced downstream C-terminal codon A^{457} of *H. polymorpha* PEX3 and fused to the *Xho*I site preceding tev-GFP to construct the *Pex3.tev.GFP* hybrid gene. The *PEX3.tev.GFP* and *PEX10.tev.GFP* hybrid genes were inserted as *Bam*HI-*Sal*I fragments into pHIPX4.

Biochemical Methods—Preparation of crude extracts of *H. polymorpha* (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 using the 27-kDa fragment of the TEV 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 Biotech) according to the manufacturers' protocols.

Microscopical Procedures—Fluorescent microscopy to localize hybrid proteins containing GFP was performed as described (46) using an Axioskop H fluorescence microscope (Zeiss Netherlands b.v., The Netherlands) 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 immunocytochemistry as described previously (11). Immunolabeling was performed on ultrathin sections of unicryl-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 II

Primers and plasmids used in this study

Primers	DNA sequence (5′-3′)
KN3	CCCAAGCTTATGGAGCAGAAGTTGATTTCTGAGGAAGACCTCGAGTCTTCTACAGAAAAGCGGC
KN11	CCCAAGCTTGGATCCATGTCCTTGTTTAAGGGACCACG
KN12	GGGGTCGACTTACGAGTACACCAATTCATTC
KN13	GGGGTCGACTTACAGCTTCGAGTACACGAGCTCATTCATGAG
KN14	CCCCTCGAGAACCTGTACTTCCAGTCGAGATCTGTGAGCAAGGGCGAGGAGC
KN15	AACCGACTGGAAGTACAGGTTCTCGTCGACAGCATCGAAATTAGAGTAG
KN20	GGGCTCGAGAGATCTTAGAGGCAACAGCTGCG

Plasmids	Relevant characteristics
pHIPX4[a]	Kan^R, *ScLEU2*, P$_{AOX}$-driven expression
pHIPZ4[a]	Amp^R, Zeo^R, P$_{AOX}$-driven expression
pHIPZ5[b]	Amp^R, Zeo^R, P$_{AMO}$-driven expression
pFEM45	pHIPZ4-*TEV*
pFEM46	pHIPZ4-*TEV.SKL*
pFEM68	pHIPZ5-*TEV*
pFEM69	pHIPZ5-*TEV.SKL*
pFEM72	pHIPX4-*Nmyc.tev.GFP*
pFEM74	pHIPX4-*Nmyc.tev.GFP.SKL*
pFEM152	pHIPX4-*PEX3.tev.GFP*
pFEM147	pHIPX4-*PEX10.tev.GFP*

[a] Plasmids reported in Refs. 61 and 45, respectively.
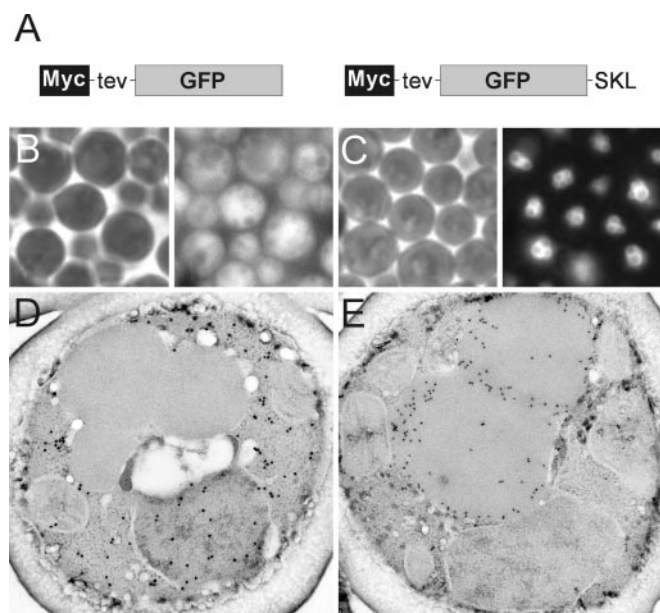[b] All other plasmids first reported in this study.

FIG. 1. **Sorting of the TEVp-substrate proteins, Nmyc.tev.GFP and Nmyc.tev.GFP.SKL.** *A*, schematic representation of the cytosolic and peroxisomal TEVp substrate proteins, Nmyc.tev.GFP and Nmyc.tev.GFP.SKL. Strains HF42 (Nmyc.tev.GFP) (*B* and *D*) and HF45 (Nmyc.tev.GFP.SKL) (*C* and *E*) were grown to the mid-exponential growth phase in methanol-containing medium. Normaski images (*B* and *C*, *left panels*), fluorescence microscopy images (*B* and *C*, *right panels*), and immunolocalization of the substrate molecules using antibodies raised against the Myc epitope (*D* and *E*) are shown.

FIG. 2. *In vivo* **processing of the cytosolic and peroxisomal TEVp-substrate proteins.** Western blot analysis of cell-free extracts of various strains synthesizing TEV-substrate proteins with or without TEV protease. *Lanes 1* and *2* are controls showing expression of the TEV-substrates alone (Nmyc.tev.GFP and Nmyc.tev.GFP.SKL, respectively). *Lanes 3-6* show the effect on the substrate proteins when combined with high level expression (by P$_{AOX}$) of the TEV protease (TEVp or TEVp.SKL) when cleavage of the Myc tag is extensive in all combinations. *Lanes 7-10* show that with reduced expression level (by P$_{AMO}$) of the TEV protease, the processing of the substrate proteins still occurs when it co-localizes with the protease (*lanes 7* and *10*) but is virtually absent when both proteins do not co-localize (*lanes 8* and *9*). *Lanes 1-6*, cells grown in methanol/ammonium sulfate medium. *Lane 1*, Nmyc.tev.GFP (strain HF42); *lane 2*, Nmyc.tev.GFP.SKL (HF45); *lane 3*, Nmyc.tev.GFP and TEVp (HF34); *lane 4*, Nmyc.tev.GFP.SKL and TEVp (HF35) *lane 5*, Nmyc.tev.GFP and TEVp.SKL (HF36); and *lane 6*, Nmyc.tev.GFP.SKL and TEVp.SKL (HF37). *Lanes 7-10*, cells grown in methanol/methylamine medium. *Lane 7*, Nmyc.tev.GFP and TEVp (HF38); *lane 8*, Nmyc.tev.GFP.SKL and TEVp (HF39); *lane 9*, Nmyc.tev.GFP and TEVp.SKL (HF40); and *lane 10*, Nmyc.tev.GFP.SKL and TEVp.SKL (HF41). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis analyzed by Western blotting using specific antisera raised against GFP (*top panel*), Myc (*middle panel*), and TEVp (*bottom panel*).

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 as well as in peroxisomes, and 3) the activity of cytosolic and peroxisomal TEVp should be selective toward cytosolic and peroxisomal substrate molecules. The experiments to analyze these prerequisites are detailed below.

Construction of the Substrate Molecules Nmyc.tev.GFP Containing or Lacking a PTS1—To establish the activity and subcellular localization of TEVp and TEVp.SKL in *H. polymorpha*
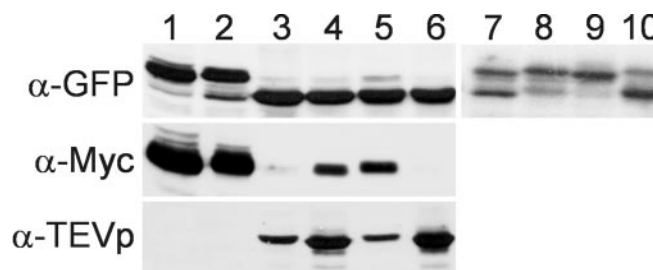
in vivo, we constructed two hybrid genes encoding either a cytosolic or a peroxisomal tev-containing substrate protein molecule (Fig. 1*A*). These proteins consist of an N-terminal Myc sequence (MEQKLISEEDL) followed by the TEV proteolytic consensus sequence (ENLYFQ↓S, abbreviated as tev in text and 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 controlled by the strong methanol-regulated alcohol oxidase promoter (P$_{AOX}$). The production of the hybrid proteins was determined by Western blot analysis using antibodies directed against GFP or the Myc epitope. Both antisera specifically

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. 1*B*), 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. 1*D*). 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. 1*C*). The peroxisomal localization of this protein was corroborated by immunocytochemical experiments (Fig. 1*E*) 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 in between the crystalline matrix and the peroxisomal membrane (Fig. 1*E*), 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 P_{AOX}. 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 E*XXYX*Q↓(S/G), 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 oc-
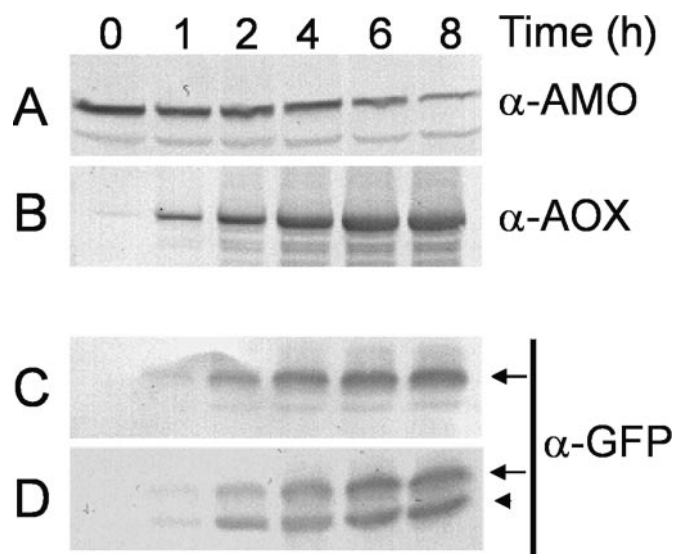

FIG. 3. **Synthesis of TEVp.SKL prior to that of TEVp-substrate molecules improves the specificity of *in vivo* processing.** Strains HF40 (P_{AOX}-*Nmyc.tev.GFP*,P_{AMO}-*TEVp.SKL* (*A*, *B*, and *C*)) and HF41 (P_{AOX}-*Nmyc.tev.GFP.SKL*,P_{AMO}-*TEVp.SKL* (*D*)) were pregrown in glucose/ethylamine-medium to induce P_{AMO}-expressed genes (*A*, *AMO* and *TEVp.SKL*, not shown). Subsequently, these cells were transferred to methanol/ammonium sulfate-medium, thus repressing further synthesis of TEVp.SKL but inducing P_{AOX}-expressed genes (*B*, *AOX*; *C*, *Nmyc.tev.GFP*; and *D*, *Nmyc.tev.GFP.SKL*). Prior to the shift (*t* = 0), P_{AMO}-expressed genes (*AMO* and *TEVp.SKL*) are synthesized and gradually decrease over time after the shift. In contrast, P_{AOX}-expressed genes (*AOX, Nmyc.tev.GFP*, and *Nmyc.tev.GFP.SKL*) only become detectable after the shift, and the protein levels increase in time. Significant amounts of the Nmyc.tev.GFP.SKL substrate protein becomes processed in time (indicated by the *arrowhead*), whereas only the unprocessed Nmyc.tev.GFP is detected after the shift.

curs 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 (P_{AMO}) 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 P_{AMO} together with either

Nmyc.tev.GFP or Nmyc.tev.GFP.SKL under the control of the P$_{AOX}$ 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 NH4$^+$), 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/NH4$^+$-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, α-*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, α-*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 protease. 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 HpPex3p and HpPex10p 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/methylamine-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 Pex3.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
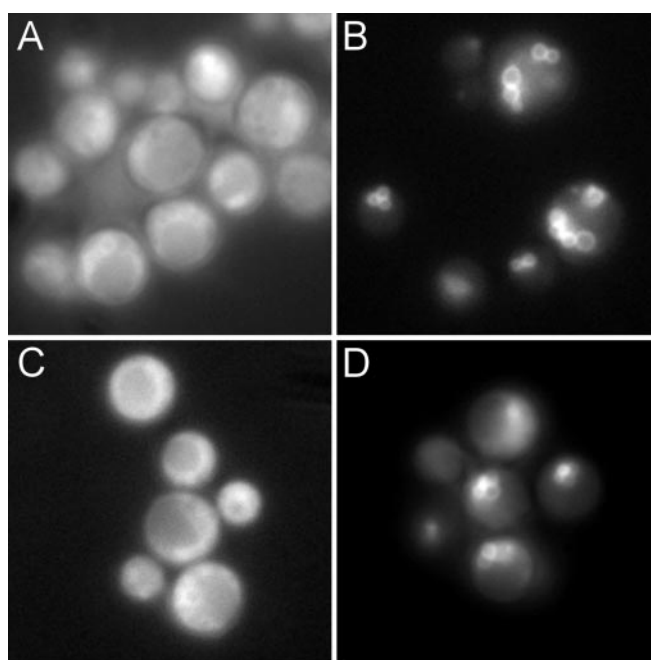

FIG. 4. **Determination of the topology of peroxisomal membrane proteins Pex3.tev.GFP and Pex10p.tev.GFP *in vivo*.** Fluorescence microscopic analysis of strains HF151 (P$_{AMO}$-*TEVp*,P$_{AOX}$-*PEX3.tev.GFP*) (*A*), HF153 (P$_{AMO}$-*TEVp.SKL*, P$_{AOX}$-*PEX3.tev.GFP*) (*B*), HF191 (P$_{AMO}$-*TEVp*,P$_{AOX}$-*PEX10.tev.GFP*) (*C*), and HF193 (P$_{AMO}$-*TEVp.SKL*,P$_{AOX}$-*PEX10.tev.GFP*) (*D*) in methanol/methylamine medium. Co-synthesis of Pex3p.tev.GFP (*A*) or Pex10.tev.GFP (*C*) with cytosolic TEVp leads to a predominant cytosolic fluorescent staining, whereas co-synthesis with peroxisomal TEVp.SKL shows a peroxisomal rim staining for Pex3.tev.GFP (*B*) and Pex10.tev.GFP (*D*).
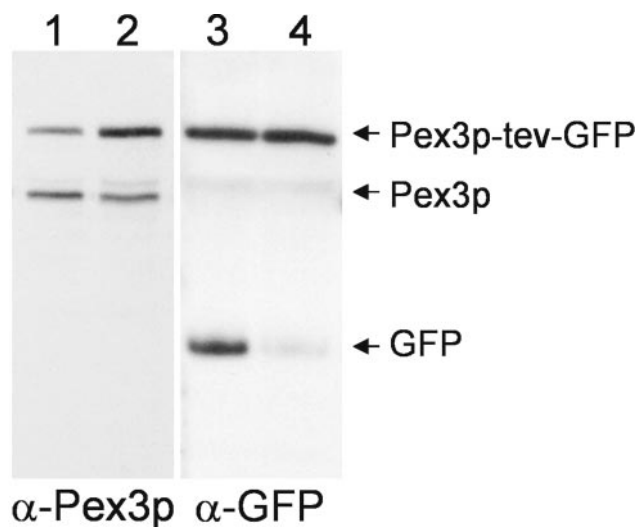

FIG. 5. **Cytosolic TEVp processes pex3p.tev.GFP.** Western blot analysis of total protein extracts prepared from HF151 (P$_{AMO}$-*TEVp*,P$_{AOX}$-*PEX3.tev.GFP*, *lanes 1* and *3*) and HF153 (P$_{AMO}$-*TEVp.SKL*, P$_{AOX}$-*PEX3.tev.GFP*, *lanes 2* and *4*) cells grown in methanol/methylamine medium using specific antibodies raised against *H. polymorpha* Pex3p (*lanes 1* and *2*) or GFP (*lanes 3* and *4*). The *arrows* indicate the hybrid protein Pex3p.tev.GFP and the processing products Pex3p and GFP as well as endogenous Pex3p.

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-

icant 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.

<div align="center">DISCUSSION</div>

In this study we describe the synthesis of the 27-kDa NIa 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 (integral) 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 bakers' 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, *e.g.* the strain co-expressing TEVp is the control for the TEVp.SKL strain and vice versa. Processing can be detected in cell free extracts and does not require organelle purification or selective permeabilization of cellular membranes. In addition, by using a cleavable GFP domain, processing (and thereby topology) can be determined by fluorescence microscopy or confocal laser scanning microscopy using living cells. Conclusive data are obtained if only one of the combinations shows a significant higher degree of processing as compared with the other, as described here for Pex3.tev.GFP and Pex10.tev.GFP.

Additional Applications of the TEV Protease in Studies on Peroxisome Biogenesis—The use of the TEV protease is clearly not restricted to protein topology determination. Firstly, the *H. polymorpha* strain expressing the peroxisomal protease is now being used to reinitiate our efforts to set up a reliable *in vitro* import system for peroxisomes. The development of such a system has been severely hampered by both the high fragility of peroxisomes and the lack of an unequivocal criterion for

import. Clearly, the processing of substrate molecules by the peroxisomal TEV protease will allow an unequivocal detection system (as for mitochondria) to monitor *in vitro* import of substrate molecules. Secondly, the TEV protease will be applicable in determining whether proteins exist transiently at a different subcellular location, which in wild type cells occurs at biochemically low or even undetectable levels. Such an approach is pre-eminently suitable to study the possible sorting of peroxisomal membrane proteins via the endoplasmic reticulum (56, 57) and the shuttling of the PTS1-receptor, Pex5p, between the cytosol and the peroxisomal matrix (58, 59). Both endoplasmic reticulum-localized TEVp and functional HpPex5p variants that are susceptible to TEVp processing have been constructed in our laboratory. Thirdly, proteins containing (internal) tev sites can be inactivated, both *in vivo* and *in vitro*, and the effect on peroxisomal biogenesis can be studied. These and probably other applications will further advance our knowledge about the specific function of the peroxins.

<div align="center">REFERENCES</div>

1. Olsen, L. J. (1998) *Plant Mol. Biol.* **38,** 163–189
2. Hannaert, V., and Michels, P. A. (1994) *J. Bioenerg. Biomembr.* **26,** 205–212
3. van de Kamp, M., Driessen, A. J., and Konings, W. N. (1999) *Antonie Leeuwenhoek* **75,** 41–78
4. van der Klei, I. J., and Veenhuis, M. (1997) *Trends Microbiol.* **5,** 502–509
5. van den Bosch, H., Schutgens, R. B., Wanders, R. J., and Tager, J. M. (1992) *Annu. Rev. Biochem.* **61,** 157–197
6. Gould, S. J., and Valle, D. (2000) *Trends Genet.* **16,** 340–345
7. Lazarow, P. B., and Fujiki, Y. (1985) *Annu. Rev. Cell Biol.* **1,** 489–530
8. Subramani, S. (1996) *J. Biol. Chem.* **271,** 32483–32486
9. Titorenko, V. I., Smith, J. J., Szilard, R. K., and Rachubinski, R. A. (1998) *J. Cell Biol.* **142,** 403–420
10. Hettema, E. H., Distel, B., and Tabak, H. F. (1999) *Biochim. Biophys. Acta* **1451,** 17–34
11. Waterham, H. R., Titorenko, V. I., Haima, P., Cregg, J. M., Harder, W., and Veenhuis, M. (1994) *J. Cell Biol.* **127,** 737–749
12. Liu, H., Tan, X., Russell, K. A., Veenhuis, M., and Cregg, J. M. (1995) *J. Biol. Chem.* **270,** 10940–10951
13. Smith, J. J., Szilard, R. K., Marelli, M., and Rachubinski, R. A. (1997) *Mol. Cell. Biol.* **17,** 2511–2520
14. Tan, X., Waterham, H. R., Veenhuis, M., and Cregg, J. M. (1995) *J. Cell Biol.* **128,** 307–319
15. Kalish, J. E., Theda, C., Morrell, J. C., Berg, J. M., and Gould, S. J. (1995) *Mol. Cell. Biol.* **15,** 6406–6419
16. Warren, D. S., Morrell, J. C., Moser, H. W., Valle, D., and Gould, S. J. (1998) *Am. J. Hum. Genet.* **63,** 347–359
17. Okumoto, K., Itoh, R., Shimozawa, N., Suzuki, Y., Tamura, S., Kondo, N., and Fujiki, Y. (1998) *Hum. Mol. Genet.* **7,** 1399–1405
18. Erdmann, R., and Blobel, G. (1995) *J. Cell Biol.* **128,** 509–523
19. Marshall, P. A., Krimkevich, Y. I., Lark, R. H., Dyer, J. M., Veenhuis, M., and Goodman, J. M. (1995) *J. Cell Biol.* **129,** 345–355
20. Marshall, P. A., Dyer, J. M., Quick, M. E., and Goodman, J. M. (1996) *J. Cell Biol.* **135,** 123–137
21. Abe, I., Okumoto, K., Tamura, S., and Fujiki, Y. (1998) *FEBS Lett.* **431,** 468–472
22. Schrader, M., Reuber, B. E., Morrell, J. C., Jimenez-Sanchez, G., Obie, C., Stroh, T. A., Valle, D., Schroer, T. A., and Gould, S. J. (1998) *J. Biol. Chem.* **273,** 29607–29614
23. Passreiter, M., Anton, M., Lay, D., Frank, R., Harter, C., Wieland, F. T., Gorgas, K., and Just, W. W. (1998) *J. Cell Biol.* **141,** 373–383
24. Lorenz, P., Maier, A. G., Baumgart, E., Erdmann, R., and Clayton, C. (1998) *EMBO J.* **17,** 3542–3555
25. Albertini, M., Rehling, P., Erdmann, R., Girzalsky, W., Kiel, J. A. K. W., Veenhuis, M., and Kunau, W. H. (1997) *Cell* **89,** 83–92
26. Girzalsky, W., Rehling, P., Stein, K., Kipper, J., Blank, L., Kunau, W. H., and Erdmann, R. (1999) *J. Cell Biol.* **144,** 1151–1162
27. Will, G. K., Soukupova, M., Hong, X., Erdmann, K. S., Kiel, J. A. K. W., Dodt, G., Kunau, W. H., and Erdmann, R. (1999) *Mol. Cell. Biol.* **19,** 2265–2277
28. Shimizu, N., Itoh, R., Hirono, Y., Otera, H., Ghaedi, K., Tateishi, K., Tamura, S., Okumoto, K., Harano, T., Mukai, S., and Fujiki, Y. (1999) *J. Biol. Chem.* **274,** 12593–12604
29. Titorenko, V. I., and Rachubinski, R. A. (1998) *Mol. Cell. Biol.* **18,** 2789–2803
30. South, S. T., and Gould, S. J. (1999) *J. Cell Biol.* **144,** 255–266
31. Huhse, B., Rehling, P., Albertini, M., Blank, L., Meller, K., and Kunau, W. H. (1998) *J. Cell Biol.* **140,** 49–60
32. Snyder, W. B., Koller, A., Choy, A. J., Johnson, M. A., Cregg, J. M., Rangell, L., Keller, G. A., and Subramani, S. (1999) *Mol. Biol. Cell* **10,** 4005–4019
33. Dougherty, W. G., and Semler, B. L. (1993) *Microbiol. Rev.* **57,** 781–822
34. Smith, T. A., and Kohorn, B. D. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 5159–5162
35. Mondigler, M., and Ehrmann, M. (1996) *J. Bacteriol.* **178,** 2986–2988

36. Sagot, I., Bonneu, M., Balguerie, A., and Aigle, M. (1999) *FEBS Lett.* **19;447,** 53–57
37. Baerends, R. J. S., Rasmussen, S. W., Hilbrands, R. E., van der Heide, M., Faber, K. N., Reuvekamp, P. T., Kiel, J. A. K. W., Cregg, J. M., van der Klei, I. J., and Veenhuis, M. (1996) *J. Biol. Chem.* **271,** 8887–8894
38. Götte, K., Girzalsky, W., Linkert, M., Baumgart, E., Kammerer, S., Kunau, W. H., and Erdmann, R. (1998) *Mol. Cell. Biol.* **18,** 616–628
39. Snyder, W. B., Faber, K. N., Wenzel, T. J., Koller, A., Luers, G. H., Rangell, L., Keller, G. A., and Subramani, S. (1999) *Mol. Biol. Cell* **10,** 1745–1761
40. van Dijken, J. P., Otto, R., and Harder, W. (1976) *Arch. Microbiol.* **111,** 137–144
41. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Sping Harbor Laboratory Press, Cold Spring Harbor, NY
42. Faber, K. N., Haima, P., Harder, W., Veenhuis, M., and AB, G. (1994) *Curr. Genet.* **25,** 305–310
43. Faber, K. N., Haima, P., Gietl, C., Harder, W., AB, G., and Veenhuis, M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 12985–12989
44. Salomons, F. A., Kiel, J. A. K. W., Faber, K. N., Veenhuis, M., and van der Klei, I. J. (2000) *J. Biol. Chem.* **275,** 12603–12611
45. Kiel, J. A. K. W., Keizer-Gunnink, I., Krause, T., Komori, M., and Veenhuis, M. (1995) *FEBS Lett.* **377,** 434–438
46. Baerends, R. J. S., Faber, K. N., Kram, A. M., Kiel, J. A. K. W., van der Klei, I. J., and Veenhuis, M. (2000) *J. Biol. Chem.* **275,** 9986–9995
47. Laemmli, U. K. (1970) *Nature* **227,** 680–685
48. Kyhse-Andersen, J. (1984) *J. Biochem. Biophys. Methods* **10,** 203–209
49. Talcott, B., and Moore, M. S. (1999) *Trends Cell Biol.* **9,** 312–318
50. Shulga, N., Mosammaparast, N., Wozniak, R., and Goldfarb, D. S. (2000) *J. Cell Biol.* **149,** 1027–1038
51. Keizer, I., Roggenkamp, R., Harder, W., and Veenhuis, M. (1992) *FEMS Microbiol. Lett.* **72,** 7–11
52. Dougherty, W. G., and Parks, T. D. (1991) *Virology* **183,** 449–456
53. Hohfeld, J., Veenhuis, M., and Kunau, W. H. (1991) *J. Cell Biol.* **114,** 1167–1178
54. Soukupova, M., Sprenger, C., Gorgas, K., Kunau, W. H., and Dodt, G. (1999) *Eur. J. Cell Biol.* **78,** 357–374
55. Ghaedi, K., Tamura, S., Okumoto, K., Matsuzono, Y., and Fujiki, Y. (2000) *Mol. Biol. Cell* **11,** 2085–2102
56. Titorenko, V. I., and Rachubinski, R. A. (1998) *Trends Biochem. Sci.* **23,** 231–233
57. Mullen, R. T., Lisenbee, C. S., Miernyk, J. A., and Trelease, R. N. (1999) *Plant Cell* **11,** 2167–2185
58. van der Klei, I. J., Hilbrands, R. E., Kiel, J. A. K. W., Rasmussen, S. W., Cregg, J. M., and Veenhuis, M. (1998) *EMBO J.* **17,** 3608–3618
59. Dammai, V., and Subramani, S. (2001) *Cell* **105,** 187–196
60. Gleeson, M. A. G., and Sudbery, P. E. (1988) *Yeast* **4,** 293–303
61. Gietl, C., Faber, K. N., van der Klei, I. J., and Veenhuis, M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 3151–3155