Cytosolic factors mediate protein insertion into the peroxisomal membrane

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Abstract Following in vitro translation of the 22 kDa peroxisomal membrane protein (Pmp22p), gel filtration analysis of the post-ribosomal supernatant revealed that Pmp22p forms two complexes. Complex I is of high molecular weight, results in a crosslinking product of 80 kDa, and by co-immunoprecipitation with anti-TCP1 antibody was identified as TRiC. In complex II Pmp22p was crosslinked to a yet unknown polypeptide of 40 kDa (P40). This complex exhibited much higher efficiency to insert Pmp22p into the peroxisomal membrane compared to complex I. In a model we suggest that newly synthesized Pmp22p is first bound to TRiC before being transferred to P40 which may function as a cytosolic Pmp22p receptor.

Key words: Peroxisomal membrane protein; Membrane insertion; TRiC

1. Introduction

The peroxisomal membrane harbours a variety of activities like enzymes, substrate transporters, receptors, the machineries for membrane protein insertion and matrix protein import, the protein apparatus forming new peroxisomes and components that interconnect peroxisomes to the cytoskeleton [1–4]. Little is known on how all the proteins that participate in these various activities are transported to and inserted into the peroxisomal membrane. Work of Fujiki et al. [5] provided evidence that the major 22 kDa peroxisomal membrane protein (Pmp22p) is synthesized on free polyribosomes and thus is inserted into peroxisomes post-translationally [6]. This mechanism of protein insertion raises questions as to (i) how the extremely hydrophobic polypeptide chains are protected from aggregation during and after synthesis, (ii) how they are transported to and recognized by the target membrane, and (iii) how they are inserted into the membrane. Dyer et al. [7] recently identified in Pmp47p of *Candida bodinii* a consensus motif mediating peroxisomal targeting. Up to now this motif has been found only in some PMPs of yeast and higher eukaryotes. Based on in vitro experiments we recently reported that insertion of Pmp22p and PAF1 proceeds dependent on time and temperature, does not require the hydrolysis of ATP, and involves a proteinaceous component of the peroxisomal membrane. Two steps of the insertion process could be distinguished: binding to and insertion into the membrane [3,8].

In the present paper we address the question how a newly synthesized transmembrane polypeptide chain is kept soluble in the cytosol, a prerequisite for their post-translational transport and insertion. Pmp22p served as the model protein, since previous observations have shown that 70–80% of Pmp22p synthesized by in vitro translation are soluble in a rabbit reticulocyte lysate. Detailed analysis of the lysate revealed that Pmp22p in the post-ribosomal supernatant exists in two polypeptide complexes, one of which could be identified as TCP1 ring complex (TRiC), the eukaryotic homolog of GroEL [9–11]. In the second complex Pmp22p is bound to a single 40 kDa polypeptide (P40).

2. Material and methods

Peroxisomes and mitochondria were isolated from the liver of male Wistar rats treated with clofibrate for 5–10 days. The light mitochondrial fraction of the liver homogenate was further separated on a linear 14–45% (w/v) Nycodenz gradient as described previously [12]. Organelles were recovered by centrifugation and resuspended at a concentration of 10 mg/ml in buffer H (165 mM potassium acetate, 3 mM magnesium acetate, 2.5 mM DTT, 10 mM HEPES–KOH pH 7.4) containing 0.2 mM antipain and 0.02 mM leupeptin. For in vitro insertion [8] 20 μl of purified organelles were incubated for 1 h at 26°C with 200 μl of the appropriate fraction obtained by gel filtration of the translation mixture.

In vitro transcription/translation was performed as described [8] using a commercial preparation of the rabbit reticulocyte lysate (Promega, Heidelberg, Germany) for translation. After removal of the ribosomes by high-speed centrifugation the supernatant was separated by gel filtration on a 10/30 Superdex G-200 column (Pharmacia, Freiburg, Germany) equilibrated with buffer H containing 80 mM potassium acetate. For in vitro transcription/translation in the *E. coli* T7/S30 extract (Promega, Heidelberg, Germany) Pmp22p-cDNA was subcloned into the pET-11a vector (Stratagene, Heidelberg, Germany).

Crosslinking of polypeptides to Pmp22p was carried out by using the hetero-bifunctional reagents, SPDP (*N*-succinimidyl 3′-(2-pyridyldithio) propionate) or Sulfo-MBS (meta-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester; Pierce, Germany) and added in concentrations of 32 μM (SPDP) or 23 μM (Sulfo-MBS) to the polypeptide complexes. Incubations (500 μl final volume) were done for 90 min (SPDP) or 10 min (Sulfo-MBS) at 4°C. Reactions were terminated by the addition of excess Tris-HCl, pH 7.4, or Tris-HCl and DTT (Sulfo-MBS) and the polypeptides were analysed by SDS-PAGE (6–20% gradient gel) and autoradiography.

Microsequencing was performed following Western blotting (Fluorotrans PALL, Portsmouth, UK), trypsinisation and separating the trypsin fragments by HPLC [13]. Two fragments were analysed exactly matching the GroEL sequences DTTTIIDGVG and AAVEEGVVAG.

The following antisera were used for immunoprecipitation: rabbit anti-Pmp22p [8], rat anti-TCP1 (Biomol, Hamburg, Germany) and rabbit anti-GroEL, anti-DnaK, anti-DnaJ, kindly provided by Dr. H. Bujard (ZMBH, Heidelberg, Germany). Variable concentrations of the antibodies were pre-adsorbed onto protein A or protein G Sepharose and added to the polypeptides.

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3. Results

3.1. Pmp22p forms two post-translational complexes

We synthesized Pmp22p by in vitro transcription/translation from its cDNA [14]. Following high-speed centrifugation of the translation mixture 70–80% of the synthesized Pmp22p were recovered from the supernatant in highly soluble form, whereas 20–30% were found in the pellet which mainly consists of ribosomes. One likely reason for the solubility of newly synthesized Pmp22p is that the polypeptide during and/or after translation associates with cytosolic constituents of the reticulocyte lysate. Therefore we analysed the supernatant by gel filtration. As shown in Fig. 1, Pmp22p was eluted from the gel filtration column within two peak fractions corresponding to apparent molecular weights of >400 kDa (complex I) and ≈ 60 kDa (complex II), respectively.

3.2. Insertion competence of the Pmp22p complexes

In order to test the insertion competence of both Pmp22p complexes, we incubated equal amounts of Pmp22p present in complex I and II with isolated rat liver peroxisomes as described previously [3,8]. The subtilisin-resistant portion of Pmp22p bound to peroxisomes was taken as correctly inserted polypeptide. According to this criterium both complexes were competent. However, complex II was about 3–4 times more efficient for Pmp22p insertion than complex I (Fig. 2A,B). Insertion was specific for peroxisomes and was not observed with isolated mitochondria.

3.3. In complex I Pmp22p is associated with TRiC

Unlike translation in the rabbit reticulocyte lysate, where 80% of the synthesized Pmp22p was soluble, synthesis in an *E. coli* in vitro translation system resulted in only about 10% soluble polypeptide. The vast majority of the synthesized Pmp22p was recovered from the pellet fraction. Gel chromatographic analysis of the soluble portion demonstrated that Pmp22p was contained in a single high molecular weight complex strikingly similar in its chromatographic behaviour to complex I of the reticulocyte lysate. A second peak corresponding to complex II was not detected in the *E. coli* system. Silver staining of the polypeptides contained in the *E. coli* translation mixture and separated by gel filtration revealed co-elution of the newly synthesized Pmp22p with a 60 kDa polypeptide (Fig. 3A,B). By microsequencing we identified the polypeptide as the chaperonin GroEL, the *E. coli* Hsp60 homolog. The direct association of Pmp22p with GroEL was demonstrated by co-immunoprecipitation of Pmp22p using a polyclonal anti-GroEL antiserum (Fig. 3C). Antibodies directed against DnaK and DnaJ, the *E. coli* Hsp70 and Hsp40 homologs, were unable to co-immunoprecipitate Pmp22p.

The eukaryotic, cytosolic equivalent of GroEL is the hetero-oligomeric TCP1 ring complex, TRiC. Therefore, we assumed that the complex I of the reticulocyte lysate represents Pmp22p associated with TRiC. This assumption was substantiated by co-immunoprecipitation of Pmp22p with a monoclonal anti-TCP1 antibody (Fig. 4A,B). Using SPDP as a

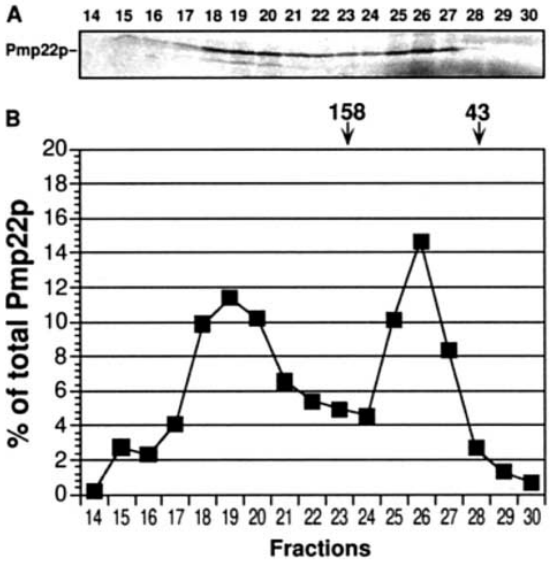

Fig. 1. Gel filtration analysis of the reticulocyte lysate post-ribosomal supernatant after in vitro translation of Pmp22p. The post-ribosomal supernatant of the lysate (300 μl) is separated by Superdex G-200 chromatography. A: Aliquotes of the fractions obtained by gel filtration were analysed by SDS-PAGE and autoradiography. Pmp22p is eluted within two peak fractions corresponding to molecular weights of >400 kDa (fractions 18–20) and 60 kDa (fractions 25–27). B: Quantitative evaluation of the autoradiography shown in (A). The peak fractions of two molecular weight standards (ovalbumin, 43 kDa and aldolase, 158 kDa) are indicated by arrows.

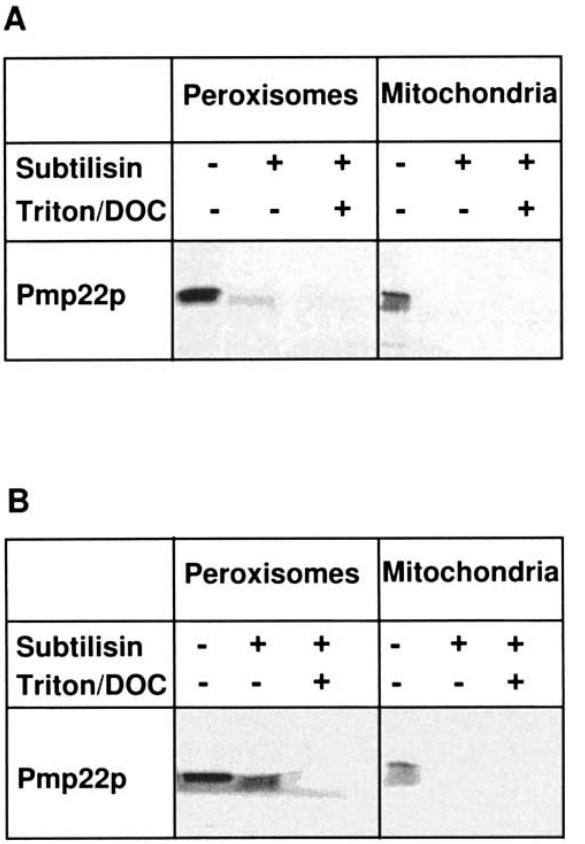

Fig. 2. Comparison of the efficiencies of complex I (A) and complex II (B) for the insertion of Pmp22p into peroxisomal membranes. Equal amounts of newly synthesized Pmp22p present in complex I and complex II (60 000 dpm) were incubated with isolated rat liver peroxisomes (200 μg) as outlined in Section 2. Mitochondria (200 μg) were used as controls demonstrating the peroxisomal specificity of the insertion.

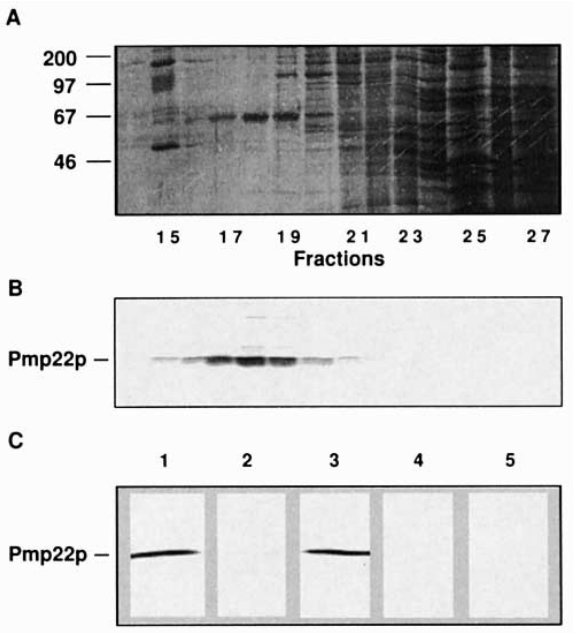

Fig. 3. Characterization of the protein complex formed after in vitro translation of Pmp22p in the *E. coli* S30 extract. The S30 post-ribosomal supernatant was separated by gel filtration and fractions were analyzed for total protein by silver staining (A) and for newly synthesized Pmp22p by autoradiography (B). C: Co-immunoprecipitation of Pmp22p from the pooled fractions 17–19, obtained by gel filtration, using anti-GroEL antiserum (lane 3). Pmp22p immunoprecipitated by a polyclonal anti-Pmp22p antiserum is shown in lane 1 and by control serum in lane 2. Antisera directed against DnaK (lane 4) and DnaJ (lane 5) were unable to co-immunoprecipitate Pmp22p.

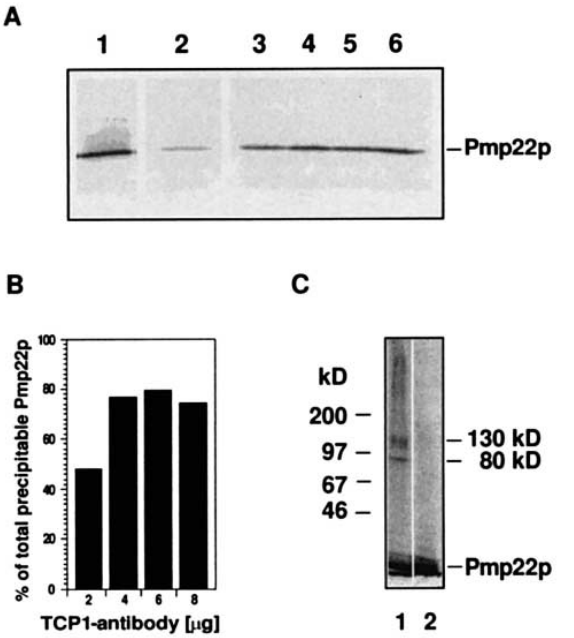

Fig. 4. Characterization of complex I formed after in vitro translation of Pmp22p in the rabbit reticulocyte lysate. A: Co-immunoprecipitation using increasing concentrations of monoclonal rat anti-TCP1 antibody is shown in lanes 3–6. Pmp22p immunoprecipitated by the polyclonal anti-Pmp22p antiserum and by control antiserum (rat IgG) is shown in lanes 1 and 2, respectively. B: Quantitative evaluation of the TCP1 co-immunoprecipitation. C: In the pooled fractions 18–20 (complex I), obtained by gel filtration of the lysate, Pmp22p is crosslinked to polypeptides of 80 and 130 kDa using the hetero-bifunctional reagent SPDP (1). In the control (2) the reagent was omitted.

hetero-bifunctional chemical crosslinker, Pmp22p present in complex I formed two crosslink products of 80 and 130 kDa (Fig. 4C) suggesting close association of Pmp22p with one or two TRiC subunits.

3.4. In complex II Pmp22p is associated with a 40 kDa polypeptide

To further characterize the composition of complex II, again chemical crosslinking was applied using the hetero-bifunctional, water soluble reagent Sulfo-MBS which provides crosslinking by both a sulfhydryl- and an amino-reactive site. Since Pmp22p does not contain cysteine residues, the appearance of Pmp22p crosslink products indicate formation of a hetero-oligomeric rather than homo-oligomeric complex. As shown in Fig. 5, Sulfo-MBS crosslinking of complex II resulted in a single product of 60 kDa suggesting Pmp22p to be associated with a single 40 kDa polypeptide.

4. Discussion

In the present study we address the question of post-translational organisation of the extremely hydrophobic peroxisomal membrane protein, Pmp22p. By using in vitro systems for synthesis and post-translational insertion of Pmp22p we provide evidence that after synthesis Pmp22p forms two cytosolic complexes. Whereas complex I has been identified as associate of Pmp22p with the cytosolic chaperonin TRiC, in complex II Pmp22p appears tightly bound to a single 40 kDa polypeptide, P40.

TRiC, the GroEL equivalent of the eukaryotic cytoplasm, has been demonstrated to exert rather high substrate specific-

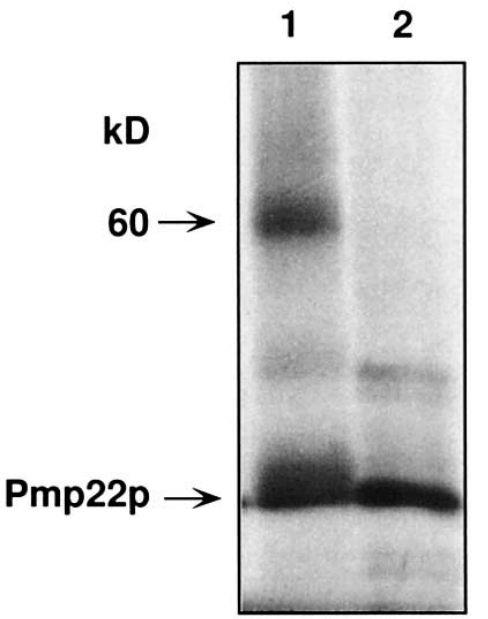

Fig. 5. Crosslinking of Pmp22p of complex II to a 40 kDa polypeptide. The pooled fractions 25–27, obtained by gel filtration of the reticulocyte lysate translation mixture, were treated with the hetero-bifunctional reagent Sulfo-MBS resulting in a crosslinking product of 60 kDa (1). In the control (2) the reagent was omitted.

ity. To date only a small subset of polypeptides, such as actin and tubulin, have been shown to require TRiC for folding in vivo [14,15]. In a recent study Frydman et al. [16] demonstrated that a peroxisomal enzyme, firefly luciferase, is also folded in vitro by TRiC. The mechanism proposed includes the coordinated activity of TRiC with Hsp70/Hsp40. These chaperones first bind to the nascent chain, and once the polypeptide chain has reached the length of about 250 residues the growing chain is transferred to TRiC where folding is completed [16]. In analogy to this model we favour a similar mechanism for the stepwise maturation of Pmp22p. Since the molecule consists of 194 amino acid residues, its transfer to TRiC may be preceded by other chaperone interactions which may include Hsp70/Hsp40 or the ribosome itself. Interaction of Pmp22p with TRiC will subsequently mediate the formation of complex II which presumably targets Pmp22p to a peroxisomal membrane receptor [3,8]. Alternatively, the newly synthesized Pmp22p, bypassing TRiC, may directly form complex II. In that case the observed interaction of Pmp22p with TRiC may reflect a more unspecific binding possibly triggered by a limited amount of P40 in the reticulocyte lysate. The observed substrate specificity of TRiC, however, may argue against this view.

Insertion competence is predominantly confined to complex II, as shown by the in vitro assay (Fig. 2). Therefore, a direct delivery of Pmp22p from TRiC to the peroxisomal membrane seems to be rather unlikely, as the release of substrate from TRiC is dependent on the hydrolysis of ATP [17] which is not required for the insertion of Pmp22p [3,8].

Preliminary attempts to identify P40 by immunological means were unsuccessful. Although Pmp22p could be immunoprecipitated from the native complex II with a polyclonal anti-Pmp22p antiserum, no co-immunoprecipitation was detected using anti-DnaK, anti-DnaJ or anti-Hsp40 antibodies indicating that the protein does not belong to these classes of chaperones. The work of several laboratories has shown that the import of peroxisomal matrix proteins is mediated by cytosolic receptors, such as, Pex5p and Pex7p [18–22]. P40 might function in a way similar to these receptors.

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