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Oligomeric complexes involved in translocation of proteins across the membrane of the endoplasmic reticulum

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Abstract Proteins involved in protein translocation across the membrane of the endoplasmic reticulum assemble into different oligomeric complexes depending on their state of function. To analyse such membrane protein complexes we fractionated proteins of mammalian rough microsomes and analysed them using blue native PAGE and immunoblotting. Among the proteins characterised are the Sec61p complex, the oligosaccharyl transferase (OST) complex, the translocon-associated protein (TRAP) complex, the TRAM and RAMP4 proteins, the signal recognition particle (SRP) and the SRP receptor (SR). Interestingly, the RAMP4 protein, SR and OST complex display more than one oligomeric form.

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Key words: Endoplasmic reticulum; Blue native PAGE; Protein targeting; Protein translocation

1. Introduction

Translocation of proteins across the membrane of endoplasmic reticulum (ER) involves targeting and translocation components [1,2]. Targeting is initiated when a signal sequence of a nascent polypeptide is recognised by the 54 kDa protein of the signal recognition particle (SRP), SRP54. SRP54 then targets the ribosome nascent chain complex to the ER membrane through its interaction with the SRP receptor (SR) [1,3]. SRP54 and the two subunits of the SR, SRα (70 kDa) and SRβ (30 kDa), are GTPases [4–6]. In their GTP bound forms SRP54 and SR form a tight complex resulting in the release of the signal sequence from SRP54 and insertion of the nascent chain into the translocon [7,8]. When GTP is hydrolysed, SRP dissociates from its receptor.

The core component of the translocon is the Sec61p complex which consists of three subunits, α, β and γ [2,9]. An evolutionally related complex is also found in bacteria. Low-resolution structures of the purified Sec61p complex have revealed a ring structure of 85 Å in diameter with a central hole of 20 Å [10]. When the ribosome-Sec61p complex was analysed by cryo-electron microscopy, a similar ring structure with a funnel-shaped pore in the centre was found for the Sec61p complex. Furthermore the central pore was aligned with the putative polypeptide exit tunnel in the large ribosomal subunit [11]. The calculation of the molecular volume of the Sec61p complex suggests that it can accommodate two Sec61p trimers. Fluorescence quenching studies have shown that the size of the translocation pore may undergo changes during translocation [12]. Furthermore it was suggested that the translocon may be able to adopt different sizes in response to different proteins being inserted or translocated [13].

Other proteins involved in translocation are the translocating chain-associated membrane protein (TRAMp) that has been implicated in the regulation of translocation [2,14], the oligosaccharyl transferase (OST) complex that transfers oligosaccharides from dolichol onto the nascent polypeptide chain [15], the TRAP (translocon-associated protein) complex with its α, β, γ and δ subunits [16], the small ribosome-associated membrane protein 4 (RAMP4) [17], and the signal peptidase complex [18]. Proteins which remain associated with membrane bound ribosomes after their solubilisation from rough microsomes (RM) with digitonin are the Sec61p complex, the OST complex, the TRAP complex and the RAMP4 protein [9].

To understand the coordinated functioning of proteins involved in protein targeting and translocation we characterised membrane protein complexes of the endoplasmic reticulum by blue native polyacrylamide gel electrophoresis (PAGE), which allows the separation of native membrane protein complexes [19,20]. With this system the multisubunit complexes of the oxidative phosphorylation system of mitochondria could be separated at a higher resolution than by the conventional sucrose density gradients or gel filtration. Similarly the protein import machinery complexes of both mitochondria outer [21] and inner membranes [22] have been characterised using this method. Blue native PAGE has also been shown to allow a fairly reliable assessment of the molecular masses of protein complexes [20]. Here we report on the characterisation of protein complexes of the ER targeting and translocation machinery by blue native PAGE.

2. Materials and methods

Serva blue G, Bis-Tris, acrylamide and bisacrylamide were from Serva, Sigma and AppliChem respectively. High molecular weight electrophoresis calibration kit was from Pharmacia. Digitonin purchased from Merck was further purified as described previously [17]. General chemicals were from Merck or Sigma.

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Abbreviations: ER, endoplasmic reticulum; RM, rough microsomes; PKRM, puromycin/high salt treated RM; RAMP, ribosome-associated membrane proteins; SRP, signal recognition particle; SR, SRP receptor; TRAMp, translocating chain-associated membrane protein; OST, oligosaccharyl transferase; TRAP, translocon-associated protein; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence

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2.1. Fractionation of RM proteins

Rough microsomes (RM) were prepared as described by Walter and Blobel (1983) [23]. For direct solubilisation (T fraction), RM were adjusted to a concentration of 0.5 eq/μl in solubilisation buffer (S) (20 mM Tris-HCl pH 7.6, 5 mM $Mg(OAc)_2$, 2 mM DTT, 10 μg/ml chymostatin/leupeptin/aprotinin/pepstatin, 12% glycerol) containing 2% digitonin and 300 mM NaCl. NaCl was used to replace the conventionally used KOAc throughout, because potassium ions are not compatible with blue native PAGE. The mixture was incubated on ice for 20 min, centrifuged at $10\,000\times g$ for 10 min to remove aggregates and then further centrifuged at $400\,000\times g$ for 1 h. The supernatant (T fraction) contains both the lumenal proteins and those membrane proteins which are not tightly associated with translating ribosomes. The pellet contains ribosomes and ribosome-associated membrane proteins.

To prepare the lumenal protein fraction (L fraction), RM were permeabilised in S buffer containing 0.2% digitonin and 50 mM NaCl at a concentration of 0.5 eq/μl. The mixture was centrifuged at $300\,000\times g$ for 40 min. The supernatant contains the majority of the lumenal proteins. The pelleted membrane fraction was resuspended in S buffer at 0.5 eq/μl containing 2% digitonin and 300 mM NaCl and incubated on ice for 20 min. Materials not solubilised and aggregates were removed by centrifugation at $10\,000\times g$ for 10 min. The ribosomes and ribosome-associated membrane proteins were then pelleted by centrifugation at $400\,000\times g$ for 1 h. The resulting supernatant (M fraction) contains the membrane proteins which are not tightly associated with translating ribosomes. The ribosomes and ribosome-associated membrane proteins were resuspended at a concentration of 1 eq/μl in a puromycin/GTP buffer (100 mM HEPES-NaOH pH 7.8, 15 mM $Mg(OAc)_2$, 150–1000 mM NaCl, 5 mM DTT, 10 μg/ml chymostatin/leupeptin/aprotinin/pepstatin, 15% glycerol, 2 mM puromycin, 2 mM GTP and 3% digitonin). The mixture was incubated at 25°C for 60 min and centrifuged at $400\,000\times g$ for 90 min to pellet the ribosomal subunits. The supernatant contains the ribosome-associated membrane proteins (RAMP fraction). We found removal of the ribosomes to be important for the subsequent blue native PAGE analysis.

2.2. Guanine nucleotide-dependent reaction

To test SRP binding to the ER membrane components, puromycin/high salt treated RM (PKRM) were prepared as described by Hauser et al. [24]. SRP was purified from RM as described by Walter and Blobel [25]. The incubation of RM with 500 μM GMPPNP or GDP was performed at 150 mM KOAc, and the membranes were solubilised in the presence of the respective nucleotide. The incubation of PKRM with purified SRP in the presence of GMPPNP or GDP was performed as described previously [24]. After incubation, the salt concentration was raised to 500 mM with concentrated KOAc and the membranes were then pelleted through a high salt sucrose cushion. The pelleted membranes were then solubilised with 1% digitonin and 150 mM NaCl.

2.3. Antibodies

The antibodies against the α, β and γ subunits of Sec61p, SRα, SRP54, and TRAMp were made by S. Hauser and K. Schröder against the peptides described previously [9,17]. The antibodies against SRβ were made by S. Hauser against amino acid residues 196–209 of SRβ. The antibodies against RAMP4 were made by B. Martoglio against the amino acid 1–34 of RAMP4. The anti-TRAPα antibodies were gifts from Dr. T. Rapoport, anti-OST48 and anti-ribophorin I antibodies were from Dr. G. Kreibich and anti-BiP antibodies were from Dr. I. Haas.

2.4. Blue native PAGE

Blue native PAGE and 2-dimensional SDS-PAGE analysis was performed largely as described by Schägger and von Jagow [19,20]. Before application to the gel, the salt and detergent concentration of the sample were adjusted below 200 mM and 2% respectively with S buffer. Single bands were excised from the blue native gel, soaked briefly in 1 mM DTT solution and then applied to a 8–18% linear gradient Laemmli gel [26]. After completion of the run, gels were subjected to either silver/Coomassie brilliant blue staining or Western blotting. Silver staining was performed according to Heukeshoven et al. [27]. For mass spectrometry, the Coomassie blue stained bands of a Laemmli gel were excised and digested with trypsin. The tryptic fragments were analysed by mass spectrometry and the collected data were used to deduce the identity of the protein. For Western blotting analysis of blue native gels, we used a transfer buffer (20 mM Tris, 150 mM glycine, 0.04% SDS, 20% methanol) and PVDF membranes. Immunoblotting was performed according to Harlow and Lane [28]. In the case of SRα detection, the blotted membrane was destained in a solution of 40% methanol and 10% acetic acid for 15 min to remove the Coomassie blue and then washed three times (15 min each) in phosphate buffered saline solution containing 0.05% Tween-20 prior to incubation with the antisera. The immunoreactive proteins were visualised using the enhanced chemiluminescence system (ECL, Boehringer).

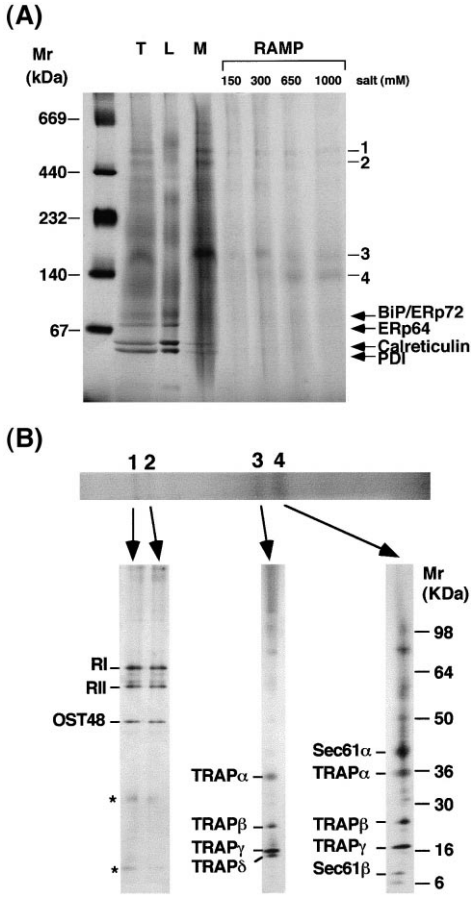

Fig. 1. A: Coomassie blue staining of RM protein complexes separated by blue native PAGE. RM were fractionated and protein complexes separated by blue native PAGE and stained with Coomassie blue. T: RM proteins after solubilisation of RM with digitonin-containing buffer and removal of ribosomal complexes; L: lumenal proteins of RM; M: RM membrane proteins after removal of lumenal proteins and ribosomal complexes; RAMP: ribosome-associated membrane proteins released from ribosomes with puromycin at three different salt concentrations. Amount loaded in each lane corresponds to 10 eq of RM. Major protein complexes seen in the RAMP fraction are labeled 1–4. The identified proteins in the lower part of the gel are also indicated. Molecular weight markers: thyroglobulin 669 kDa, ferritin 440 kDa, catalase 232 kDa, lactose dehydrogenase 140 kDa, bovine serum albumin 67 kDa. B: Separation of RAMP protein complexes 1 to 4 by SDS-PAGE. Protein bands 1 to 4 from the RAMP fraction were excised from a blue native gel as shown in A and separated by SDS-PAGE. Proteins indicated are either identified by mass spectrometry: TRAPα, β, γ and δ subunits of the TRAP complex; or specific antibodies: RI, ribophorin I; RII, ribophorin II; OST48, 48 kDa protein of the oligosaccharyl transferase; Sec61α, β: subunits of the Sec61p complex. Additional proteins of the OST complex whose identities were not determined are indicated (*).

3. Results

3.1. Membrane protein complexes of the ER characterised by blue native PAGE

For the characterisation of membrane protein complexes from the rough ER by blue native PAGE, we used rough microsomes (RM) prepared from canine pancreas. RM contain integral membrane proteins, lumenal proteins, and proteins peripherally associated with the membrane. Ribosomes are associated on the cytoplasmic side of the membrane and interact with the membrane either via electrostatic interactions alone or in addition via a nascent chain that traverses the membrane. Membrane proteins engaged in translocation can be found in the ER membrane in association with ribosomes or in a free form. Proteins that can be found in tight association with translating ribosomes and in a free form are the Sec61p, OST and TRAP complex [29]. Components such as TRAM protein and SR are not associated tightly with ribosomes. To characterise protein complexes involved in protein translocation we fractionated RM and separated the proteins by blue native PAGE. The following fractions were used: (a) total RM proteins (T) that were solubilised in 2% digitonin, 300 mM NaCl and depleted of ribosomes and associated components; (b) lumenal ER proteins (L) that were released from RM by treatment with 0.2% digitonin and 50 mM NaCl; (c) RM membrane proteins (M) obtained after removal of RM lumenal proteins, ribosomes and associated components; (d) ribosome-associated membrane proteins (RAMP) that were released from digitonin solubilised ribosomal complexes with puromycin at different salt concentrations.

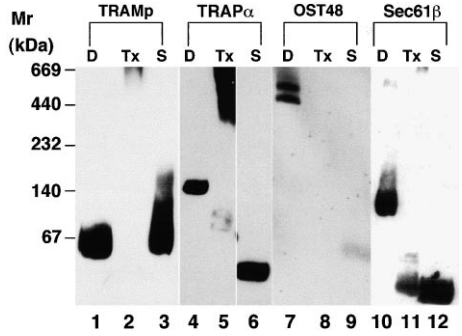

Fig. 3. Analysis of TRAM protein and OST, TRAP and Sec61p complexes by blue native PAGE. RM membrane proteins were solubilised in digitonin (D). Triton X-100- (T) or SDS- (S) containing buffers as described in the legend to Fig. 2A and analysed by blue native PAGE and immunoblotting. Amounts analysed with anti-OST48 and anti-TRAPα antibodies were 5 eq for D and T and 3 eq for S; with anti-TRAMp antibody 2 eq for D and T and 1 eq for S.

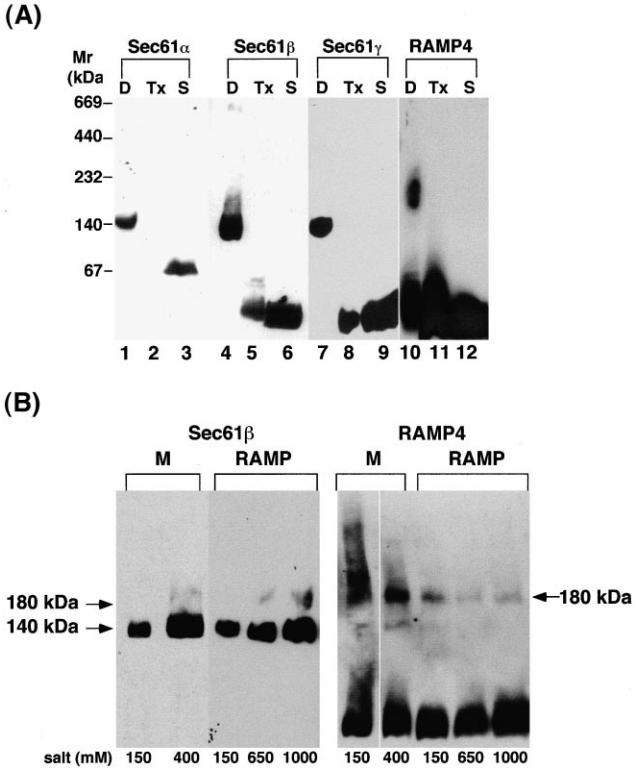

Fig. 2. The Sec61p complexes and RAMP4 analysed by blue native PAGE. A: RM proteins were solubilised with three different detergents, 1% digitonin, 150 mM NaCl (D), 1% Triton X-100, 250 mM NaCl (T) or 2% SDS buffer (S) and ribosomal complexes removed by centrifugation. Proteins in the supernatant were separated by blue native PAGE and further characterised by immunoblotting with antibodies against Sec61α, Sec61β, Sec61γ and RAMP4. The following amounts were loaded: with anti-Sec61α and Sec61β antibodies: 3 eq for D and T and 2 eq for S; with anti-Sec61γ and anti-RAMP4 antibodies: 5 eq for D and T and 3 eq for S. B: Comparison of Sec61p complexes in the M fraction of RM and in the RAMP fraction. RM were solubilised with 1% digitonin and 150 mM or 400 mM NaCl and ribosomal complexes removed by centrifugation. To obtain RAMP, RM were solubilised with 2% digitonin, 400 mM NaCl and ribosomal complexes pelleted. RAMP were then released from ribosomes with puromycin at the salt concentrations indicated in the figure. Amounts analysed by blue native PAGE were 3 eq (anti-Sec61β antibodies) and 5 eq (anti-RAMP4 antibodies) respectively.

The four RM fractions were separated by blue native PAGE and proteins visualised by Coomassie blue staining (Fig. 1A). Generally a diffuse pattern and some distinct bands are seen for the proteins in the T, L and M fractions. This is indicative of a complex protein pattern with some abundant protein complexes. In the T and L fractions, four distinct, sharply resolved bands can be seen in the lower part of the gel (Fig. 1A). Using 2-dimensional SDS-PAGE analysis and immunoblotting (data not shown) the bands were identified as BiP/ERp72, ERp64, calreticulin and protein disulphide isomerase (PDI) respectively. Some less sharply resolved bands can be seen in the high molecular mass range of the gel. In contrast to the T, L and M fraction, the RAMP show a more distinct protein pattern. Four major bands representing complexes with apparent molecular masses of 480 kDa, 450 kDa, 150 kDa and 140 kDa (bands 1, 2, 3, 4) can be distinguished. Protein complexes of similar electrophoretic mobility are also present in the M and T fractions but not in the L fraction, suggesting that they represent integral membrane protein complexes. The sizes and intensities of bands 1, 2 and 3 in the RAMP fraction do not change significantly when the salt concentration during their dissociation from the ribosome is increased. It appears that the amount of complex 4 increases with increasing salt concentration (Fig. 1A).

To characterise the subunit composition of the RAMP complexes we analysed proteins in the four bands by 2-dimensional SDS-PAGE (Fig. 1B). Where indicated, subunit proteins were identified by mass spectrometry and immunoblotting (data not shown). Bands 1 and 2 from the RAMP fraction were found to contain ribophorin I (65 kDa), ribo-

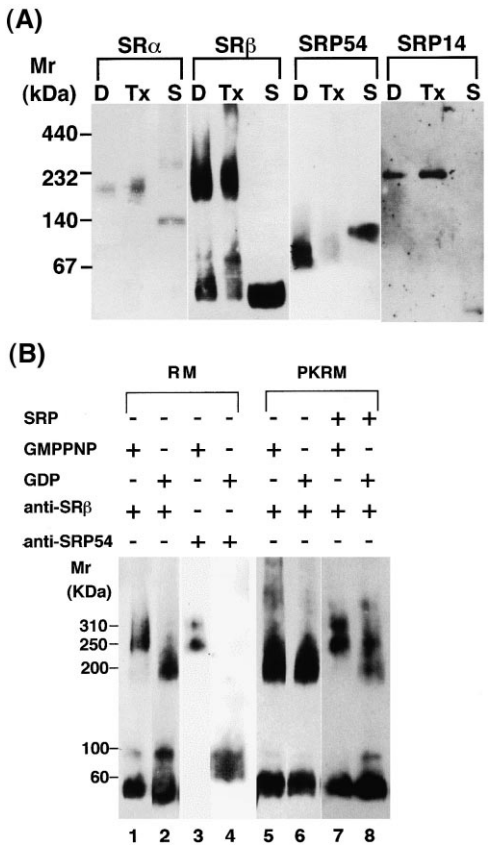

Fig. 4. A: Analysis of SR, SRP and SR/SRP complexes by blue native PAGE. SR and SRP complexes probed with anti-SRα, SRβ, SRP54 and SRP14 antibodies. RM membrane proteins or purified SRP were solubilised in digitonin (D), Triton X-100- (T) or SDS- (S) containing buffers as described in the legend to Fig. 2A and analysed by blue native PAGE and immunoblotting. Amounts analysed with anti-SRα anti-SRP54 and anti-SRP14 antibodies were 5 eq for D and T and 3 eq for S; with anti-SRβ antibody 2 eq for D and T and 1 eq for S. B: GMPPNP-dependent complex formation between SR and SRP (SRP54). RM, PKRM or PKRM and SRP were incubated with 500 μM GMPPNP or GDP at 150 mM salt. Membranes were pelleted through a high salt cushion and membrane proteins solubilised in 1% digitonin, 150 mM NaCl in the presence of the respective guanine nucleotide. After removal of ribosomal complexes proteins were analysed by blue native PAGE and immunoblotted with anti-SRβ or anti-SRP54 antibodies. Amounts analysed with anti-SRβ antibody are 2 eq and with anti-SRP54 antibody 5 eq. Calculated molecular masses of protein complexes identified by the antibodies are indicated on the left.

phorin II (63 kDa) and OST48 (48 kDa), suggesting that they represent two distinct oligomeric forms of the OST complex. Proteins of about 10 kDa and 30 kDa in the larger OST complex have not yet been identified. Protein subunits contained in band 3 of the RAMP fraction were identified as TRAPα (34 kDa), TRAPβ (22 kDa), TRAPγ (20 kDa) and TRAPδ (18 kDa) (Fig. 1B). In band 4 the α and β subunits of the Sec61p complex and also some of the subunits of the TRAP complex were identified.

3.2. Characterisation of RM protein complexes by blue native PAGE and immunoblotting

To more directly characterise the membrane protein complexes in RM, we used immunoblotting with specific antibodies against known targeting and translocation components. We also investigated the effect of different detergents on the solubilisation, separation and integrity of the protein complexes. We used three different detergents, digitonin, Triton X-100 and SDS. Digitonin at physiological salt concentration was chosen as a mild condition to maintain oligomeric complexes of the translocation machinery. Digitonin has previously been shown to solubilise membrane components of RM (Sec61p complex, SR and TRAMp) in such a way that they could functionally be reconstituted into liposomes [17]. Triton X-100 is also a mild detergent, however, it is known to partially disassemble some membrane protein complexes such as the Sec61p complex. SDS would be expected to dissociate membrane protein complexes and denature the constituent subunits.

Total RM were solubilised with digitonin-, Triton X-100- or SDS-containing buffers, ribosomes were removed by centrifugation and proteins separated by blue native PAGE. Translocation components were identified by Western blotting with specific antibodies. The Sec61p complex was identified with antibodies against its known subunits, Sec61α, Sec61β and Sec61γ. As can be seen in Fig. 2A (lane 1), Sec61α solubilised in digitonin buffer migrates in a protein complex of apparent molecular mass of 140 kDa. The same mobility is observed for Sec61β and Sec61γ (Fig. 2A, lanes 1, 4 and 7). When Triton X-100 was used to solubilise protein complexes, Sec61α could not be detected in the separating gel. Sec61β and Sec61γ migrate as small molecular weight proteins consistent with their monomeric state (Fig. 2A, lanes 5 and 8). After SDS solubilisation Sec61α migrates with an apparent molecular mass of 50 kDa. Sec61β and Sec61γ migrate as small molecular weight proteins.

The small ribosome-associated membrane protein RAMP4 has been found to cofractionate in various amounts with the Sec61p complex [17]. After separation by blue native PAGE we find digitonin solubilised RAMP4 migrating at two positions on the separating gel: a large proportion migrates as an apparent monomer at the same position as the Triton X-100 and SDS solubilised RAMP4. However, a small amount is found in an oligomeric complex of 180–200 kDa, clearly larger than the Sec61p complex (see Fig. 2B, cf. lanes 1, 4, 7, with 10). No RAMP4-containing higher molecular weight complexes are seen when Triton X-100 or SDS are used for the solubilisation (Fig. 2A, lanes 11 and 12).

We next compared the Sec61p and RAMP4 complexes of the M fraction with those of the RAMP fraction using digitonin solubilisation at different salt concentrations (Fig. 2B). The Sec61p complex was detected by Western blotting with antibodies against Sec61β. As can be seen in Fig. 2B, the apparent size of the major form of the Sec61p complex (140 kDa) of the M and the RAMP fraction is the same at all salt concentrations used. A small amount of the Sec61p complex ($<5\%$ of total) from the M and RAMP fraction can also be found in a higher molecular weight complex of around 180 kDa. As the M fraction mainly contains unengaged Sec61p complexes and the RAMP fraction contains those engaged in protein translocation, our data suggest that the core Sec61p complexes from the two fractions are of the same size.

We similarly compared RAMP4 from the M fraction with that of the RAMP fraction. In both fractions, RAMP4 is found mainly in its apparent monomeric form (Fig. 2B). However, some of the RAMP4 molecules of the RAMP fraction can also be found in a complex of about 180 kDa, in partic-

ular when released at low salt concentration. In the M fraction there may be more than one form in the higher molecular weight complexes when solubilisation is carried out at 150 mM salt (Fig. 2B). In contrast to the higher molecular weight form of the Sec61p complex, the amount of the high molecular weight RAMP4 complex is reduced with increasing salt concentration, suggesting that these two complexes are not the same.

3.3. Other ribosome- or translocon-associated membrane protein complexes

Other proteins which are found associated with membrane bound ribosomes or the translocon are the TRAM protein, the TRAP complex and the OST complex. These proteins or complexes were identified in RM (T fraction) after solubilisation with digitonin, Triton X-100 or SDS. Antibodies against the TRAM protein recognised a 60 kDa protein in the digitonin and SDS solubilised RM proteins (Fig. 3, lanes 1 and 3). The Triton X-100 solubilised TRAM protein apparently does not enter the gel. Identification of the TRAP complex with anti-TRAPα antibodies revealed a complex of about 150 kDa in digitonin solubilised RM, high molecular weight aggregates in Triton X-100 solubilised RM and a small protein after SDS solubilisation. This suggests that the TRAP complex can be solubilised with digitonin in a homogeneous form. The digitonin solubilised TRAP and Sec61 complexes can be clearly distinguished by their different sizes, 150 kDa and 140 kDa respectively. Antibodies against OST48 identify two sharply separated OST complexes in digitonin solubilised RM with 480 and 450 kDa (Fig. 3, lane 7). Like the TRAM protein the Triton X-100 solubilised OST48 does not enter the gel. When digitonin solubilised RAMP proteins instead of the T fraction of RM proteins were probed with antibodies against TRAPα, OST48 or TRAMp, the same size complexes as found in the T fraction were revealed (data not shown).

3.4. Oligomeric forms of the SRP receptor (SR)

To see whether the SR complex and SRP can be characterised by blue native PAGE, we probed digitonin, Triton X-100 and SDS solubilised RM proteins and SRP after Western blotting with antibodies against SRα, SRβ, SRP54 and SRP14. Anti-SRα antibodies reacted with a complex of about 200 kDa when digitonin or Triton X-100 solubilised RM proteins were used in the analysis (Fig. 4A). After SDS denaturation, anti-SRα antibodies recognised a protein migrating with an apparent molecular mass of 140 kDa. When analysed by SDS-PAGE SRα migrates as a 70 kDa protein [30] consistent with its calculated monomeric molecular weight. Thus the migration of SRα in blue native gels may not reflect the true molecular mass of the SRα protein. Anti-SRβ antibodies recognised among digitonin solubilised RM proteins a 200 kDa protein complex, suggesting this to be the oligomeric SR complex. Furthermore, anti-SRβ antibodies recognised small amounts of 60 kDa and 100 kDa proteins. Among the SDS solubilised RM proteins anti-SRβ antibodies recognised a protein of small molecular weight. Taken together these data confirm previous findings that SR occurs in a heterodimeric form consisting of SRα and SRβ and that SRβ can also exist in a free form [31,32].

To identify SRP in blue native gels we used antibodies to two of the SRP proteins, SRP54 (54 kDa) and SRP14 (14 kDa). SRP is a ribonucleoprotein particle assembled from SRP 7S RNA and six different polypeptides of 9, 14, 19, 54, 68 and 72 kDa. SRP54 binds to the central part of SRP RNA while SRP14 binds to the so-called Alu domain of SRP formed by the 3′ and 5′ regions of SRP RNA. The calculated molecular weight of SRP is about 310 kDa [3]. Purified SRP was separated on blue native gels and after Western blotting probed for SRP54. Independent of the detergent used, a protein with a molecular weight of around 60 kDa, consistent with the monomeric form of SRP54 was detected (Fig. 4A). SRP14 was found in digitonin- and Triton X-100-containing samples in a complex of about 230 kDa. In SDS-containing buffer SRP14 is revealed as a small molecular weight protein. This indicates that SRP54 is released from SRP in the Coomassie blue-containing buffer whereas SRP14 and probably some other SRP proteins remain associated with the 7S RNA.

SR is known to interact with SRP54 of SRP and also free SRP54 in a GTP-dependent manner. In the presence of the non-hydrolysable GTP analogue GMPPNP, a tight complex between SRα and SRP54 of SRP is formed [24,8]. In the presence of GDP, SRP is released from its receptor. To examine the GTP dependence of the complex formation between SR and SRP, we incubated RM with GMPPNP or GDP and identified SR/SRP54 complexes with anti-SRβ and anti-SRP54 antibodies after Western blotting of blue native gels. As revealed by the anti-SRβ antibody, in the presence of GDP SR migrates as a complex with apparent molecular mass of 200 kDa (Fig. 4B, lane 2). In the presence of GMPPNP a major complex of 250 kDa together with a minor form of 300 kDa can be seen (Fig. 4B, lane 1). Complexes of the same molecular weights are also seen when GMPPNP treated RM are probed with an anti-SRP54 antibody (Fig. 4B, lane 3). However in GDP treated RM anti-SRP54 antibodies recognise only a 60 kDa protein consistent with monomeric SRP54 (Fig. 4B, lane 4; cf Fig. 4A). RM from which SRP had been removed by high salt extraction (PKRM) showed in the presence of GMPPNP or GDP only the 200 kDa SR complex when probed with anti-SRβ antibodies. When SRP was added to GMPPNP treated PKRM, complexes of 250 and 300 kDa can again be seen (Fig. 4B, lanes 7 and 8). Taken together these data show that SR/SRP54-containing complexes of 250 kDa and 300 kDa are formed in the presence of GMPPNP.

4. Discussion

Using blue native PAGE we have characterised protein complexes involved in the translocation of proteins across the membrane of the endoplasmic reticulum. In our approach we combined fractionation of RM proteins with immunodetection of subunits of the Sec61p complex, TRAP complex, OST complex, RAMP4, TRAMp, SRP and SR. In particular, we compared membrane protein complexes unengaged in translocation (T fraction) with those associated with ribosomes and engaged in nascent chain translocation (RAMP) [9]. The Sec61p complex, the core component of the translocon, is resolved as a major 140 kDa complex and a minor one of 180 kDa. The 140 kDa complex contains all three Sec61p subunits. Given the calculated mass of a single Sec61p trimer of about 60 kDa, the 140 kDa complex may consist of two or three copies of the heterotrimer. This is consistent with calculations made on the basis of electron

microscopic analysis of ribosome bound Sec61p complexes [11]. No size differences could be detected between the unengaged Sec61p complex and the ribosome bound one suggesting that the Sec61p complex does not disassemble into its subunits after completion of translocation [10]. The finding of a near homogeneous size of Sec61p complexes does not exclude the possibility that the Sec61p complex is able to change its size when engaged in the insertion of certain types of membrane proteins [13]. Indeed we consistently find a small amount of larger size Sec61p complexes.

Proteins which have been found associated with the Sec61p complex are the ER lumenal chaperone BiP [33] and the small membrane protein RAMP4 [17]. Anti-BiP did not detect a complex of similar size as the Sec61p complex (data not shown). This suggests that the interaction between BiP and the Sec61p complex may be weak and not maintained under the electrophoresis conditions used.

RAMP4 is a major constituent of the RAMP fraction and has been shown to cofractionate to some degree with the Sec61p complex [17]. Furthermore RAMP4 has been shown to interact with the invariant chain of MHC class II molecules during its insertion into the translocon. (Schröder et al., EMBO J., in press). We find RAMP4 mainly in an apparent monomeric form and also to some degree in oligomeric complexes of 180 kDa and higher. The subunit composition of these complexes remains unclear. The presence of RAMP4 in mono- and oligomeric form may reflect differential engagement of RAMP4 in the translocation of only some proteins or at only some stages of translocation.

Besides the Sec61p complex and RAMP4, the TRAP and the OST complex are found in the RAMP fraction [9]. We find the four subunits of the TRAP complex after digitonin solubilisation in a single oligomeric complex of about 150 kDa. The subunits of the TRAP complex have together a calculated molecular mass of about 100 kDa [16]. Whether the higher apparent molecular weight of the TRAP complex in blue native gels reflects the presence of two copies of each subunit or only an unusual migration behaviour remains to be determined. Using antibodies and Western blotting the TRAP complex (150 kDa) could be clearly separated from the major Sec61p complex (140 kDa). Compared with the 2-dimensional SDS-PAGE analysis method (Fig. 1B) a higher resolution of the TRAP and Sec61p complex is achieved by Western blotting. Analysis by 2-dimensional PAGE revealed TRAP complex in complexes 3 and 4 while Western blotting clearly separated the 140 kDa Sec61p complex from the 150 kDa TRAP complex.

Mammalian oligosaccharyl transferase (OST) has been shown to be an oligomeric complex composed of ribophorins I and II and OST48 [34] and probably a fourth component DAD1 [35]. The OST complex of yeast *S. cerevisiae* has been shown to contain in addition subunits of about 10 and 30 kDa (reviewed by Silberstein, [15]). Digitonin solubilised OST of the M and RAMP fraction can be separated into two distinct oligomeric forms of about 480 and 450 kDa. Both contain the two ribophorins and OST48. The 480 kDa form of the OST contains in addition two proteins with apparent molecular weights of around 30 and 10 kDa. Whether these proteins are subunits of the larger OST complex remains to be seen. Considering that the sum of the molecular weights of the three largest OST subunits is about 200 kDa, it is conceivable that the OST contains two copies of each subunit.

Consistent with previous findings, we show that SRα and SRβ assemble into a tight complex and that SRβ in addition exists in monomeric form. As the subunits of the SR migrate unusually in blue native PAGE, it is not possible to estimate the copy number of the subunits. SRP partially disassembles under the conditions of blue native PAGE with SRP54 being released from the particle. In the presence of GMPPNP, complex formation between SR and SRP can be demonstrated confirming previous biochemical binding studies [8,24,36]. The apparent molecular weight of the 250 kDa complex suggests that it may result from one SRP54 molecule associating with the 200 kDa SR complex. An additional ER protein may be present in the 300 kDa complex.

Taken together we show here that several of the components involved in the targeting or translocation are in oligomeric forms which can be identified by blue native PAGE. We are now in the position to determine the physiological conditions under which the different complexes are generated. This may be particularly relevant to RAMP4, SR and OST complexes which display more than one oligomeric form.

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