Ribosome-independent Regulation of Translocon Composition and Sec61α Conformation*

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In this study, the contributions of membrane-bound ribosomes to the regulation of endoplasmic reticulum translocon composition and Sec61α conformation were examined. Following solubilization of rough microsomes (RM) with digitonin, ribosomes co-sedimented in complexes containing the translocon proteins Sec61α, ribophorin I, and TRAPα, and endoplasmic reticulum phospholipids. Complexes of similar composition were identified in digitonin extracts of ribosome-free membranes, indicating that the ribosome does not define the composition of the digitonin-soluble translocon. Whereas in digitonin solution a highly electrostatic ribosome-translocon junction is observed, no stable interactions between ribosomes and Sec61α, ribophorin I, or TRAPα were observed following solubilization of RM with lipid-derived detergents at physiological salt concentrations. Sec61α was found to exist in at least two conformational states, as defined by mild proteolysis. A protease-resistant form was observed in RM and detergent-solubilized RM. Removal of peripheral proteins and ribosomes markedly enhanced the sensitivity of Sec61α to proteolysis, yet the readdition of inactive ribosomes to salt-washed membranes yielded only modest reductions in protease sensitivity. Addition of sublytic concentrations of detergents to salt-washed RM markedly decreased the protease sensitivity of Sec61α, indicating that a protease-resistant conformation of Sec61α can be conferred in a ribosome-independent manner.

Beginning with early morphological and biochemical studies defining the rough endoplasmic reticulum as the site of protein entry into the secretory pathway (1–3), an understanding of the mechanism of ribosome binding to the endoplasmic reticulum (ER) membrane has been considered essential to the elucidation of the molecular mechanism of protein translocation. A theme consistent through the past decades of research into protein translocation has been the hypothesis that the membrane-bound ribosome performs regulatory functions that govern the structural and functional state of the translocon, the site of protein translocation in the ER (4–9). In these models, the ribosome is thought to interact with protein components of the ER membrane and thereby initiate the assembly of a protein-conducting channel through which translocation proceeds (5–8, 10, 11). In addition to a role in the regulation of channel assembly, the ribosome is also thought to regulate the structural state of the protein-conducting channel during the translocation event itself and in this manner assist the assembly of proteins of complex topologies, such as apolipoprotein B-100, the prion protein, and polytopic membrane proteins (9, 12–14).

Diverse experimental approaches have provided evidence indicating a role for the ribosome in the regulation of translocon assembly. For example, following solubilization of rough microsomes (RM) with digitonin, ribosomes co-fractionate with Sec61α, the core component of the protein-conducting channel (6, 15). Furthermore, inactive ribosomes elicit Sec61p oligomerization in proteoliposomes comprising lipids and the purified Sec61p complex (7). In agreement with these observations, a direct physical interaction between inactive yeast ribosomes and yeast Sec61p was recently identified by cryo-electron microscopy (8). It remains uncertain, however, whether the Sec61p-ribosome interactions identified in these studies are identical to the ribosome-membrane junction as characterized in vitro (8, 16–19).

Current experimental evidence supports the identification of Sec61α as the ribosome receptor, although substantial literature exists regarding the identification of ribosome receptor proteins other than Sec61α (20–29). Surprisingly, although significant disagreement regarding the identification and characterization of ribosome receptor proteins persists, there is near unanimity in the choice of assay system used for their discovery. In this assay, radiolabeled biosynthetically inactive ribosomes are incubated with ribosome-stripped microsomal vesicles and the bound and free fractions are separated by flotation centrifugation (20). This assay identifies saturable and high affinity ribosome binding, although at physiological salt concentrations the observed ribosome binding stoichiometries are approximately 10% of those found in native membranes (20, 28). For this reason, it is uncertain whether the ribosome binding activity observed in this assay is identical to that present during ribosome/nascent chain targeting and translocation. In fact, the hypothesis that ER membrane components other than Sec61α contribute to ribosome binding was presented in a recent study demonstrating the binding of ribosomes bearing nascent secretory chains to sites on the ER membrane other than Sec61α (30).

We report that solubilization of RM with digitonin yields macromolecular complexes comprising ribosomes, a diverse population of integral membrane proteins, and phospholipids. Such complexes were also observed following solubilization of ribosome-free membranes, indicating that the ribosome is not required for complex stability in detergent solution. In contrast to the results seen with digitonin, solubilization of RM with lysophosphatidylcholine or 1,2-diheptanoyl-sn-phosphatidylcholine (DHPC) yields monodisperse solutions of RM proteins...
that did not reside in stable association with ribosomes. In studies designed to assess the role of Sec61α in the regulation of ribosome interactions with the ER membrane, Sec61α was observed to exist in two conformational states. In native RM and detergent extracts of native RM, Sec61α assumed a protease-resistant conformation. Removal of bound ribosomes and peripheral proteins yielded a dramatic increase in the sensitivity of Sec61α to proteolysis; readDITION of inactive ribosomes did not efficiently restore the protease-resistant conformation. However, the addition of sublytic concentrations of detergents to ribosome-depleted membranes markedly enhanced the resistance of Sec61α to proteolysis, indicating that Sec61α conformation can be regulated in a ribosome-independent manner.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Digitonin was obtained from Wako Chemicals USA (Richmond, VA). DHPC was from Avanti Polar Lipids (Alabaster, AL). Purumycin and BigChAP were obtained from Calbiochem (San Diego, CA). 5-2,3-Dihydroxypropylacetalamido-2,4,6-triido-N,N'-bis(2,3-dihydroxypropyl)-isopthalamide (Nycodenz), phosphatidylcholine, lysophosphatidylcholine, phosphatidylethanolamine, phosophatydylglycerol, and dinitrophenol were obtained from Sigma. Chymotrypsin was supplied by Worthington. Rabbit reticulocyte lysate was from Promega (Madison, WI). [35S]Pro-Mix ([35S]methionine/cysteine) was obtained from Amerham Pharmacia Biotech.

**Preparation of Rough Microsomes, Ribo- somed Stripped Microsomes, and Ribosomes**—Canine rough microsomes (RM), prepared as in Ref. 31, were stripped of ribosomes by treatment with EDTA and KOAc (EKRM), as described previously (30). Alternatively, microsomes were stripped of ribosomes by treatment with puromycin and KOAc (PKRM) as described previously (6). To prepare ribosomes, rabbit reticulocyte lysate (200 μl) was centrifuged for 30 min at 80,000 rpm in a TLA100 rotor (Beckman Instruments, Palo Alto, CA) at 4 °C. The ribosomal pellet, containing ribosomes, was resuspended in 130 μl of ribosome buffer (25 mM K-HEPES (pH 7.2), 50 mM KOAc, 5 mM Mg(OAc)2, 1 mM DTT), loaded onto a 70-μl Nycodenz (pH 7.2), 150 mM KOAc, 5 mM Mg(OAc)2, 1 mM DTT and 0.5 mM PMSF. Samples were then subjected to centrifugation for 30 min at 80,000 rpm in a SW55 rotor (Beckman) at 4 °C. The ribosomal pellet was collected and precipitated by addition of trichloroacetic acid to 10%. Samples were processed for SDS-PAGE and immunoblot analysis as described above.

**Analysis of Protein and Lipid Association with Ribosomes**—Detergent solubilization of microosomal vesicles was performed as follows: 10 equivalents (eq) of digitonin, or an equal volume of ribosome-stripped membranes, were suspended in buffer containing 20 mM detergent (unless otherwise specified), 25 mM K-HEPES (pH 7.2), 150 mM KOAc, 5 mM Mg(OAc)2, 1 mM DTT, and 0.5 mM PMSF in a final volume of 200 μl. Samples were mixed with 4 volumes of 0% Nycodenz, all in 25 mM K-HEPES (pH 7.2), 150 mM KOAc, 5 mM Mg(OAc)2, 1 mM DTT and 0.5 mM PMSF. Samples were subjected to centrifugation and fractionation through 10–30% sucrose gradients as described above. Ribosomes were resuspended in ribosome buffer, and concentrations determined by UV spectrometry (1 A260 unit = 21.4 pmol of 80 S ribosomes; Ref. 32).

**Lipid Extraction and Detergent-solubilized Membranes**—Membranes were left untreated or solubilized with digitonin or DHPC as described above, and chymotrypsin was added at the concentrations indicated in the figures. Digestions were performed for 30 min on ice, after which time the trichloroacetic acid was added to 10%. Samples were collected by centrifugation and processed for SDS-PAGE and immunoblotting. When SDS was used to solubilize membranes, the above procedures were carried out at 37 °C to avoid precipitation of the SDS. In experiments involving ribosome binding to membranes, the concentration of digitonin was increased to 200 μl of 0.1% digitonin, which contained the flocculated vesicles, was collected and precipitated by addition of trichloroacetic acid to 10%. Samples were processed for SDS-PAGE and immunoblot analysis as described above.

**Proteolysis of Intact and Detergent-solubilized Membranes**—Membranes were left untreated or solubilized with digitonin or DHPC as described above, and chymotrypsin was added at the concentrations indicated in the figures. Digestions were performed for 30 min on ice, after which time the trichloroacetic acid was added to 10%. Samples were collected by centrifugation and processed for SDS-PAGE and immunoblotting. When SDS was used to solubilize membranes, the above procedures were carried out at 37 °C to avoid precipitation of the SDS. In experiments involving ribosome binding to membranes, the concentration of digitonin was increased to 200 μl of 0.1% digitonin, which contained the flocculated vesicles, was collected and precipitated by addition of trichloroacetic acid to 10%. Samples were processed for SDS-PAGE and immunoblot analysis as described above.

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**Protein Translation Activity of Detergent-isolated Ribosomes**—2 pmol of ribosomes, derived from either reticulocyte lysate or RM solubilized with 20 mM DHPC, were added to 8 μl of a rabbit reticulocyte lysate mixture containing 5% of ribosomes supplemented with 500 ng preprolactin mRNA, 16 Ci of [35S]Pro-Mix, 0.05 unit/ml RNasin, 1 mM DTT, and 20 μM amino acid mix (minus methionine) in a total volume of 20 μl. Following incubation for the indicated time periods at 25 °C, 5 μl of the translation reaction was spotted onto filter paper and protein synthesis levels were measured as described previously (33).

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used at 1:1500 dilution, as described previously (30). Following transfer to nitrocellulose, membranes were blocked in phosphate-buffered saline containing 5% nonfat milk and 0.1% Tween 20 for 1 h at room temperature. Primary incubations were performed for 1 h at room temperature in phosphate-buffered saline containing 1% nonfat milk and 0.1% Tween 20, and secondary incubations performed for 30 min in the identical buffer using the appropriate secondary antibodies at 1:3000 dilution.

RESULTS

Composition of Detergent-soluble Ribosome-Translocon Complexes—To investigate the role of membrane-bound ribosomes in the regulation of translocon composition, native RM were solubilized with either digitonin or DHPC, and ribosome-associated membrane components were resolved by sedimentation of the detergent-soluble fraction on sucrose gradients. The selection of detergents was based on published studies identifying either ribosome-membrane protein complexes (digitonin; Ref. 6) or high recovery of native protein structure and activity (DHPC; Ref. 34). As depicted in Fig. 1 (A and C), when RM were solubilized with digitonin, the ribosome fraction sediments as a heterogeneous fraction containing Sec61α, ribophorin I, TRAPα, and ER phospholipids. Quantitation of the ribosome-associated lipid fraction by chemical phosphate analysis indicated that phospholipids were present at approximately a 50-fold molar excess to ribosomes (data not shown). When RM were solubilized with DHPC, none of the assayed membrane components (Sec61α, ribophorin I, TRAPα, TRAM, or signal peptidase complex) co-sedimented with ribosomes (Fig. 1B). In addition, although phospholipids could be identified with the DHPC-solubilized ribosomes (Fig. 1C), the quantities of ribosome-associated phospholipids were approximately 2% of that seen with digitonin-soluble ribosomes.

Translocon Structure Persists in the Absence of Bound Ribosomes—The data presented in Fig. 1 indicated that, when RM were solubilized with digitonin, membrane-bound ribosomes sedimented in association with a large, heterogeneous complex of integral membrane proteins and phospholipids. When RM were solubilized with DHPC, however, stable ribosome-membrane protein interactions were not detected. To further investigate these differences, RM were stripped of bound ribosomes in puromycin/high salt buffers (6) and experiments similar to those depicted in Fig. 1 were performed (Fig. 2). In the absence of bound ribosomes, the digitonin-soluble membrane proteins would be predicted to sediment with a considerably smaller sedimentation coefficient than that depicted in Fig. 1. Thus, to adequately resolve the detergent-soluble protein fraction derived from ribosome-free membranes, the detergent extracts were centrifuged on sucrose gradients for extended time periods. As shown in Fig. 2A, when ribosome-free membranes were solubilized with digitonin, Sec61α, ribophorin I, and TRAPα were again observed to migrate as a complex. Analysis of the total protein composition of these fractions indicated that the digitonin-soluble translocon complex was heterogeneous and consisted of at least 20 silver stain-reactive polypeptides (Fig. 2B).

FIG. 1. Association of rough microsome components with ribosomes in detergent solution. 30 eq of RM were solubilized at 150 mM KOAc with 20 mM digitonin (A) or DHPC (B) and the soluble fraction subjected to velocity sedimentation in 10–30% sucrose gradients. Gradient fractions were collected and processed for immunoblot analysis with antibodies against the indicated proteins. The immunoblot for L3/L4 indicates the position of ribosomes within the gradient. In panel C, the ribosome peak from each gradient was collected and the ribosomes recovered by centrifugation. Following organic extraction of the pelleted ribosomes, the phospholipid composition of the organic phase was determined by thin layer chromatography and detection by iodine staining.

FIG. 2. Ribosome-independent macromolecular complexes of ER membrane proteins exist in digitonin solution, but not in DHPC solution. In panel A, a digitonin-soluble PKRM extract was resolved on a 5–20% sucrose gradient. Fractions were collected and subjected to immunoblot analysis for the indicated proteins. In panel B, a digitonin-soluble PKRM extract was resolved as in A, gradient fractions were collected, the proteins were concentrated by trichloroacetic acid precipitation, and following resolution on a 12.5% SDS-PAGE gel, the protein components were identified by silver stain detection. A digital image of the silver-stained gel is shown. In panel C, a DHPC-soluble RM extract was processed as in panel A, except smaller fraction sizes were collected to obtain higher resolution.
Ribosome-independent Translocon Regulation

2040

Ribosome-independent Translocon Regulation

Effects of DHPC on Oligomeric Protein Structure, Ribosome Binding, and Ribosome Function—DHPC is well documented to solubilize a diverse array of integral membrane proteins while maintaining both native structure and enzymatic activity (34, 35). Nonetheless, the inability to detect stable translocon protein-ribosome interactions in DHPC solution could result from DHPC-induced disruptions in the structural integrity of ER oligomeric protein complexes, inactivation of ribosome binding activity, and/or DHPC-induced structural perturbations in the ribosome itself. To determine whether the inability to detect ribosome-membrane protein interactions in DHPC solution was a consequence of general disruptions in oligomeric protein integrity, the DHPC-soluble fraction from RM was fractionated by velocity sedimentation and the oligomeric structure of the Sec61, TRAP, and signal peptidase complexes determined. As shown in Fig. 3, the α and β subunits of Sec61 undergo partial dissociation in DHPC solution, whereas other membrane protein complexes, such as the TRAP and signal peptidase complexes, remain wholly intact. From these data, it can be concluded that DHPC does not elicit a general disruption of oligomeric membrane protein complexes. Furthermore, because it has been demonstrated that the α and β subunits of Sec61 readily dissociate in detergent solution and that the β subunit of Sec61 is not required for ribosome binding (36), the partial dissociation of the α and β subunits of Sec61 observed in DHPC solution does not provide a convincing explanation for the lack of stable membrane protein-ribosome interactions in DHPC-solubilized membranes.

The ribosome-Sec61α interaction, as assayed in digitonin solution, is highly electrostatic and is stable at salt concentrations approaching 1 M (6). Consistent with this observation, when native RM were solubilized in digitonin at 0.5 M salt and centrifuged, Sec61α was recovered in the ribosome-rich pellet fraction, whereas when ribosome-stripped membranes were solubilized with digitonin, Sec61α was recovered in the supernatant fraction (Fig. 4A, lanes 5 and 6). In contrast, solubilization of native RM with either DHPC or lysophosphatidylcholine at physiological salt concentrations yielded efficient recovery of Sec61α in the supernatant fraction (Fig. 4B, lanes 3–6) and, as illustrated in Fig. 4C, the DHPC-soluble Sec61α was not associated with ribosomes. This phenomenon is further illustrated in Fig. 4D, where the ability of the two detergents to release Sec61α from native RM was assayed as a function of detergent concentration. As is clear from the data in Fig. 4D, whereas solubilization of Sec61α with DHPC exhibits a clear maxima at a DHPC concentration of 16–24 mM (detergent:RM of 400–600 nmol detergent: eq RM, with 1 eq RM containing ~4 nmol of phospholipid), solubilization of Sec61α with increasing concentrations of digitonin does not alter the distribution of Sec61α into large (>100 S) complexes that are recovered in a ribosome-rich pellet upon centrifugation. Thus, if ribosome association with the ER membrane is conferred via electrostatic interactions with Sec61α, such interactions are disrupted in the presence of DHPC. Alternatively, ribosome binding to the ER membrane may require multiple weak interactions with ER membrane components and, under conditions in which the ER membrane is efficiently solubilized (addition of DHPC or lysophosphatidylcholine), weak bi-molecular interactions between ribosomes and individual membrane components are not captured.

To determine if the lack of stable ribosome-membrane protein interactions seen in DHPC solution were due to disruption of the presumed electrostatic ribosome-membrane interaction by the choline headgroup of DHPC, RM were treated with 0.5 M choline and ribosome release was assayed. Under these conditions no ribosome release was observed (data not shown). In additional experiments, RM were solubilized with digitonin and choline was subsequently added to 0.5 M. Again, ribosome-membrane component interactions remained intact (data not shown). These data indicate that the inability to detect stable ribosome-Sec61α interactions following solubilization with DHPC was not solely due to the choline headgroup, and, because lysophosphatidylcholine treatment of RM also yielded soluble, ribosome-free Sec61α at moderate salt concentrations, not a unique consequence of the relatively short (7 carbon) acyl chains present on DHPC.

To directly evaluate the effect of DHPC treatment on the ribosome binding activity of microsomal membranes, the following experiments were performed. In the first series of experiments, proteoliposomes were reconstituted from DHPC-solubilized RM components and their ribosome binding activity determined. Reconstituted vesicles were incubated with ribosomes at physiological salt concentrations, and the membrane-bound ribosomes separated by gradient flotation on Nycodenz step gradients. Bound ribosomes were then quantified by immunoblotting for the large ribosomal subunit proteins L3/L4.

**Fig. 3. Effects of DHPC on membrane protein quaternary structure.** RM were solubilized at physiological salt concentrations in 20 mM DHPC and the soluble fraction resolved by velocity sedimentation in sucrose gradients as described in the legend to Fig. 1. The oligomeric structures of the Sec61 complex, the TRAP complex, and the signal peptidase complex were determined by immunoblot analysis of the SDS-PAGE resolved gradient fractions.

2B). In contrast, when RM were solubilized with DHPC, Sec61α, ribophorin I, and TRAPα exhibited overlapping, but non-identical sedimentation profiles (Fig. 2C). From these experiments, it can be concluded that the isolation of digitonin-soluble translocon complexes does not require bound ribosomes. Furthermore, when RM were solubilized with DHPC, the integral membrane protein components of the ER behaved in a monodisperse manner and no stable membrane protein-ribosome interactions were observed.

In current views, ribosome binding to the ER membrane is maintained primarily through interactions with Sec61α (7, 28). The data depicted in Figs. 1 and 2 support this view, although the molecular heterogeneity of the digitonin-soluble translocon complexes, as seen in both the presence and absence of bound ribosomes, prevents unequivocal identification of Sec61α as the ribosome receptor. Alternatively, the results obtained with DHPC can be interpreted to indicate that neither Sec61α, ribophorin I, nor TRAPα alone constitute the primary site of ribosome-membrane interaction. To reconcile these conflicting interpretations, additional experiments were performed to critically assess the significance of the DHPC data and to further evaluate ribosome-Sec61α interactions.

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At maximal binding stoichiometries, 2 pmol of ribosomes were bound to reconstituted proteoliposomes containing the amount of Sec61α found in 3–4 eq of RM (Fig. 5A). Native RM contain approximately 1 pmol of bound ribosomes/eq (data not shown), and so proteoliposomes prepared from DHPC extracts of RM display approximately 50% of the ribosome binding activity observed in native membranes. A 50% relative binding stoichiometry is consistent with a stochastic reconstitution of Sec61α in the proper and inverse topologies. Thus, solubilization of RM in DHPC and subsequent reconstitution of the detergent extract into proteoliposomes does not substantially alter the binding of inactive ribosomes.

To assess whether DHPC elicited an alteration in ribosome structure, as reflected in translation activity, the effects of DHPC on protein synthesis were examined. In these experiments, a post-ribosomal supernatant of reticulocyte lysate was supplemented with identical quantities of ribosomes obtained either by centrifugation of DHPC-solubilized RM, or resuspension of the reticulocyte ribosomes remaining from preparation of the lysate post-ribosomal supernatant. The capacity of the ribosome-supplemented post-ribosomal supernatant to translate preprolactin was then assayed. As shown in Fig. 5B, the translation activity of the DHPC-treated ribosomes was virtually identical to that seen with the reticulocyte ribosomes, and thus DHPC does not alter ribosome structure in a manner that alters ribosome function. Furthermore, ribosomes recovered from DHPC-solubilized RM are capable of binding to PKRM in a manner similar to that depicted in Fig. 5A (data not shown).

Sec61α Exists in Multiple Structural States Differing in Protease Accessibility—Although the results depicted in Fig. 5 support the conclusion that solubilization of RM with DHPC preserves inactive ribosome binding activity upon reconstitution, it was necessary to address the hypothesis that Sec61α, in DHPC solution but not in digitonin solution, assumed a conformation unsuitable for ribosome binding. To test this hypothesis, experiments were performed to examine the conformation of Sec61α in the detergent-solubilized state, using the protease-independent translocon regulation assay.
These data indicate that the protease resistance of Sec61α in native membranes cannot be attributed solely to bound ribosomes and furthermore in native RM Sec61α exists in at least two conformations, which differ in their relative sensitivity to chymotrypsin digestion.

The protease sensitivities of Sec61α in detergent extracts of ribosome-stripped membranes (PKRM) were identical (Fig. 6B, lanes 9–12), yet distinct from those observed in intact PKRM (Fig. 6B, lanes 7 and 8). In PKRM, virtually the entire population of Sec61α had been converted to the chymotrypsin sensitive form, and was recovered as the 28-kDa form (Fig. 6B, lanes 7 and 8). In contrast, when PKRM were solubilized with either digitonin or DHPC, a third limit digestion product was identified. In this conformational state, Sec61α was cleaved to yield a form of intermediate mobility to the full-length and 28-kDa forms, and is indicated by the single asterisk (Fig. 6B). Because the antisera used in these experiments was raised against an N-terminal epitope, the mobility of this form on SDS-PAGE is consistent with proteolysis occurring in a domain of Sec61α C-terminal to that generating the 28-kDa limit digestion product. As will be demonstrated in Fig. 7, this digestion product is likely an intermediate in the formation of the 28-kDa form. In summary, the data presented in Fig. 6 indicate that: 1) the conformation of Sec61α in digitonin solution is similar to that observed in DHPC solution; 2) removal of bound ribosomes and peripheral proteins elicits a conformational change in Sec61α; 3) the inability of proteases to cleave Sec61α in native membranes cannot be attributed solely to bound ribosomes, and 4) Sec61α can exist in at least two conformations, both of which are present in native RM.

Previous studies demonstrating that the addition of inactive ribosomes to PKRM elicits a protease-insensitive conformation of Sec61α support the conclusion that ribosome binding to Sec61α is the primary determinant governing protease accessibility (28). As the data presented in Fig. 6 indicate that the conformational status of Sec61α can be altered in a ribosome-independent manner, the effects of ribosome addition on the conformation of Sec61α were investigated. In these experiments, protease titrations were performed on RM, PKRM, and PKRM to which excess inactive ribosomes were added (Fig. 7A). Comparison of the proteolysis patterns indicates that, as demonstrated previously, Sec61α in RM exists in two conformational states, with the predominant form being resistant to chymotrypsin cleavage (Fig. 7A, lanes 1–5). Addition of increasing concentrations of chymotrypsin to PKRM results in the appearance of two forms: one of intermediate mobility, which is identical to that seen upon digestion of detergent-solubilized PKRM (Fig. 6B, lanes 10 and 12, single asterisk), and one of higher mobility (Fig. 6, A and B, double asterisk), which is identical to that seen in low abundance in native RM (Fig. 7A, lanes 6–10). When chymotrypsin titrations were performed on PKRM supplemented with excess ribosomes, the overall pattern of digestion was similar to that seen in the absence of ribosomes (Fig. 7A, lanes 7–10 versus lanes 12–15). Comparison of the proteolysis patterns at intermediate concentrations of chymotrypsin did indicate, however, a modest influence of the inactive ribosomes on the conformation of Sec61α, primarily with regard to the intermediate digestion product seen most readily in detergent solubilized PKRM (Fig. 7A, compare lanes 8 and 9 with lanes 13 and 14). Clearly, however, the conformation of Sec61α in PKRM supplemented with a 5-fold molar excess of free ribosomes is markedly different from that present in RM. Additional experiments provided evidence for a ribosome-independent regulation of Sec61α conformation. As shown in Fig. 7B, following treatment of PKRM with a sublytic concentration of DHPC, Sec61α was markedly resist-
ant to proteolytic digestion (Fig. 7B, lanes 1–7 versus lanes 8–14). At the concentration of DHPC used in these experiments (0.75 mM), integral membrane proteins, such as Sec61α, TRAM, and the signal peptidase complex were not solubilized (data not shown), and luminal proteins such as BiP were not released from the membrane vesicles (Fig. 7C). Because the protease accessibility characteristics of Sec61α as seen in intact RM were displayed upon addition of sublytic concentrations of detergent to ribosome stripped membranes, it appears that low concentrations of detergent either alter membrane structure in a manner that effects a change in Sec61α conformation and/or alter protein-protein interactions that contribute to the regulation of Sec61α conformation. The observed DHPC-dependent changes in Sec61α protease accessibility could also be obtained by addition of a structurally unrelated detergent (BigCHAP) or a compound that preferentially distributes in the outer membrane leaflet (dinitrophenol), indicating that this phenomenon is not unique to DHPC (data not shown). These data clearly demonstrate that the conformation of Sec61α, as displayed by limited proteolysis, can be modulated in a ribosome-independent manner.

DISCUSSION

In this study, the role of membrane-bound ribosomes in the regulation of the macromolecular organization of the endoplasmic reticulum translocon and the conformation of Sec61α were investigated. Six conclusions can be drawn from these studies. 1) In digitonin solution, the translocon is a heterogeneous complex consisting of multiple integral membrane proteins, phospholipids and ribosomes. 2) The molecular composition of such complexes is ribosome-independent. 3) When solubilized under conditions in which ER membrane proteins are recovered in monodisperse solution, membrane protein-ribosome interactions are not maintained. 4) Sec61α can be found in at least two conformational states. 5) The proteolytic sensitivity of Sec61α is enhanced upon removal of peripheral proteins and bound ribosomes. 6) Conversion of Sec61α to a protease-resistant conformation, as is predominantly found in native RM, can be elicited by sublytic concentrations of detergent and with very modest efficiency by inactive ribosomes.

With regard to the role of the ribosome in regulating translocon structure, it was observed that following solubilization of RM with digitonin, ribosomes remained in association with large macromolecular complexes containing Sec61α, ribophorin I, TRAP, and approximately a 50-fold molar excess of phospholipid to ribosomes. Related studies by Chevet et al. (37) indicate that similar detergent-derived complexes also contain calnexin and the ER-to-Golgi recycling protein α2p24. The isolation of such macromolecular complexes is in agreement with previous studies (48), and was not dependent on bound ribosomes, as complexes of similar composition were obtained following solubilization of ribosome-free microsomes (PKRM) with digitonin. Because the digitonin-soluble translocon complexes are heterogeneous and apparently not dependent upon the ribosome for their higher order structure, it is difficult to determine whether the ribosome binding activity displayed by such complexes reflects the activity of any single component of the complex, as would be expected from published data (6, 8, 15, 28, 38).

Previous studies have postulated that the interaction between the Sec61 complex and the ribosome occurs through stable electrostatic interactions (6). In support of this hypothesis, the release of Sec61α from digitonin-solubilized ribosome pellets required salt concentrations in excess of 1 M (6). However, when RM were solubilized with DHPC or lysophosphatidylcholine, no stable interactions between ribosomes and Sec61α were observed. Rather, following solubilization in DHPC at physiological salt concentrations, resident integral membrane proteins behaved in a monodisperse manner and were readily resolved from membrane-derived ribosomes. This observation was unexpected and thus necessitated a series of
control experiments to evaluate the effects of DHPC treatment on the structure and activity of the two assumed binding partners, ribosomes and Sec61α. In these experiments, it was demonstrated that DHPC did not elicit a general disruption of membrane protein quaternary structure, that ribosome binding activity could be efficiently recovered in proteoliposomes reconstituted from DHPC extracts of RM, that the protease accessibility of Sec61α in either digitonin or DHPC extracts was similar, and that DHPC treatment did not alter the protein translation activity or membrane binding activity of ribosomes. Solubilization of RM with DHPC does not, therefore, appear to alter the structural or functional characteristics of the ER components that are presumed responsible for ribosome binding. It should be noted, however, that although extensive control experiments indicated that solubilization of RM with DHPC did not irreversibly affect ribosome binding, we cannot eliminate the possibility that DHPC elicits a heretofore uncharacterized conformational change in Sec61α that disrupts Sec61α-ribosome interactions. Equally as important, the substantial molecular heterogeneity of the digitonin-soluble translocon complexes and the observation that translocon composition in digitonin is ribosome-independent makes the identification of the ribosome receptor(s) in digitonin extracts of RM difficult.

To reconcile our experimental findings with previous studies, we propose that the differences in the mechanism of membrane solubilization displayed by the two detergents determine whether ribosome association with the translocon can be captured. It is clear that digitonin and DHPC solubilize biological membranes by different mechanisms (39–43). Digitonin displays numerous structural similarities with the bile acid class of detergents. Principally, digitonin is a planar, heterocyclic nonionic detergent containing a polyglycidic side chain. Previous studies have shown that detergents of this structural class act by releasing membrane fragments enclosed at their periphery with detergent molecules (44, 45). These studies also noted that solubilization of membranes with such detergents preserves the structural organization of the bilayer in the mixed micelle (44, 45). In contrast to digitonin, DHPC is a short-chain phosphatidylcholine derivative, which, because of its pronounced structural similarities to native membrane lipids and solubility in aqueous media, efficiently disperses biological membranes (34, 46). In so doing, DHPC is remarkable in its ability to preserve native membrane protein structure and function in the solubilized state (34, 35). With these differences in solubilization mechanism in mind, the data presented herein can be interpreted to indicate that digitonin treatment of native or stripped RM yields the release of large macromolecular complexes constituting a defined mixed detergent/protein/lipid domain. DHPC, because it is not subject to the solubilization constraints imposed by the structural characteristics of heterocyclic planar detergents such as digitonin, efficiently disperses the protein and lipid components of the membrane bilayer (34). Thus, whereas in digitonin the ribosome can maintain multiple contacts with the membrane components that comprise the digitonin-soluble membrane domain, in DHPC such membrane domains are not captured and thus stable ribosome-membrane protein interactions are not maintained. On the basis of these data, we postulate that membrane-bound ribosomes maintain their association with the ER membrane through multiple, weak interactions, rather than via a high affinity homomolecular interaction. In other words, the kinetic basis for ribosome binding to protein components of the ER membrane is an avidity rather than an affinity-based process. The hypothesis that ribosome binding to the ER membrane is regulated through interactions involving multiple membrane components is supported by reconstitution studies, where it was observed that proteoliposomes containing the minimal translocation machinery display approximately 10% of the ribosome binding observed in native membranes, and by studies of the binding of biosynthetically active ribosome nascent chain complexes, where ribosome binding to sites other than Sec61α was reported (28, 30). Proteoliposomes containing the SRP receptor and Sec61α function in translocation, and so Sec61α likely figures prominently in the regulation of ribosome binding (15).

As an experimental approach, detergent-based co-fractionation studies did not provide unequivocal data identifying a stable, stoichiometric association between an ER translocon component(s) and membrane-bound ribosomes, and so additional criteria were examined. As reported by Kalies et al. (28), evidence for the identification of Sec61α as the ribosome receptor was obtained in studies demonstrating that Sec61α is protected from proteolysis by membrane-bound ribosomes, and that the addition of inactive ribosomes to ribosome-striped microsomes restores the protease resistance of Sec61α. To further examine this conclusion, the protease sensitivity of Sec61α was compared in native membranes, native membranes solubilized with digitonin (where a stable ribosome-membrane junction is maintained), and native membranes solubilized with DHPC (where the ribosome-membrane junction is lost). Contrary to prediction, the protease sensitivity of Sec61α did not increase following solubilization with DHPC. If native membranes were stripped of bound ribosomes with high salt and puromycin prior to solubilization and proteolysis, however, Sec61α was readily accessible to chymotrypsin digestion. These data indicate that the conditions used to extract membrane-bound ribosomes from ER microsomes alter Sec61α protease accessibility, but as the data obtained with DHPC extracts depicts, the protease-resistant conformation seen in native membranes need not be conferred by the bound ribosome. Furthermore, when ribosome-stripped membranes are supplemented with free ribosomes, only a very modest restoration of protease resistance is observed. From these data, it appears that Sec61α can assume at least two conformations and that the removal of peripheral proteins and bound ribosomes alters Sec61α conformation in a manner that is only inefficiently reversed by the addition of excess inactive ribosomes. It follows that the binding of inactive ribosomes to ribosome-stripped membranes is mechanistically different from that occurring during the physiological targeting event and so inactive ribosomes, should they bind to Sec61α, do not protect it from proteolysis.

Because the protease-resistant conformation of Sec61α could be efficiently elicited by addition of sublytic concentrations of detergent (Fig. 7) or the amphiphile dinitrophenol (data not shown), we speculate that the differences in Sec61α protease accessibility seen following the extraction of peripheral proteins represent, at least in part, a conformational change in the protein. The observation that Sec61α conformation is sensitive to the addition of sublytic concentrations of amphiphiles suggests that the physical state of the membrane can influence Sec61α conformation. When viewed with respect to previous studies demonstrating that membrane-bound ribosomes increase the structural order parameters of microsomal membranes (47), these data identify a mechanism whereby membrane-bound ribosomes, by influencing the physical properties of the membrane, could elicit changes in Sec61α conformation in the absence of direct, stable interactions.

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Ribosome-independent Translocon Regulation

REFERENCES