Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 9931–9935, December 1989
Cell Biology

Reconstitution of translocation-competent membrane vesicles from detergent-solubilized dog pancreas rough microsomes

(endoplasmic reticulum/protein translocation/signal peptide cleavage/N-glycosylation)

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Contributed by David D. Sabatini, September 18, 1989

ABSTRACT **Dog pancreas rough microsomes were solubilized in 1% octyl β-glucoside, and membrane vesicles were reconstituted by slow 30-fold dilution with a buffer of low ionic strength. Asymmetric assembly of the membranes occurred during reconstitution since the vesicles formed contained ribosomes bound only to the vesicular outer surfaces. The reconstituted vesicles were similar in protein composition to native rough microsomes, although these vesicles were largely devoid of luminal-content proteins. These reconstituted vesicles could translocate and process nascent secretory (human placental lactogen) and membrane proteins (influenza hemagglutinin and rat liver ribophorin I) synthesized in cell-free translation systems programmed with the corresponding mRNAs. Signal cleavage and N-glycosylation only occurred when the reconstituted membranes were present during translation, providing evidence that the translocation apparatus was asymmetrically assembled into the reconstituted membranes. When a supernatant lacking ribosomes and particles >50S from centrifuging the detergent-solubilized microsomes at high speed was used for reconstitution, smooth-surfaced membrane vesicles were obtained that, except for the absence of ribosomal proteins, were similar in protein composition to that of the reconstituted vesicles from total solubilized rough microsomes. The reconstituted smooth-surfaced vesicles, however, were totally inactive in cotranslational processing and translocation of nascent polypeptides. These findings suggest that ribosomes and/or large macromolecular complexes, not dissociated under our solubilization conditions, are essential for *in vitro* assembly of a functional translocation apparatus.**

Translocation of a polypeptide chain across the hydrophobic phospholipid bilayer of the rough endoplasmic reticulum membrane occurs during polypeptide elongation and is accomplished by a multicomponent molecular apparatus (for reviews see refs. 1–3). In recent years, several specific rough endoplasmic reticulum membrane proteins have been implicated in the process of targeting nascent polypeptides to the endoplasmic reticulum and in effecting their subsequent insertion into and translocation across the membrane. These proteins include two polypeptides that constitute the signal-recognition particle (SRP) receptor (4, 5), two glycoproteins, ribophorins I and II (RI and RII), that are closely associated with membrane-bound ribosomes (6, 7), a 34-kDa glycoprotein that may serve as a signal sequence receptor (8, 9) and a complex of six polypeptides that manifests signal peptidase activity (10). In addition, other proteins involved in modifying nascent chains by glycosylation (11), hydroxylation of proline residues (12), or disulfide rearrangement (13, 14) must also be closely associated with the translocation apparatus. Except for the SRP receptor, which participates in the targeting process and is only transiently associated with the site of translocation (15), the other putative components of the translocation apparatus appear in a 1:1 molar ratio with bound ribosomes and, therefore, are probably part of the macromolecular assembly that effects translocation itself and is largely restricted to the rough domains of the endoplasmic reticulum (5–7, 16).

Elucidation of the function of specific components of the translocation apparatus is likely to require their separation and reassembly into membrane vesicles, the composition of which can be experimentally controlled. As a step towards this goal, we developed a procedure for reconstituting membrane vesicles from octyl β-glucoside-solubilized dog pancreas rough microsomes (RM); these reconstituted vesicles display the capacity of native membranes to translocate and process nascent polypeptides in a cotranslational manner.

MATERIALS AND METHODS

Solutions. Membrane suspension buffer was 50 mM Tris·HCl (pH 7.4)/250 mM sucrose/1 mM dithiothreitol; octyl β-glucoside stock solution was 10% (wt/vol) octyl β-glucoside/0.5 M NaCl.

Reconstitution of Membrane Vesicles from Octyl β-Glucoside-Solubilized Dog Pancreas RM. Dog pancreas RM (17) resuspended in membrane suspension buffer (10 mg of protein per ml; 0°C) were solubilized by adding 1/9 vol of the octyl β-glucoside stock solution. The sample was incubated in ice for 30 min and centrifuged either at low speed in an Eppendorf centrifuge (15,600 × *g* for 15 min) to remove rapidly sedimenting undissolved material (P1 in Table 1, representing only 1% of total protein) or at high speed (400,000 × *g* for 15 min) to remove ribosomes and other macromolecular complexes with sedimentation coefficients >50S (P2 in Table 1). Membrane vesicles were reconstituted from both supernatants (S1 and S2, respectively) by a 30-fold dilution with buffer [50 mM Tris·HCl, pH 7.4/10% (vol/vol) glycerol containing protease inhibitors Leu-Leu-Leu at 1 μg/ml, leupeptin at 0.5 μg/ml, Trasylol at 10 units/ml, and 1 mM dithiothreitol] added dropwise under a constant N_2 stream over a 90-min period while the sample was kept on ice. The reconstituted membrane vesicles were then recovered by sedimentation (110,000 × *g* for 30 min at 4°C). Vesicles obtained from the low-speed supernatant, which contains essentially the total complement of microsomal proteins, are designated R_t, whereas those obtained from the postribosomal supernatant are designated R_s.

Assays for Functional Competence of the Reconstituted Vesicles. Rabbit reticulocyte lysates and wheat germ translation systems were programmed with *in vitro*-transcribed

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Abbreviations: RM, rough microsomes; R_t, membrane vesicles reconstituted from total solubilized RM; R_s, smooth-surfaced membrane vesicles reconstituted from a postribosomal supernatant fraction; RI and RII, ribophorins I and II, respectively; HA, hemagglutinin; SRP, signal-recognition particle; HPL, human placental lactogen.

Table 1. Recovery of protein and cotranslational signal cleavage activity in microsomal subfractions and reconstituted vesicles

Fraction	Protein yield		Cotranslational processing	
	mg	(%)	Activity*, %	Recovery†, %
RM	22.8	(100)	25	100
S1	22.6	(99)	—	—
P1	0.2	(1)	0	0
S2	17.6	(77)	—	—
P2	5.0	(22)	0	0
R_t	4.3	(19)	74	56
R_s	2.7	(12)	4	2

Fractions are as defined in *Materials and Methods*.

*Translocation activity was measured as percentage of pre-HPL nascent chains processed to HPL [HPL/(HPL + pre-HPL); see ref. 18]. For each fraction the cotranslational processing assay contained 5 μg of protein in 25 μl of reticulocyte translation system programmed with total placental RNA. The amount of processin that occurred posttranslationally (<5%) was subtracted in eac case.

†Recovery is calculated as ratio of the product of activity and protein yield of each sample to the same product for native RM.

mRNAs coding for influenza hemagglutinin (HA) (19), RI (20), or with mRNA extracted from human placenta (18). Dog pancreas microsomes and SRP were prepared according to Walter and Blobel (17, 21). When cotranslational translocation was assayed, membranes (5–10 μg of protein when the reticulocyte lysate was used and 0.5–5 μg of protein supplemented with 20 nM purified SRP when the wheat germ extract was used) were added to the translation mixtures (final vol, 25 μl) before starting translation.

Translation of placental or HA mRNA was carried out for 1 hr at 30°C in the reticulocyte system, and translation of RI mRNA was carried out for 1.5 hr at 25–26°C in the wheat germ system. For posttranslational assays, after translation was done in the absence of membranes, the sample was incubated with 3 mM 7-methylguanosine 5′-phosphate for 0.5 hr at the translation temperature. Membranes were then added, and the incubation was continued at the same temperature and for the same length of time as in the cotranslational assay.

RESULTS

Solubilization of Dog Pancreas RM by Octyl β-Glucoside. Addition of 1% octyl β-glucoside to a suspension of dog pancreas RM (10 mg of protein per ml) in low-salt buffer efficiently solubilized the membranes. Solubilization was apparent from the immediate decrease in turbidity and the fact that centrifugation for 15 min in an Eppendorf centrifuge (15,600 × *g*), a procedure that leads to quantitative recovery of native RM, removed <1% of total microsomal protein (P1 in Table 1). A sediment (P2) obtained from the resulting supernatant (S1) by high-speed centrifugation mainly contained ribosomal particles (Fig. 1*D*), although Western (immunologic) blotting analysis (data not shown) indicated that substantial amounts of RI, RII, and the α subunit of the SRP receptor also sedimented with the ribosomes.

The state of aggregation of the proteins in the detergent-solubilized membranes was assessed by sucrose gradient centrifugation analysis of solubilized microsomes from which luminal-content proteins and ribosomes had been removed before solubilization. SDS gel electrophoretic and Western blotting analysis of the sucrose density gradient fractions (Fig. 2) showed that many membrane proteins, including the α subunit of the SRP receptor, RI, and RII were distributed throughout the gradient. This result suggests that, in the native membrane, components of the translocation apparatus may be part of large macromolecular complexes that are only partially disassembled by the detergent. Indeed, immunoprecipitates obtained with anti-RI antibody from fractions from the lower half of the gradient contained, in addition to RI, both RII and the α subunit of the SRP receptor (data not shown).

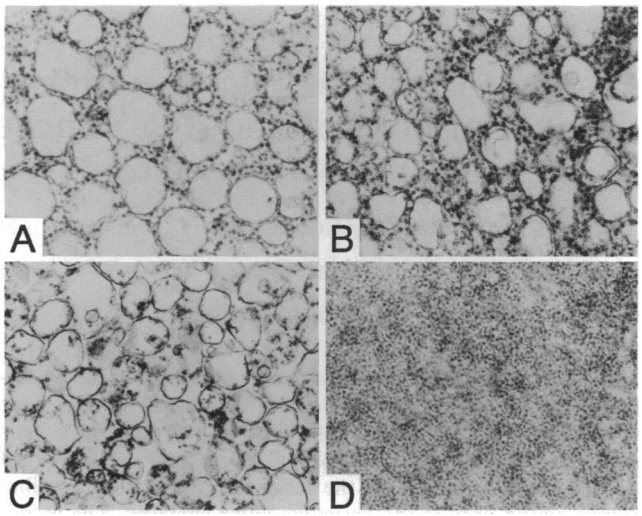

FIG. 1. Electron micrographs of native RM and reconstituted membrane vesicles. (*A*) Native dog pancreas RM; (*B*) R_t vesicles; (*C*) R_s vesicles; (*D*) Sediment (P2) containing particles (mainly ribosomes) removed from the octyl β-glucoside-solubilized RM before reconstitution of R_s. (×21,400.)

Reformation of Membrane Vesicles from Solubilized Dog Pancreas Microsomes by Detergent Dilution. A slow 30-fold dilution of the total octyl β-glucoside-soluble fraction led to the reformation of membrane vesicles (R_t) that had ribosomes attached only on the outer surface of their membranes (Fig. 1*B*). These vesicles contained 19% of the total amount of the microsomal protein (Table 1). As expected, smooth vesicles (R_s) devoid of ribosomes (Fig. 1*C*) were obtained when the same procedure was applied to a supernatant obtained after high-speed centrifugation of the octyl β-glucoside-solubilized microsomes containing 77% of total microsomal protein. The R_s vesicles contained 12% of the initial microsomal protein. Both R_t and R_s vesicles had an average diameter of ≈200 nm (Fig. 1).

The membranes of R_t and R_s (Fig. 3*A*), have similar protein compositions and differ from native RM in their lack of content proteins, which normally represent ≈25% of RM protein (25). In fact, the Coomassie blue staining pattern of R_t vesicles was very similar to that of RM pretreated with 0.05% deoxycholate to release content proteins (data not shown). Western blotting analysis showed that both types of reconstituted vesicles contain the α subunit of the SRP receptor, RI, and RII (Fig. 3 *B* and *C*) in slightly higher concentrations than in native RM.

Functionality of the Reconstituted Vesicles. The capacity of the reconstituted vesicles to effect signal cleavage, translocation, and cotranslational glycosylation of nascent polypeptides was assessed in *in vitro* translation systems programmed with various mRNAs. Human placental lactogen (HPL) was used to obtain a quantitative measure of cotranslational signal cleavage, because pre-HPL and HPL can be well separated by gel electrophoresis, and the translocation of HPL is not accompanied by other cotranslational modifications that could complicate interpretation of the electrophoretic patterns of the protein products. R_t vesicles were found to have a 2- to 3-fold higher capacity to effect cotranslational signal cleavage of nascent pre-HPL molecules than RM, whereas R_s were inactive in this assay (Table 1). The enhanced specific activity of R_t relative to RM can be

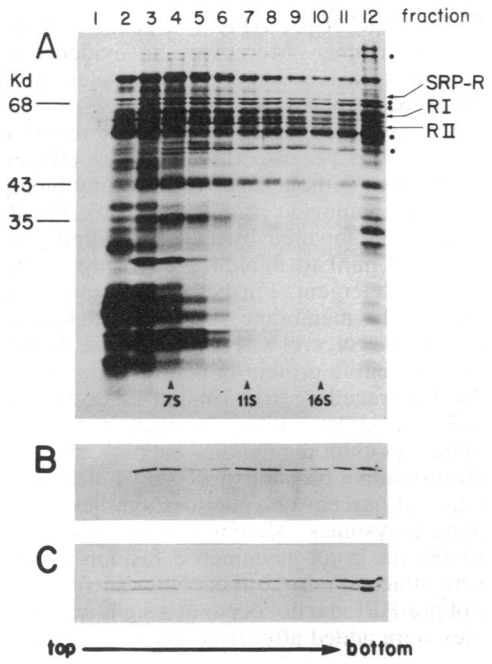

FIG. 2. Analysis of detergent-solubilized dog pancreas microsomes by rate zonal centrifugation. Dog pancreas RM stripped of ribosomes with EDTA in a high-salt medium (22) were resuspended in membrane suspension buffer at protein concentration 10 mg/ml, and content proteins were removed (23) by adding octyl β-glucoside to a final concentration of 0.2%, followed by centrifugation (100,000 × *g* for 30 min). Sedimented membrane fraction was resuspended in membrane suspension buffer (10 mg/ml) and solubilized by adding 1/9 vol of 10% octyl β-glucoside/0.5 M NaCl. After 30 min on ice, a sample (2.5 mg of protein) was loaded onto sucrose gradient (5–20%) prepared over 2.25 M sucrose cushion. All sucrose solutions contained 50 mM Tris (pH 7.4), the mixture of protease inhibitors, and 0.8% octyl β-glucoside. After centrifugation in an SW41 rotor at 35,000 rpm for 20 hr, the gradient was fractionated. (*A*) Aliquots (15 μl) of each fraction were analyzed by electrophoresis in 12% SDS-containing polyacrylamide slab gel followed by silver staining. (*B* and *C*) Aliquots of each fraction (50 μl) were also analyzed by Western blotting with anti-SRP receptor (α subunit) antibody (*B*), or a combination of anti-RI and anti-RII antibodies (*C*). Dots in *A* indicate those membrane proteins, other than RI, RII, and the α subunit of the SRP receptor (arrows), distributed throughout the gradient.

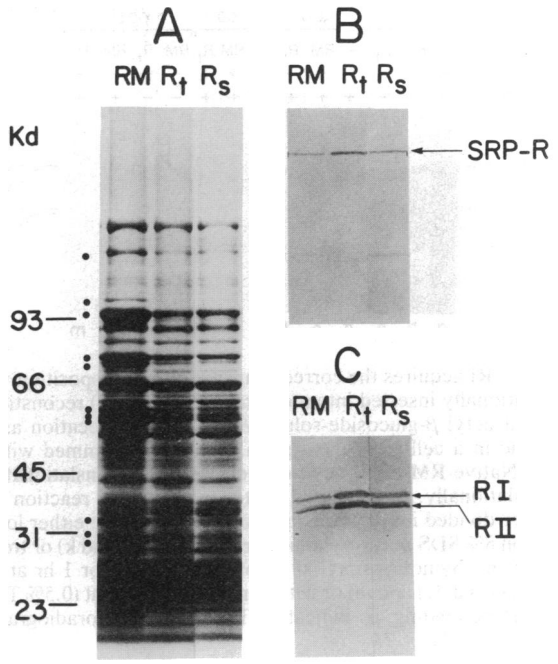

FIG. 3. Protein composition of native RM and reconstituted membrane vesicles. Native RM, R_t, and R_s were stripped of ribosomes by the puromycin–KCl procedure (24), and aliquots (50 μg of protein) were analyzed by electrophoresis in a 6–12% polyacrylamide gradient slab gel. (*A*) Coomassie blue staining; (*B* and *C*) Western blots using polyclonal anti-SRP receptor antiserum (*B*) or a mixture of monoclonal anti-RI and anti-RII antibodies (*C*). Dots in *A* mark bands largely absent from the reconstituted membrane vesicles and represent RM content proteins. Arrows in *B* and *C* indicate α subunit of the SRP receptor (SRP-R), RI, and RII.

partially accounted for by the higher content of membrane proteins, such as RI, RII, and SRP receptor in R_t. The lack of activity of R_s is surprising, given the fact that these vesicles are nearly identical in protein composition to R_t (Fig. 3). It, therefore, appears that ribosomes, or other rapidly sedimenting components from the octyl β-glucoside-solubilized microsomal preparation are essential for reformation of translocation-competent vesicles.

The capacity of reconstituted vesicles to cotranslationally glycosylate and translocate nascent polypeptides was studied by using translation systems programmed with mRNAs for influenza HA (Fig. 4) or for RI (Fig. 5). These are type I membrane glycoproteins that contain cleavable insertion signals, acquire N-linked oligosaccharides during translocation, and, after translocation, remain membrane-anchored with large luminal segments inaccessible to the attack of added proteases (cf. 19, 20). With HA mRNA as template, two glycosylated products (Fig. 4, lanes b and c), migrating significantly more slowly than the pre-HA synthesized in the absence of membranes (lane a), were seen when translation was done with either RM (lane b) or R_t (lane c), but not when translation was done with R_s (lane d) or when any vesicles were added posttranslationally (lanes e–g). The proportion of glycosylated molecules was similar when either RM or R_t were used (compare lanes b and c), although R_t more significantly inhibited translation than RM and, therefore, yielded smaller amounts of glycosylated products. After membrane insertion only a small segment of HA (11 amino acid residues) remains exposed on the cytoplasmic side of the membrane (26). Hence, incubation with proteases causes no significant decrease in size of the glycosylated HA molecules inserted *in vitro* into native RM (lane h). Despite their high capacity to effect cotranslational glycosylation, R_t vesicles were appar-

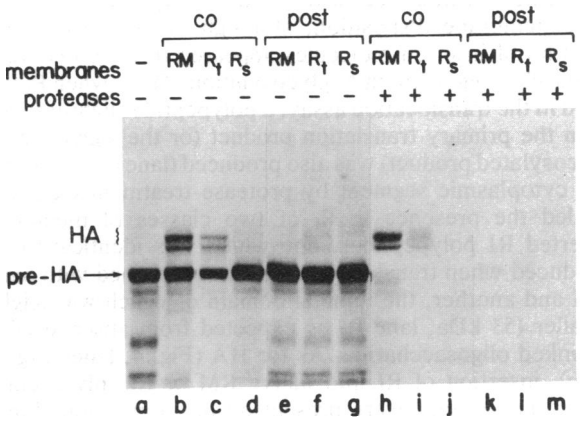

FIG. 4. R_t but not R_s are capable of cotranslational glycosylation and translocation of HA. Translocation assays were done in a rabbit reticulocyte protein synthesis system programmed with HA mRNA. Native RM, R_t, or R_s membrane vesicles were added cotranslationally (co) or posttranslationally (post), as indicated. After incubation for co- and posttranslational translocation, equal portions of each sample were either loaded directly or digested with a trypsin/chymotrypsin mixture (100 μg of each per ml) for 1 hr at 0°C, as indicated, before analysis by SDS/8% PAGE and autoradiography (12 hr).

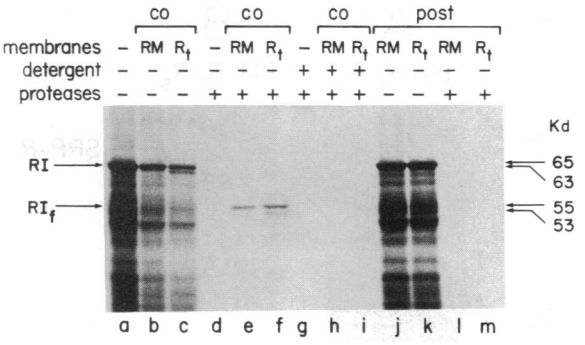

FIG. 5. RI acquires the correct transmembrane disposition when cotranslationally inserted into membrane vesicles (R_t) reconstituted from total octyl β-glucoside-solubilized RM. Translocation assays were done in a cell-free wheat germ system programmed with RI mRNA. Native RM or R_t vesicles were added cotranslationally or posttranslationally, as indicated. After incubations, reaction mixtures were divided into three equal portions that were either loaded directly on 8% SDS polyacrylamide gel (lanes a–c, j and k) or treated with trypsin/chymotrypsin (100 μg of each per ml for 1 hr at 0°C) without (lanes d–f, l and m) or with (lanes g–i) detergent (0.5% Triton X-100) before loading, as indicated. The gel was autoradiographed for 24 hr. Kd, kDa.

ently less effective than native RM in protecting the luminal portion of the glycosylated HA molecules from protease attack (compare lanes c and i). Thus, some glycosylated molecules may be translocated into reconstituted vesicles that are more fragile than native RM or into vesicles that are not totally sealed.

With RI mRNA as template (Fig. 5), we could determine that translocation with R_t leads to the normal anchoring of polypeptide in membrane. Luminal and cytoplasmic domains of RI consist of 414 and 150 amino acid residues, respectively (20). Cotranslational cleavage of the insertion signal sequence in RI is normally accompanied by addition of a single N-linked oligosaccharide, so that no net change in M_r is seen (27). RI molecules synthesized in the presence of native RM are properly inserted in the membrane so that treatment of RM with proteases leads to the same reduction in size of the polypeptide (from 65 to 55 kDa; Fig. 5, lanes b and e) that is seen with microsomes containing RI molecules inserted *in vivo* (Y.Y., D.D.S., and G.K., unpublished work; 27). RI molecules synthesized with R_t also acquired the normal transmembrane disposition, although in this case a small fraction of inserted molecules appeared to have undergone signal cleavage without N-glycosylation. Thus, when R_t were used in the translocation assay, a polypeptide slightly smaller than the primary translation product (or the signal-cleaved glycosylated product) was also produced (lane c). Removal of the cytoplasmic segment by protease treatment clearly revealed the presence in R_t of two classes of membrane-inserted RI polypeptides, one which was identical to that produced when translocation was accomplished with native RM and another, the luminal domain of which was slightly smaller (53 kDa, lane f), as expected from absence of the N-linked oligosaccharide. As for HA (Fig. 4, lanes e–g and k–m), insertion of RI into native RM or R_t only occurred when the vesicles were present during translation (Fig. 5, lanes j–m).

DISCUSSION

In previous work we attempted to reconstitute functional microsomal membranes from rat liver RM dissolved with the detergent sodium deoxycholate, by using a dialysis procedure to remove the detergent (28). Although single-walled vesicles were obtained by this method, the membranes were not asymmetrically reconstituted because they could effect the signal cleavage of pre-HPL in a post-, as well as in a cotranslational manner. Moreover, no evidence could be obtained for the translocation of the signal-cleaved HPL into the lumen of the reconstituted vesicles.

In the reconstitution procedure of this report octyl β-glucoside was used because this detergent efficiently dissolves membranes without inactivating many biological activities of their component proteins (29, 30). The detergent concentration was lowered by dilution within a relatively short time (≈90 min) to minimize exposure of membrane components to detergent. Furthermore, protease inhibitors were added to the membrane suspension before detergent solubilization, and glycerol was included in the dilution buffer to stabilize the native protein structure (31). The R_t reconstituted by this procedure from solubilized dog pancreas RM manifested several key activities characteristic of rough endoplasmic reticulum membrane—namely, the capacity to effect cotranslational translocation, signal cleavage, and N-glycosylation of nascent polypeptides synthesized on membrane-bound polysomes. Membranes of these vesicles appeared to reform in an asymmetric fashion because ribosomes were attached only to their outer surface, and signal cleavage of pre-HPL did not occur at a significant level when the vesicles were added after translation was completed.

By various criteria, such as signal cleavage of pre-HPL, glycosylation of influenza HA, and sequestration of the luminal domain of RI, activity of the reconstituted vesicles (R_t) for a fixed amount of vesicle protein was greater than or equal to activity of native RM. It was apparent, however, that not all cotranslational processes took place with equal efficiency. Thus, not all signal-cleaved and membrane-inserted RI molecules were glycosylated when translocation was accomplished by reconstituted vesicles. This result may stem either from partial loss or inactivation of the oligosaccharyltransferase or from incomplete reassociation of this enzyme with the translocation apparatus, which, based on our observations, does not require the oligosaccharyltransferase activity for function.

Strikingly, membrane vesicles (R_s) reconstituted from post-ribosomal supernatant from the detergent-treated microsomes were essentially inactive in cotranslational processing of nascent polypeptides. Although a fraction of the rough endoplasmic reticulum-specific proteins, including RI, RII and the α subunit of the SRP receptor, was removed from the supernatant with the sedimented ribosomes, the membranes of R_s vesicles were, nevertheless, compositionally nearly identical to those of R_t and contained at least as much of the aforementioned three proteins as native RM. Although it is conceivable that an as-yet-unidentified critical polypeptide was totally removed with the ribosomes, more probably the restoration of translocation function during reconstitution requires ribosomes and/or the integrity of sedimentable large protein complexes that are part of the translocation apparatus and remain intact after detergent treatment. Indeed, from analyzing fractions obtained by sucrose gradient sedimentation, many proteins in the detergent-solubilized microsomes clearly remain within oligomeric complexes with different degrees of aggregation. Perhaps only when complexes with the highest degree of aggregation (those removed with the ribosomes) are incorporated in the reassembled membranes, do these vesicles regain their translocation capacity. During reconstitution, ribosomes and/or attached undissociated membrane components may serve as nucleation sites for the asymmetric reassembly of the membrane and for the proper reincorporation of essential components into the translocation apparatus.

The functional reconstituted vesicles lacked the microsomal content proteins, which remained soluble after dilution of the detergent. We can, therefore, conclude that these proteins are not essential for the translocation and cotransla-

tional processing activities of rough endoplasmic reticulum membrane. Recently, the oligosaccharyltransferase activity of hen oviduct RM, assayed with an exogenous tripeptide substrate, has been demonstrated to require participation of a luminal protein that binds to the glycosylation recognition site and can be released from microsomes by sonication or treatment with the detergent saponin (32). Our finding that the vesicles reconstituted from dog pancreas microsomes have substantial N-glycosylation activity suggests that, at least in these microsomes, all components of the oligosaccharyltransferase complex are in some form (perhaps indirectly through a peripheral protein type of interaction) associated with membrane constitutents. Also the cotranslational glycosylation of a nascent chain could require much lower levels of the "glycosylation site-binding protein" than the glycosylation of soluble exogenous tripeptide substrates.

Recently, other investigators (33) have reported the reconstitution of membranes capable of ribosome binding from a nonglycoprotein subfraction of rat liver RM that was incorporated into liposomes. Although this work allowed the authors to exclude the possibility that glycoproteins are essential for ribosome binding, no other functional activity of the vesicles was shown.

Our results represent an advance toward the goal of complete reconstitution of a functional translocation apparatus from specific sets of its components. Whether it will be possible to obtain functional vesicles reconstituted from purified individual components or whether the integrity of large molecular complexes must be maintained to reestablish translocation in reconstituted vesicles, however, remains to be determined. Indeed, it is possible that assembly of a functional translocation apparatus can only occur within the endoplasmic reticulum membrane from components incorporated into the membrane during or soon after their synthesis.

We thank Dr. P. Walter (University of California at San Francisco) for his gift of anti-SRP receptor antibodies, P.-V. Luc for SRP, I. Gumper for the electron microscopy, and J. Culkin and H. Plesken for the illustrations. We also give special thanks to Dr. M. Adesnik for his help in preparing the manuscript. This work was supported by National Institutes of Health Grants GM21971 and GM20277 and by the Cancer Center Core Support Grant P30CA-16087.

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