# Formation of a Functional Ribosome–Membrane Junction during Translocation Requires the Participation of a GTP-binding Protein

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*Abstract.* The requirement for ribonucleotides and ribonucleotide hydrolysis was examined at several distinct points during translocation of a secretory protein across the endoplasmic reticulum. We monitored binding of in vitro–assembled polysomes to microsomal membranes after removal of ATP and GTP. Ribonucleotides were not required for the initial low salt–insensitive attachment of the ribosome to the membrane. However, without ribonucleotides the nascent secretory chains were sensitive to protease digestion and were readily extracted from the membrane with either EDTA or 0.5 M KOAc. In contrast, nascent chains resisted extraction with either EDTA or 0.5 M KOAc and were insensitive to protease digestion after addition of GTP or nonhydrolyzable GTP analogues. Translocation of the nascent secretory polypeptide was detected only when ribosome binding was conducted in the presence of GTP. Thus, translocation-competent binding of the ribosome to the membrane requires the participation of a novel GTP-binding protein in addition to the signal recognition particle and the signal recognition particle receptor. The second event we examined was translocation and processing of a truncated secretory polypeptide. Membrane-bound polysomes bearing an 86-residue nascent chain were generated by translation of a truncated preprolactin mRNA. Ribonucleotide-independent translocation of the polypeptide was detected by cleavage of the 30-residue signal sequence after puromycin termination. Nascent chain transport, per se, is apparently dependent upon neither ribonucleotide hydrolysis nor continued elongation of the polypeptide once a functional ribosome–membrane junction has been established.

THE amino-terminal signal sequence of a secretory protein contains sufficient information to direct the specific attachment of ribosomes to the rough endoplasmic reticulum (RER),[1] to promote vectorial transport of the nascent polypeptide across the membrane bilayer, and to specify endoproteolytic cleavage by signal peptidase (20). The process of protein translocation can be regarded as a sequential series of events beginning with initiation of protein synthesis upon free ribosomes within the cytoplasm and ending with folding of the translocated polypeptide inside the lumen of the RER (4, 5). Some, if not all, of the intervening events are mediated by protein translocation components which function to decode the information contained in the RER signal sequence (3). Translation of an mRNA containing an RER signal sequence induces high affinity binding of the signal recognition particle (SRP) (38) to the ribosome via a direct interaction between the 54-kD polypeptide of SRP and the signal sequence (18). High affinity binding of SRP often induces a site-specific elongation arrest of translation (25, 36). The SRP receptor (13) or docking protein (25) functions as an endoplasmic reticulum–specific (16) receptor for the SRP–ribosome complex. The resultant attachment of the ribosome to the membrane is accompanied by SRP displacement from the ribosome by the SRP receptor (10) and by a direct interaction between the signal sequence and a membrane component (11). Thus, current evidence indicates that ribosome binding requires at a minimum the participation of SRP (35, 36, 38) and the SRP receptor (10, 25, 33).

An essential role for the ribosome during nascent chain translocation was initially implied by the obligate coupling between synthesis and translocation (5). Recently, ribosome-dependent posttranslational translocation and membrane integration of polypeptides has been elegantly demonstrated (26, 29), and found to be dependent upon the inclusion of ribonucleotides (29). Puromycin-induced release of the nascent polypeptide from the ribosome before the addition of microsomal membranes prevented posttranslational translocation (29).

We have now determined whether ribonucleotides are essential for several distinct events in translocation of proteins across the endoplasmic reticulum. The first translocation event we examined corresponded to the binding of SRP-arrested polysomes to microsomal membrane vesicles. A synchronized wheat germ translation system containing SRP was programmed with prolactin mRNA to produce elongation-arrested polysomes. After gel filtration to remove ribonucleotides, the elongation-arrested polysomes were incubated with salt-extracted (i.e., SRP-depleted) microsomal

1. *Abbreviations used in this paper*: AF, arrested fragment; AMPPNP, adenylyl-5′-imidodiphosphate; GMPPNP, guanylyl-5′-imidodiphosphate; K-RM, salt-extracted microsomal membranes; PPO, diphenyloxazole; RER, rough endoplasmic reticulum; SRP, signal recognition particle.

membranes (K-RM) to allow polysome binding either in the presence or absence of added ribonucleotides. The formation of a functional ribosome–membrane junction was assayed by several different criteria including EDTA extraction, protease protection, and puromycin-dependent translocation and processing. Our results demonstrated an essential role for GTP as a cofactor during the formation of a functional ribosome–membrane junction. Thus, the protein translocation apparatus of the endoplasmic reticulum contains a novel GTP-binding protein. The second stage of translocation corresponded to the transit of a nascent polypeptide across the membrane bilayer after termination of protein synthesis. A truncated mRNA containing the first 86 codons of preprolactin was translated in the presence of SRP and K-RM to yield a partially translocated polypeptide (pPL-86). After ribonucleotide depletion, puromycin was added to induce nascent chain termination. Ribonucleotide-independent translocation of pPL-86 was accompanied by removal of the 30-residue signal sequence to yield PL-56. Nascent chain transit across the membrane bilayer is apparently neither coupled to ribonucleotide hydrolysis nor dependent upon the continued elongation of the polypeptide chain.

## Materials and Methods

### Materials

[$^{35}$S]methionine (1,000 Ci/mmol) was obtained from New England Nuclear (Boston, MA). The nonionic detergent Nikkol (octaethyleneglycol-mono-*N*-dodecyl ether) was obtained from Nikko Chemical Co., Ltd. (Tokyo, Japan). Protease inhibitors, cycloheximide, emetine dihydrochloride, and 7-methylguanosine-5′-monophosphate were from Sigma Chemical Co. (St. Louis, MO). Sephacryl S-200, Sepharose CL-2B, guanylyl-5′-imidodiphosphate (GMPPNP) and adenylyl-5′-imidodiphosphate (AMPPNP) were from Pharmacia Fine Chemicals (Piscataway, NJ). Puromycin dihydrochloride, ATP, GTP, guanylyl (β, γ methylene)-diphosphonate, GDP, guanosine-5′-O-(2-thiodiphosphate), dGTP, and calf liver tRNA were from Boehringer Mannheim Biochemicals (Indianapolis, IN). The vector pGEM-4 and RNasin (placental RNase inhibitor) were from Promega Biotec (Madison, WI), antibody to sheep prolactin was from United States Biochemical Corp. (Cleveland, OH).

### Preparation of Microsomal Membranes, SRP, and Salt-extracted Microsomal Membranes

The triethanolamine buffer used in all preparative and analytical procedures was prepared as a 1 M stock solution adjusted with acetic acid to pH 7.5 at 25°C, and is referred to as TEA. SRP and K-RM were prepared from canine pancreas rough microsomal membranes as described previously (38).

### Cell-free Protein Synthesis

The standard 100-μl cell-free translation system contained 30 μl of staphylococcal nuclease–treated wheat germ S 23 (8), 100 μCi of [$^{35}$S]methionine, human placental RNase inhibitor, and a mixture of protease inhibitors described previously (38). All synchronized cell-free translation systems were adjusted to 140 mM KOAc, 2.5 mM $Mg(OAc)_2$. Translations containing SRP were supplemented with 0.002% Nikkol to stabilize SRP activity (38). 100-μl synchronized translations were incubated at 25°C for 2 min before the addition of 0.4 U, $A_{260}$, of bovine pituitary RNA. After an additional 2 min of incubation at 25°C, the translation was adjusted to 2 mM 7-methylguanosine-5′-monophosphate. Elongation-arrested polysomes were allowed to accumulate during an additional 8 min of synthesis at 25°C. After considering several methods for removing ribonucleotides from the elongation-arrested polysomes, we chose gel filtration chromatography in preference to enzymatic hydrolysis of ribonucleotide triphosphate pools. Preliminary experiments using [α-$^{32}$P]GTP revealed a residual GTP content in excess of 10 μM in translation reactions treated with hexokinase and glucose as assayed by thin layer chromatography on polyethyleneimine cellulose. The translation was adjusted to 250 μm cycloheximide to prevent further protein synthesis and chilled on ice before fractionation at 4°C using a 1.0-ml Sephacryl S-200 column equilibrated with 50 mM TEA, 150 mM KOAc, 2.5 mM $Mg(OAc)_2$, 0.002% Nikkol, 1 mM dithiothreitol (DTT). The void volume fraction (175 μl) containing elongation-arrested polysomes was collected and adjusted to 250 μM cycloheximide (or 1 mM emetine) before incubation with K-RM.

The plasmid pSP6BP3 was provided by Drs. William Hansen and Peter Walter, (University of California, San Francisco). pSP6BP3 contains a bovine preprolactin cDNA insert derived from pBRPL72 (32), downstream from the 5′ untranslated region of *Xenopus* β-globin in the plasmid pSP64T (17). Digestion of pSP6BP3 with Hind III and Pst I yielded a 945-base pair (bp) fragment containing both sequences which we inserted in the plasmid pGEM-4 to obtain pGEMBP1. T7 RNA polymerase was purified from the bacterial strain BL21/pAR 1219. The purification method for T7 RNA polymerase and BL21/pAR 1219 was generously provided by Dr. William Studier (Brookhaven National Laboratories, Upton, NY). The plasmid pGEMBP1 was linearized with the restriction enzyme Pvu II and transcribed at a concentration of 0.125 mg/ml in 40 mM Tris-Cl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 12.5 mM NaCl, 10 mM DTT, 0.5 mM each of ATP, GTP, CTP, and UTP, 0.5 U/μl RNase inhibitor, and 15 μg/ml of T7 RNA polymerase. After transcription, mRNA was purified by phenol-chloroform extraction and by precipitation with ethanol, and lithium chloride. The mRNA transcript was translated at a concentration of 300 ng/25 μl reaction. Translations containing mRNA transcripts were supplemented with calf liver tRNA at a final concentration of 200 μg/ml.

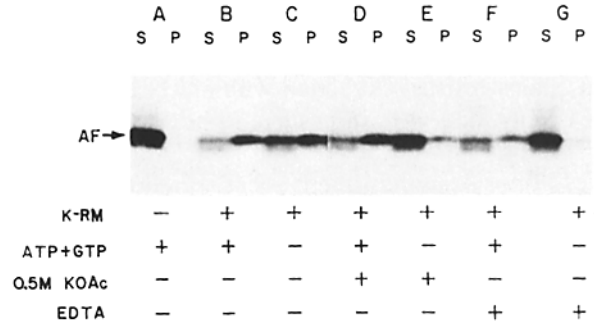


*Figure 1.* Ribonucleotide-dependent binding of elongation-arrested polysomes to K-RM. Bovine pituitary RNA was translated for 10 min in a synchronized wheat germ system supplemented with 16 nM SRP to assemble elongation-arrested polysomes. A 100-μl aliquot of the translation was adjusted to 250 μM cycloheximide, chilled on ice, and applied to a 1-ml Sephacryl S-200 gel filtration column equilibrated as described in Materials and Methods. The 175-μl void volume fraction containing elongation-arrested polysomes was adjusted to 250 μM cycloheximide, and divided into aliquots for incubation with K-RM. (*A*) A 25-μl aliquot containing 2.5 mM ATP and 1.0 mM GTP but no K-RM was fractionated using a physiological salt (150 mM $K^+$, 2.5 mM $Mg^{++}$) sucrose step gradient into supernatant (*S*) and pellet (*P*) fractions as described in Materials and Methods. (*B–G*) Two 75-μl aliquots of the ribonucleotide-depleted polysomes were incubated with K-RM (9 equivalents/75 μl; equivalents [eq] are defined in reference 38) for 5 min at 25°C either in the absence (*C*, *E*, and *G*) or presence (*B*, *D*, and *F*) of 2.5 mM ATP and 1.0 mM GTP, and then each binding reaction was divided into three equal aliquots. A pair of aliquots from each binding reaction (*B* and *C*) was fractionated as in *A*. A second pair of aliquots (*D* and *E*) was adjusted to high salt (500 mM KOAc, 5.0 mM $Mg[OAc]_2$), incubated for 10 min at 0°C, and fractionated on a high salt sucrose step gradient. The third pair of aliquots (*F* and *G*) were adjusted to 25 mM EDTA, incubated for 10 min at 0°C, and fractionated on an EDTA sucrose step gradient. The radioactive band corresponding to the arrested fragment (*AF*) was resolved on a 12–17% gradient polyacrylamide gel in SDS and visualized by fluorography of the diphenyloxazole (PPO)-impregnated gel.

### *Fractionation of Translation Products on Sucrose Step Gradients*

Translation products from membrane-binding experiments were separated into a membrane-bound or pellet (P) fraction and a free or supernatant (S) fraction by centrifugation at 4°C in a Beckman airfuge using a A-100/30 rotor (Beckman Instruments, Inc., Fullerton, CA). The binding reactions were layered over a sucrose cushion (for composition, see below) before centrifugation. The centrifugation times listed do not include the 10-s acceleration time or the 2-min deceleration period. After centrifugation, the supernatant including the cushion was removed and precipitated with an equal volume of 20% TCA. The pellet fraction was dissolved in 10 μl of 0.5 M Tris base, 6.25% SDS. The sample resuspension method removes tRNA from nascent polypeptides in both the supernatant and pellet fractions.

***Physiological Salt Step Gradient.*** 50 μl cushion of 0.5 M sucrose, 50 mM TEA, 150 mM KOAc, 2.5 mM $Mg(OAc)_2$, 1 mM DTT. Centrifugation was for 3 min at 20 psi.

***High Salt Step Gradient.*** 50 μl cushion of 0.5 M sucrose, 50 mM TEA, 500 mM KOAc, 5 mM $Mg(OAc)_2$, 1 mM DTT. Centrifugation was for 4 min at 20 psi.

***EDTA Step Gradient.*** 50 μl cushion of 0.25 M sucrose, 50 mM TEA, 150 mM KOAc, 25 mM EDTA, 1 mM DTT. Centrifugation was for 5 min at 20 psi.

### *Protease Digestion of Translation Products*

Translation products were chilled on ice and adjusted to a volume of 50 μl in 50 mM TEA, 150 mM KOAc, 2.5 mM $Mg(OAc)_2$ either in the presence or absence of 1% Triton X-100. Freshly prepared stock solutions of proteinase K in the above buffer were added to the translation products to obtain final protease concentrations of between 25 and 1,000 μg/ml. After 1 h of digestion on ice, the samples were adjusted to 2 mM PMSF for 15 min at 0°C to inactivate the proteinase K.

### *General Methods*

Antibody raised against sheep prolactin was used to immunoprecipitate prolactin-specific translation products (21). Peptidyl-tRNA was precipitated with cetyltrimethylammonium bromide as described previously (11). The method for preparation of samples for SDS gel electrophoresis and subsequent fluorography (21) has been described previously. Quantitation of radioactivity in specific polypeptides was accomplished as described previously (39).

## *Results*

### *Ribonucleotides Are Essential during Ribosome Binding*

The assembly of a ribosome–membrane junction has been examined (11) using a synchronized wheat germ translation system supplemented with SRP and salt-extracted microsomal membranes (K-RM). In a wheat germ translation system, SRP induces a site-specific elongation arrest of preprolactin synthesis (36) to produce a discrete nascent polypeptide termed the arrested fragment (AF) or 70 mer (10, 36). The addition of K-RM to elongation-arrested polysomes reconstitutes the ribosome-binding event (10) and the subsequent translocation of mature prolactin (36). Because ribosome binding is not dependent upon continued protein synthesis (10, 11), we could separate ribonucleotides from preassembled polysomes to directly address the role of ATP and GTP during subsequent events in protein translocation.

SRP-arrested polysomes were allowed to accumulate during 10 min of synthesis in a 100-μl synchronized wheat germ system before removal of ATP and GTP by gel filtration chromatography. The ribonucleotide-depleted polysomes were recovered in the excluded volume fraction from a Sephacryl S-200 column. After adjustment to 250 μM cycloheximide to prevent further protein synthesis, the in vitro–assembled polysomes were incubated with microsomal membranes at 25°C either in the absence or presence of 2.5 mM ATP and 1 mM GTP. The ribosome-binding reactions were fractioned by differential centrifugation using an airfuge into a membrane-bound (pellet, P) fraction and into an unbound (supernatant, S) fraction as described previously (11). The two fractions were recovered after centrifugation and analyzed by SDS PAGE. Ribosome binding to the membrane was indicated by the presence of AF in the pellet fraction when K-RM were included (Fig. 1 *B*, cf. *S* and *P*), but not when K-RM were deleted (Fig. 1 *A*, cf. *S* and *P*). An unbound polypeptide co-migrating with the AF (Fig. 1 *B*, *S*) is a completed translation product of bovine pituitary RNA unrelated to prolactin (11). A significant difference in ribosome binding was not detected upon deletion of ATP and GTP when the binding reactions were fractionated under physiological salt conditions (cf. Fig. 1 *B* with 1 *C*). After binding to the membrane in the presence of ribonucleotides, the arrested fragment continued to sediment with the membrane fraction after high salt extraction (0.5 M KOAc, Fig. 1 *D*) or after disruption of the ribosome with 25 mM EDTA (Fig. 1 *F*). Binding of elongation-arrested polysomes to K-RM in samples supplemented with ATP and GTP was indistinguishable by these extraction criteria from that observed previously using unfractionated translation reactions (11). Entirely different results were obtained when binding assays lacking ribonucleotides were analyzed by these latter two extraction criteria. A substantial proportion of the arrested polysomes were sensitive to high salt extraction when ribonucleotides were not included in the binding mixture (cf. Fig. 1 *D*, lane *P*, with Fig. 1 *E*, lane *P*). The salt-sensitive attachment observed without ribonucleotides was consistent with attachment of AF, in this case, being mediated primarily by the ribosome and not by insertion of the nascent polypeptide into the membrane (1, 11, 14). To confirm this proposal, membrane-bound polysomes were treated with EDTA before fractionation. Disruption of the ribosome with EDTA (31) completely extracted AF from the membrane when ribonucleotides were deleted from the binding mixture (cf. Fig. 1 *F*, lane *P*, with Fig. 1 *G*, lane *P*).

### *GTP or Hydrolysis-resistant GTP Analogues Promote Tight Ribosome Binding*

EDTA extraction clearly discriminated between two forms of membrane-bound ribosome: a "loosely" bound ribosome obtained without ribonucleotides and a "tightly" bound ribosome obtained when ATP and GTP were included. To further define the ribonucleotide requirement, we incubated elongation-arrested polysomes with K-RM in binding mixtures supplemented with individual ribonucleotides. After a 5-min incubation at 25°C, the samples were treated with EDTA to remove loosely bound ribosomes and fractionated by differential centrifugation. The inclusion of ATP (1.75 mM) in the ribosome-binding assay led to a marginal increase in the quantity of membrane-bound AF compared to the control assay lacking ribonucleotides (compare Fig. 2 *A* with Fig. 2 *B*). When GTP was included in the binding assay, the quantity of AF remaining bound after EDTA extraction was comparable to that observed when both ATP and GTP were present (compare Fig. 2 *C* with Fig. 1 *F*). Although GTP was substantially more effective than ATP in promoting tight ribosome binding, several questions remained unaddressed.

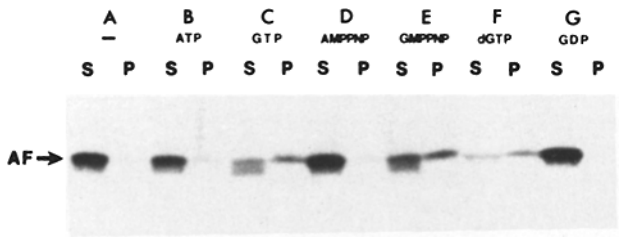


*Figure 2.* Guanine ribonucleotides promote tight ribosome binding. Bovine pituitary RNA was translated for 10 min in a synchronized wheat germ system supplemented with 16 nM SRP. Elongation-arrested polysomes were separated from ribonucleotides as described in Fig. 1. The void volume fraction was adjusted to 250 μM cycloheximide and divided into 25-μl aliquots for a 5-min incubation with K-RM (2.5 eq/25 μl) either in the absence (*A*) or presence (*B–G*) of the following ribonucleotides: (*B*) 1.75 mM ATP, (*C*) 1.75 mM GTP, (*D*) 1.75 mM AMPPNP, (*E*) 1.75 mM GMPPNP, (*F*) 1.75 mM dGTP, (*G*) 1.75 mM GDP. The binding reactions were adjusted to 25 mM EDTA, incubated for 10 min at 0°C, and fractionated on an EDTA–sucrose step gradient. The radioactive band corresponding to AF was resolved on a 12–17% polyacrylamide gradient gel in SDS and visualized by fluorography of the PPO-impregnated gel.

Was hydrolysis of GTP required for the ribosome binding event? Why did ATP appear to be marginally effective? To address these issues we supplemented binding assays with the hydrolysis-resistant imidodiphosphate analogues of ATP and GTP. Adenylyl-5′-imidodiphosphate (AMPPNP) was completely inactive in promoting EDTA-insensitive binding of the 70 mer (Fig. 2 *D*), while the hydrolysis-resistant GTP analogue, guanylyl-5′-imidodiphosphate (GMPPNP), could substitute for GTP (Fig. 2, cf. *C* and *E*). Another hydrolysis-resistant GTP analogue was also tested and found to be active in the binding assay: guanylyl (β, γ methylene)-diphosphonate (data not shown). dGTP promoted AF binding (Fig. 2 *F*), unlike GDP (Fig. 2 *G*) or guanosine-5′-O-(2-thiodiphosphate) (data not shown). The series of experiments using ribonucleotide analogues demonstrated a structural specificity for GTP as a cofactor during ribosome attachment. We can conclude that GTP is neither serving as an energy source nor as a monophosphate donor based upon the activity of hydrolysis-resistant GTP analogues.

The ribonucleotide concentrations used in the previous experiments were comparable to that present in in vitro translation systems, yet substantially higher in the case of GTP than the concentration found in intact cells. If the GTP requirement detected here is physiologically significant, then a lower concentration of a guanine ribonucleotide should elicit an identical result. Because GTP could potentially be hydrolyzed by other components in the ribonucleotide-depleted polysome fraction, we used the hydrolysis-resistant compound GMPPNP to determine the minimum concentration of guanine ribonucleotide needed to promote AF attachment. Ribonucleotide-depleted polysomes were incubated with K-RM and further supplemented with GMPPNP at concentrations ranging between 0.1 μM and 2 mM. The quantity of AF sedimenting with the membrane fraction after EDTA extraction was determined by scintillation counting of the excised AF polypeptide band after resolution on SDS polyacrylamide gels. A GMPPNP concentration of 10 μm yielded identical binding to that obtained with 1.75 mM GMPPNP (cf. Fig. 3 *B* and Fig. 2 *E*), while 1 μM GMPPNP yielded ~40% binding relative to 1.75 mM GMPPNP (data not shown). A low concentration of GTP (i.e., <1 μM) in the ribosome-binding mixture could readily account for the marginal stimulation of binding observed with ATP (Fig. 2 *B*). Low levels of GTP could be present either as a contaminant in the ATP preparation (<0.01% as assayed by the supplier) or as produced by nucleotide diphosphate kinase from ATP and protein-bound GDP.

GTP-binding proteins display an intrinsic affinity for GDP as a competitive inhibitory ligand (28, 41). We analyzed the ability of GDP to inhibit "tight" ribosome binding in assays containing 10 μM GMPPNP. As the concentration of GDP increased from 0.25 μM (Fig. 3 *C*) to 250 μM (Fig. 3 *I*) we observed a pronounced decrease in the quantity of AF which sedimented with the membrane fraction. Scintillation counting of excised gel slices revealed a 50% inhibition of AF binding at a GDP concentration of 1 μM (Fig. 3 *D*). Control experiments were conducted to assess the background sedimentation of AF in binding assays containing no added ribonucleotides (Fig. 3 *A*) or 250 μM GDP (Fig. 3 *J*).

## *Protease Sensitivity of Membrane-bound Nascent Chains*

We had previously proposed that resistance of the 70 mer to EDTA extraction was induced by a direct interaction between the signal sequence and a proteinaceous component of the microsomal membrane (11). Prevention of such an interaction by deletion of GTP should lead to other detectable alterations in the ribosome–membrane junction. Incubation of SRP-arrested polysomes with proteinase K revealed that the 70 mer was relatively sensitive to protease digestion in the absence of K-RM (data not shown). Based upon this preliminary observation, membrane-bound polysomes were subjected to digestion with several different concentrations of proteinase K. After inactivating the protease with PMSF, the 70 mer was recovered by immunoprecipitation with antibody

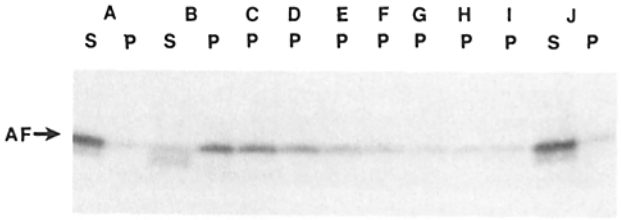


*Figure 3.* Inhibition of ribosome binding by GDP. Bovine pituitary RNA was translated for 10 min in a synchronized wheat germ system supplemented with 16 nM SRP. Elongation-arrested polysomes were separated from ribonucleotides as described in Fig. 1. The void volume fraction was adjusted to 250 μM cycloheximide and divided into 10 aliquots for incubation with K-RM (2.5 eq/25 μl) in the absence (*A* and *J*) or presence (*B–I*) of 10 μM GMPPNP. In addition, binding assays contained GDP at the following concentrations: (*A* and *B*) 0.0 μM, (*C*) 0.25 μM, (*D*) 1.0 μM, (*E*) 2.5 μM, (*F*) 10 μM, (*G*) 25 μM, (*H*) 100 μM, (*I* and *J*) 250 μM. After a 10-min incubation at 25°C, the binding reactions were adjusted to 25 mM EDTA, incubated for 10 min at 0°C, and fractionated on an EDTA–sucrose step gradient. The radioactive band corresponding to AF was resolved on a 12–17% polyacrylamide gel in SDS and visualized by fluorography of the PPO-impregnated gel.

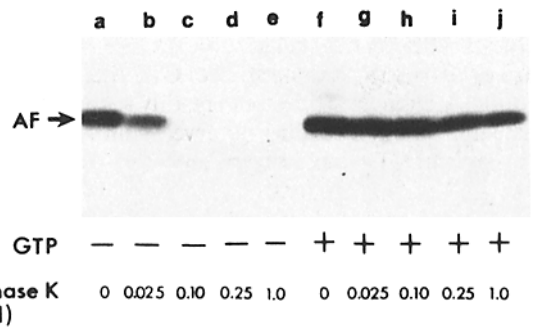


*Figure 4.* Protease resistance of membrane-bound nascent polypeptides. Bovine pituitary RNA was translated for 10 min in a synchronized wheat germ system supplemented with 16 nM SRP. Elongation-arrested polysomes were separated from ribonucleotides as described in Fig. 1. The void volume fraction was adjusted to 250 μM cycloheximide and incubated with K-RM (7.5 eq/75 μl) for 10 min at 25°C either in the absence (lanes *a–e*) or presence (lanes *f–j*) of 1 mM GTP. The 75-μl binding reactions were subdivided into five 15-μl aliquots which were chilled on ice, and diluted to 50 μl with 50 mM TEA, 150 mM KOAc, 2.5 mM $Mg(OAc)_2$. Proteinase K was added to the samples to obtain the concentrations listed in the chart. After a 60-min incubation on ice, proteinase K was inactivated by adjustment to 2 mM PMSF. The arrested fragment (*AF*) was recovered by immunoprecipitation with antibody raised against prolactin, resolved by PAGE in SDS, and visualized by fluorography of the PPO-impregnated gel.

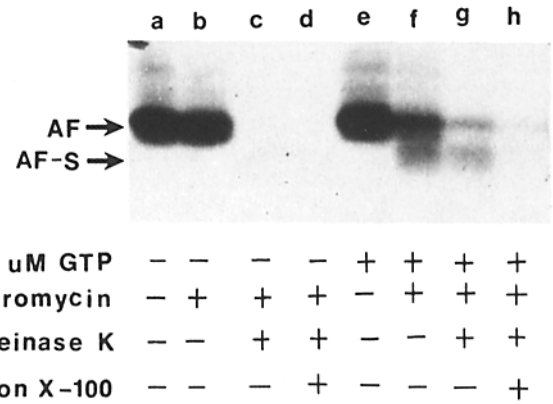


*Figure 5.* Ribosomes bound to K-RM without GTP are not translocation competent. Bovine pituitary RNA was translated for 10 min in a synchronized wheat germ system supplemented with 16 nM SRP. After gel filtration, the ribonucleotide-depleted polysome fraction was adjusted to 1 mM emetine and incubated for 5 min at 25°C with K-RM (3 eq/75 μl) either in the absence (lanes *a–d*) or presence (lanes *e–h*) of 100 μM GTP. Aliquots (lanes *a* and *e*) were removed for immunoprecipitation before adjustment of the remaining samples to 100 μM puromycin (lanes *b–d, f–h*). After a 15-min incubation at 25°C, the reactions were chilled on ice and either immunoprecipitated (lanes *b* and *f*) or further incubated with proteinase K (200 μg/ml) either in the absence (lanes *c* and *g*) or presence (lanes *d* and *h*) of 1% Triton X-100. After a 60-min incubation on ice, proteinase K was inactivated by adjustment to 2 mM PMSF. The arrested fragment (*AF*) and the processed form of arrested fragment (*AF-S*) were recovered by immunoprecipitation with antibody to prolactin, resolved by electrophoresis on a 15–20% polyacrylamide gradient gel, and visualized by autoradiography of the PPO-impregnated gel. The sample preparation method hydrolyzes the tRNA linkage to AF in those samples that did not receive puromycin.

to prolactin. The nascent polypeptide remained protease sensitive and therefore presumably exposed on the surface of the ribosome when elongation-arrested polysomes were bound to K-RM in the absence of GTP (Fig. 4, lanes *a–e*). Incubation of the "loosely" bound ribosomes with proteinase K led to the appearance of a faint but detectable limit digestion product (Fig. 4, lanes *d* and *e*) which represents that portion of the nascent polypeptide shielded from digestion by the ribosome (6, 22). No increase in the protease resistance of AF was detected in control experiments containing GTP but lacking K-RM (data not shown). In contrast, the arrested fragment was minimally 10-fold more resistant to protease digestion after inclusion of both GTP and K-RM (Fig. 4, lanes *f–j*). The extraordinary protease resistance of AF in this latter case is consistent with the formation of a tight junction between the ribosome and the membrane, thereby shielding the entire nascent polypeptide from proteolytic digestion.

Although the two criteria described above (protease protection and EDTA-insensitive AF binding) allow differentiation between two forms of membrane-bound ribosome, we have not established whether "loosely" bound and "tightly" bound ribosomes represent sequential intermediates in protein translocation. To address this issue, we incubated GTP-depleted polysomes with K-RM to prepare "loosely" bound ribosomes. The subsequent addition of GTP converted the elongation-arrested ribosome into the "tightly" bound form as assayed by both criteria (data not shown). In the converse experiment, elongation-arrested polysomes were bound to K-RM before removal of GTP by gel filtration. Once bound to the membrane in the presence of GTP, AF remained insensitive to protease digestion (data not shown). These two experiments strongly suggest that the "loosely" bound ribosome obtained without GTP represents an intermediate in the formation of a tight ribosome–membrane junction.

### *Translocation of Arrested Fragment*

If the GTP-dependent interaction of the signal sequence with the membrane is a prerequisite for protein translocation, then ribosomes bound to the membrane without GTP should be translocation defective. Alternatively, if close proximity between the ribosome and the membrane is sufficient for nascent chain transport, we would anticipate that both "loosely" and "tightly" bound ribosomes would be translocation competent. Discriminating between these two possibilities was complicated by the dependence of protein synthesis upon GTP. However, we could circumvent the GTP requirement for protein synthesis by assaying translocation of AF after puromycin termination. Elongation-arrested polysomes were bound to K-RM in the presence of emetine instead of cycloheximide. Emetine is a protein synthesis elongation inhibitor that does not interfere with the transpeptidation reaction (7). It was shown previously that elongation-arrested polysomes react rapidly with puromycin (11). When GTP was absent during ribosome attachment, puromycin termination did not diminish the intensity of the AF band or lead to the appearance of a translocated and processed polypeptide with a more rapid mobility (Fig. 5, cf. lanes *a* and *b*). Precipitation of peptidyl-tRNA with cetyltrimethylammonium bromide in control samples identical to lanes *a* and *b* demonstrated that GTP deletion did not prevent puromycin termination (data not shown). After puromycin termination, the 70 mer remained sensitive to digestion with proteinase K either in the

absence (Fig. 5, lane *c*) or presence (Fig. 5, lane *d*) of Triton X-100. Thus, in the absence of GTP we were unable to detect any translocated and protected form of the arrested fragment. Puromycin treatment of the "tightly" bound ribosomes led to a substantial decrease in intensity of the 70 mer (Fig. 5, cf. lanes *e* and *f*). The decreased intensity of AF was accompanied by the appearance of a more rapidly migrating polypeptide (AF-S; i.e., AF minus signal [Fig. 5, lane *f*]). The more rapid mobility of the processed polypeptide is consistent with the removal of the 30-residue signal sequence (32). The processed polypeptide (AF-S), as well as some of the precursor, was protected by the membrane from protease digestion (Fig. 5, lane *g*). Permeabilization of the membrane bilayer with Triton X-100 allowed access of the protease to both polypeptides (Fig. 5, lane *h*). The identification of the more rapidly migrating polypeptide (AF-S) as a processed form of the arrested fragment is based upon immunoprecipitation of the polypeptide with antisera to prolactin, the appearance of AF-S in reactions containing both GTP and K-RM, and by protection of AF-S from protease digestion. The 70 mer which remained protease resistant after addition of puromycin (Fig. 5, lane *g*) may in part arise from incomplete puromycin termination, as well as inefficient signal peptidase processing of the short translocated polypeptide. We also observed an incomplete recovery of AF-S relative to the quantity of AF present before addition of puromycin (Fig. 5, compare lanes *e* and *f*). This latter phenomena was also consistently observed after puromycin termination of AF using an unfractionated translation system. Thus, we believe the incomplete recovery of AF-S can be ascribed to technical problems associated with the recovery and electrophoresis of low molecular weight polypeptides.

### *Nascent Chain Transport Does Not Require Continued Ribonucleotide Hydrolysis*

The GTP-dependent translocation and processing of AF neither demonstrated nor disproved the participation of ribonucleotides during translocation events occurring subsequent to ribosome binding. Preassembly of membrane-bound ribosomes before ribonucleotide depletion should allow an investigation of the role of ATP and GTP during these latter translocation events. Translation of an mRNA truncated at a discrete point within the coding region yields a peptidyl-tRNA that predominately remains bound to the ribosome (26, 29). A plasmid containing a cDNA clone of bovine preprolactin (pGEMBP1) was linearized with Pvu II and transcribed with T7 RNA polymerase to yield an mRNA transcript truncated within codon 87 of preprolactin. SDS gel analysis of the translation product of the mRNA transcript revealed a single polypeptide (pPL-86) after immunoprecipitation with antibody to prolactin (Fig. 6, lane *a*). Approximately 90% of the pPL-86 cosedimented with the polysome fraction when applied to a 10–30% sucrose density gradient (data not shown). Translation of the mRNA transcript in the presence of SRP and K-RM was not accompanied by a mobility shift due to signal sequence cleavage (Fig. 6, lane *d*). The signal peptidase cleavage site of short nascent polypeptides is apparently not accessible to the active site of the signal peptidase complex. Therefore, we could use the criteria of signal sequence cleavage after puromycin termination to detect nascent chain transport. The membrane-bound polysomes bearing the 86-residue nascent chain were separated

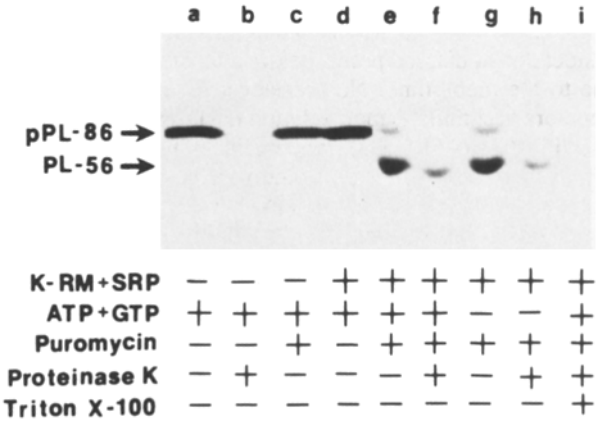


*Figure 6.* Nascent chain translocation does not require ribonucleotide hydrolysis. A truncated preprolactin mRNA (prepared by T7 RNA polymerase transcription of pGEMBP1 linearized with Pvu II) was translated for 20 min at 25°C in a wheat germ translation system either in the absence (lanes *a–c*) or presence (lanes *d–i*) of 16 nM SRP and K-RM (4 eq/100 μl). A 100-μl aliquot of the reaction containing SRP and K-RM was applied to a 1.0-ml Sepharose CL-2B column equilibrated with 50 mM TEA, 150 mM KOAc, 2.5 mM $Mg(OAc)_2$, 0.002% Nikkol, 1 mM DTT. The void volume fraction was adjusted to 100 μM puromycin and incubated for 10 min either in the presence (lanes *e*, *f*, and *i*) or absence (lanes *g* and *h*) of 1 mM ATP and 1 mM GTP. An aliquot lacking K-RM and SRP (lane *c*) also received puromycin. Several samples were incubated with proteinase K (100 μg/ml) (lanes *b*, *f*, *h*, and *i*) for 60 min on ice either in the absence (lanes *b*, *f*, and *h*) or presence (lane *i*) of 1% Triton X-100. The translation products (pPL-86 and PL-56) were recovered by immunoprecipitation with antibody to prolactin, resolved by PAGE in SDS, and visualized by fluorography of the PPO-impregnated gels. Lanes *a–d* and lanes *e–i* were resolved on separate 12–17% polyacrylamide gradient gels and aligned to show a common migration of pPL-86 in the composite photograph.

from ribonucleotides by chromatography on Sepharose CL-2B. The membrane-bound polysome fraction was further incubated at 25°C in the presence of puromycin to induce termination. Signal sequence cleavage occurred to yield PL-56 both in the presence (Fig. 6, lane *e*) or absence (Fig. 6, lane *g*) of added ribonucleotides. The processed polypeptide (PL-56) was protected from protease digestion in both cases (Fig. 5, lanes *f* and *h*) unless the nonionic detergent Triton X-100 was added to disrupt the permeability barrier of the membrane (Fig. 6, lane *i*). The nontranslocated precursor (pPL-86) is protease sensitive both before (Fig. 6, lane *b*) or after (Fig. 6, lanes *f* and *h*) puromycin termination. Control experiments confirmed that the mobility shift we detected corresponds to translocation and processing. For example, no mobility shift was observed after puromycin termination of pPL-86 when K-RM and SRP were deleted (Fig. 6, lane *c*). We can conclude that ongoing hydrolysis of ribonucleotides is not essential for transport of a nascent secretory polypeptide across the membrane bilayer.

## *Discussion*

The SRP and the SRP receptor function together as an endoplasmic reticulum–specific sorting apparatus to promote membrane attachment of ribosomes bearing nascent poly-

peptides with RER signal sequences. Deletion or inactivation of either SRP or its receptor block secretory protein translocation at defined points before attachment of the ribosome to the membrane. Nontranslocated secretory protein precursors accumulate upon deletion (34), inactivation (38), or modification of SRP (33). Inactivation of the SRP receptor prevents the release of the elongation arrest, thereby leading to the accumulation of elongation-arrested polysomes in a wheat germ in vitro system (12, 25). Moreover, proteolytic dissection of the SRP receptor prevents detectable binding of the SRP–ribosome complex to the microsomal membrane (11). We have now shown that GTP is essential for a subsequent event in the formation of a functional junction between the ribosome and the membrane. When GTP was deleted from ribosome-binding assays, the nascent polypeptide was readily accessible to protease digestion and could be extracted from the membrane with either EDTA or high salt. In contrast, the arrested fragment was extraction resistant and remarkably insensitive to protease digestion when GTP was included during ribosome binding. Furthermore, the "tightly" bound ribosomes obtained with GTP were competent for nascent chain translocation and were indistinguishable by these three criteria from membrane-bound ribosomes assembled using an unfractionated translation system.

The "loosely" bound ribosome described here presumably represents an initial intermediate in ribosome attachment to the membrane. Based upon the affinity between detergent-solubilized SRP receptor and SRP coupled to Sepharose (13), we would anticipate that GTP deletion would not preclude the initial interaction between the SRP–ribosome complex and the membrane-bound SRP receptor. The salt-sensitive interaction between SRP and the membrane-bound SRP receptor (37) may account for the GTP-independent initial attachment of the ribosome to the membrane without requiring the participation of additional proteins as ribosome receptors. Irreversible displacement of SRP from the ribosome by the SRP receptor, although independent of continued protein synthesis, appeared to be potentiated by currently unidentified membrane components (10), and as such may require GTP. Based upon current observations, we cannot precisely define which translocation components remain associated with the ribosome upon binding to membranes in the absence of guanine ribonucleotides.

We were able to define the specificity of the ribonucleotide requirement by testing a series of ribonucleotide analogues. Nonhydrolyzable GTP analogues promoted the EDTA-insensitive attachment of AF to the membrane. Moreover, the concentration of GMPPNP required to elicit "tight" ribosome attachment is consistent with the use of the guanine ribonucleotide as a cofactor for a GTP-binding protein. ATP could not substitute for GTP, nor was ATP (in contrast to GDP) an inhibitor of ribosome binding in a competition assay (Connolly, T., and R. Gilmore, unpublished observation). Since hydrolysis-resistant GTP analogues inhibit protein synthesis (27), the use of GTP to support continued protein synthesis is not an explanation for the GTP requirement detected here. Therefore, we can conclude that a GTP-binding protein functions during ribosome attachment to the endoplasmic reticulum.

The resistance of the arrested fragment to extraction from the membrane with EDTA or high salt was previously ascribed to a direct interaction between the signal sequence and an unidentified, yet presumably proteinaceous, component of the microsomal membrane (11). Approximately 40 amino acid residues of a nascent polypeptide are shielded within a protease-inaccessible domain of the ribosome (2, 6, 22) leaving ~30 residues exposed for interaction with the membrane bilayer. The extraordinary protease resistance of the membrane-bound 70 mer presumably arises from a close juxtaposition of the ribosome and the membrane surface to render the entire nascent polypeptide inaccessible to externally added protease. Although both of the above criteria monitor the location of the nascent chain with respect to the membrane surface, it would be premature to conclude that the GTP-binding protein participates directly in signal sequence binding.

The experiments we have described were specifically designed to detect ribonucleotide-dependent translocation components residing in the microsomal membrane fraction. Soluble proteins present in the wheat germ translation system were allowed ample opportunity to bind GTP before gel filtration chromatography, unlike the membranes which were maintained in a GTP-depleted state. Nonetheless, could a previously identified GTP-binding protein be responsible for the results we obtained? A role for protein synthesis elongation factors (EF-1α or EF-2) in ribosome binding would appear unlikely due to the 150 Å distance between the peptidyltransferase site and the nascent chain exit site on the ribosome (2). Because GTP-dependent ribosome binding will occur under conditions which destabilize microtubules (at 0°C or in the presence of 100 μM $Ca^{++}$; our unpublished observations), we believe that tubulin is not involved. However, additional research will be required to conclusively eliminate previously described GTP-binding proteins from consideration. We also cannot unequivocally eliminate any previously identified components of the RER translocation apparatus (i.e., SRP, SRP receptor, or the recently isolated signal peptidase complex [9]) as the GTP-binding protein. However, we believe that SRP and the SRP receptor are unlikely candidates.

Based upon our results, and upon analogies to previously described GTP-binding proteins, we propose that the initial interaction between the SRP–ribosome complex and the membrane-bound SRP receptor induces a GDP–GTP exchange in the nucleotide binding site of a novel translocation component. GTP-binding leads to an increased affinity for an additional ligand, which may be either the ribosome or the nascent signal sequence. Thus, ribosome binding and the subsequent insertion of the signal sequence into the membrane is dependent upon a translocation component which modulates the affinity of the membrane for a ribosome bearing an RER signal sequence. Consequently, the GTP-dependent translocation component should be present in microsomal membranes in a quantity equivalent to that of membrane-bound ribosomes. Protein sequence analysis of the novel translocation component should reveal sequence homology to other GTP-binding proteins, particularly with respect to sequences implicated in ribonucleotide binding (19). In this regard, the identification of the LepA protein of *Escherichia coli* as a GTP-binding membrane protein displaying sequence homology to elongation factors Tu and G is particularly significant (23). The lepA protein is encoded by the promoter proximal gene of the bicistronic leader pep-

tidase operon, and as such has been suggested to be involved in protein secretion in *E. coli* (24).

The third criteria we used to differentiate between GTP-dependent and GTP-independent ribosome–membrane junctions was translocation competence after puromycin termination. A similar observation was made by Perara et al. (29), who detected a requirement for ATP, GTP, and an energy-regenerating system for the ribosome-dependent posttranslational translocation of a secretory protein using the reticulocyte lysate system. Taken together, these two observations raised the question of whether GTP is required only during the ribosome-binding event, or whether ribonucleotide hydrolysis (either ATP or GTP) serves as an energy source to transport the nascent polypeptide across the membrane bilayer. To address the role of ribonucleotide hydrolysis during nascent chain transport, a truncated mRNA was used to direct synthesis of a discrete preprolactin polypeptide of 86 residues. Signal sequence cleavage of the partially translocated pPL-86 did not occur. Similar results were obtained upon translation of a 131-codon truncated preprolactin mRNA (Connolly, T., and R. Gilmore, unpublished observation). Therefore, we could use signal sequence cleavage, and protection from protease digestion with proteinase K, as assays for translocation after puromycin termination. We concluded that continued ribonucleotide hydrolysis was not required during transport of a nascent chain across the microsomal membrane. We must emphasize that our results to date do not eliminate a role for ribonucleotide hydrolysis subsequent to attachment of the elongation-arrested ribosome bearing the 70 mer and before synthesis of the 86-residue preprolactin truncation product. Ribosome-independent posttranslational translocation of yeast prepro-α-factor occurs in a yeast in vitro translocation system and was shown to require ribonucleotide hydrolysis (15, 30, 40). Further research will be required to determine whether the apparently different involvement of the ribosome and ribonucleotide hydrolysis in yeast as compared to higher eukaryotic in vitro systems is indicative of a fundamentally diverse translocation mechanism, or instead reflects more subtle differences in a mechanistically similar translocation apparatus.

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### *References*

1. Adelman, M., D. Sabatini, and G. Blobel. 1973. Ribosome–membrane interactions: nondestructive disassembly of rat liver rough microsomes into ribosomal and membranous components. *J. Cell Biol.* 56:206–229.

2. Bernabeu, C., and J. A. Lake. 1982. Nascent polypeptide chains emerge from the exit domain of the large ribosomal subunit: immune mapping of the nascent chain. *Proc. Natl. Acad. Sci. USA.* 79:3111–3115.

3. Blobel, G. 1980. Intracellular protein topogenesis. *Proc. Natl. Acad. Sci. USA.* 77:1496–1500.

4. Blobel, G., and B. Dobberstein. 1975. Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. *J. Cell Biol.* 67:835–851.

5. Blobel, G., and B. Dobberstein. 1975. Transfer of proteins across membranes. II. Reconstitution of functional rough membranes from heterologous components. *J. Cell Biol.* 67:852–869.

6. Blobel, G., and D. D. Sabatini. 1970. Controlled proteolysis of nascent polypeptides in rat liver cell fractions. I. Location of the polypeptides within ribosomes. *J. Cell Biol.* 45:130–145.

7. Carrasco, L., A. Jimenez, and D. Vazquez. 1976. Specific inhibition of translocation by tubulosine in eukaryotic polysomes. *Eur. J. Biochem.* 64:1–5.

8. Erickson, A. H., and G. Blobel. 1983. Cell-free translation of messenger RNA in a wheat germ system. *Methods Enzymol.* 96:38–50.

9. Evans, E. A., R. Gilmore, and G. Blobel. 1986. Purification of microsomal signal peptidase as a complex. *Proc. Natl. Acad. Sci. USA.* 83:581–585.

10. Gilmore, R., and G. Blobel. 1983. Transient involvement of the signal recognition particle and its receptor in the membrane prior to protein translocation. *Cell.* 35:677–685.

11. Gilmore, R., and G. Blobel. 1985. Translocation of secretory proteins across the microsomal membrane occurs through an environment accessible to aqueous perturbants. *Cell.* 42:497–505.

12. Gilmore, R., G. Blobel, and P. Walter. 1982. Protein translocation across the endoplasmic reticulum. I. Detection in the microsomal membrane of a receptor for the signal recognition particle. *J. Cell Biol.* 96:463–469.

13. Gilmore, R., P. Walter, and G. Blobel. 1982. Protein translocation across the endoplasmic reticulum. II. Isolation and characterization of the signal recognition particle receptor. *J. Cell Biol.* 96:470–477.

14. Harrison, T. M., G. G. Brownlee, and C. Milstein. 1974. Studies on polysome–membrane interactions in mouse myeloma cells. *Eur. J. Biochem.* 47:613–520.

15. Hansen, W., P. D. Garcia, and P. Walter. 1986. *In vitro* protein translocation across the yeast endoplasmic reticulum. ATP-dependent posttranslational translocation of the prepro-α-factor. *Cell.* 45:397–406.

16. Hortsch, M., and D. I. Meyer. 1985. Immunochemical analysis of rough and smooth microsomes from rat liver. Segregation of docking protein in rough membranes. *Eur. J. Biochem.* 150:559–564.

17. Krieg, P. A., and D. A. Melton. 1984. Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. *Nucleic Acids Res.* 12:7057–7069.

18. Kurzchalia, T. V., M. Wiedmann, A. S. Girschovich, E. S. Bochkareva, H. Bielka, and T. A. Rapaport. 1986. The signal sequence of nascent preprolactin interacts with the 54K polypeptide of the signal recognition particle. *Nature (Lond.).* 320:634–636.

19. Leberman, R., and U. Egner. 1984. Homologies in the primary structure of GTP-binding proteins: the nucleotide binding site of EF-Tu and p21. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:339–341.

20. Lingappa, V., V. Chaidez, C. S. Yost, and J. Hedgpeth. 1984. Determinants for protein localization: β-lactamase signal sequence directs globin across microsomal membranes. *Proc. Natl. Acad. Sci. USA.* 81:456–460.

21. Lingappa, V. R., A. Devillers-Thiery, and G. Blobel. 1977. Nascent prehormones are intermediates in the biosynthesis of authentic bovine pituitary growth hormone and prolactin. *Proc. Natl. Acad. Sci. USA.* 74:2432–2436.

22. Malkin, L. I., and A. Rich. 1967. Partial resistance of nascent polypeptide chains to proteolytic digestion due to ribosomal shielding. *J. Mol. Biol.* 26:329–346.

23. March, P. E., and M. Inouye. 1985. GTP-binding membrane protein of *Escherichia coli* with sequence homology to initiation factor 2 and elongation factors Tu and G. *Proc. Natl. Acad. Sci. USA.* 82:7500–7504.

24. March, P. E., and M. Inouye. 1985. Characterization of the lep operon of *Escherichia coli*. *J. Biol. Chem.* 260:7206–7213.

25. Meyer, D. I., E. Krause, and B. Dobberstein. 1982. Secretory protein translocation across membranes–the role of the "docking protein." *Nature (Lond.).* 297:503–508.

26. Mueckler, M., and H. F. Lodish. 1986. The human glucose transporter can insert posttranslationally into microsomes. *Cell.* 44:629–637.

27. Nolan, R. D., H. Grasmuk, and J. Drews. 1975. The binding of tritiated elongation factors 1 and 2 to ribosomes from Krebs II mouse ascites tumor cells. *Eur. J. Biochem.* 50:391.

28. Northup, J. K., M. D. Smigel, and A. Gilman. 1982. The guanine nucleotide activating site of the regulatory component of adenylate cyclase. *J. Biol. Chem.* 257:11416–11423.

29. Perara, E., R. E. Rothman, and V. R. Lingappa. 1986. Uncoupling translocation from translation: implications for transport of proteins across membranes. *Science (Wash. DC).* 232:348–352.

30. Rothblatt, J. A., and D. I. Meyer. 1986. Secretion in yeast: translocation and glycosylation of prepro-α-factor *in vitro* can occur via an ATP-dependent post-translational mechanism. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1031–1036.

31. Sabatini, D. D., Y. Tashiro, and G. E. Palade. 1966. On the attachment of ribosomes to microsomal membranes. *J. Mol. Biol.* 19:503–524.

32. Sasavage, N. L., J. H. Nilson, S. Horowitz, and F. M. Rottman. 1982. Nucleotide sequence of bovine prolactin messenger RNA. Evidence for sequence polymorphism. *J. Biol. Chem.* 257:678–681.

33. Siegel, V., and P. Walter. 1985. Elongation arrest is not a prerequisite for secretory protein translocation across the microsomal membrane. *J. Cell Biol.* 100:1913–1921.

34. Walter, P., and G. Blobel. 1980. Purification of a membrane-associated protein complex required for protein translocation across the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA.* 77:7112–7116.

35. Walter P., and G. Blobel. 1981. Translocation of proteins across the endoplasmic reticulum. II. Signal recognition protein (SRP) mediates the selective binding to microsomal membranes of in vitro-assembled polysomes synthesizing secretory proteins. *J. Cell Biol.* 91:551–556.

36. Walter, P., and G. Blobel. 1981. Translocation of proteins across the endoplasmic reticulum. III. Signal recognition protein (SRP) causes signal sequence-dependent and site-specific arrest of chain elongation that is released by microsomal membranes. *J. Cell Biol.* 91:557–561.

37. Walter, P., and G. Blobel. 1983. Subcellular distribution of signal recognition particle and 7SL RNA determined with polypeptide specific antibodies and cDNA probe. *J. Cell Biol.* 97:1693–1700.

38. Walter, P., I. Ibrahimi, and G. Blobel. 1981. Translocation of proteins across the endoplasmic reticulum. I. Signal recognition protein (SRP) binds to in vitro-assembled polysomes synthesizing secretory proteins. *J. Cell Biol.* 91:545–550.

39. Walter, P., R. C. Jackson, M. M. Marcus, V. R. Lingappa, and G. Blobel. 1979. Tryptic dissection and reconstruction of translocation activity for nascent presecretory proteins across microsomal membranes. *Proc. Natl. Acad. Sci. USA.* 76:1795–1799.

40. Waters, M. G., and G. Blobel. 1986. Secretory protein translocation in a yeast cell-free system can occur posttranslationally and requires ATP hydrolysis. *J. Cell Biol.* 102:1543–1550.

41. Yamanaka, G., F. Eckstein, and L. Stryer. 1986. Stereochemistry of the guanyl nucleotide binding site of transducin probed by phosphorothioate analogues of GTP and GDP. *Biochemistry.* 24:8094–8101.