Antiribophorin Antibodies Inhibit the Targeting to The ER Membrane of Ribosomes Containing Nascent Secretory Polypeptides

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Abstract. Polyclonal antibodies directed against ribophorins I and II, two membrane glycoproteins characteristic of the rough endoplasmic reticulum, inhibit the cotranslational translocation of a secretory protein growth hormone into the lumen of dog pancreas or rat liver microsomes. As expected, site-specific antibodies to epitopes located within the cytoplasmic domain of ribophorin I, but not antibodies to epitopes in the luminal domain of this protein, were effective in inhibiting translocation. Since monovalent Fab fragments were as inhibitory as intact IgG molecules, ribophorins must be closely associated with the translocation site and, therefore, are likely to function at some stage in the translocation process. In all cases, the antibodies that inhibited translocation also caused a significant reduction in total protein synthesis and treatments that neutralized their capacity to inhibit translocation also prevented their inhibitory effect on protein synthesis. This would be expected if the antibodies blocked the membrane-mediated relief of the SRP-induced arrest of polypeptide elongation. The antibodies were effective only when added before translocation was allowed to begin. In this case, they prevented the targeting of active ribosomes containing mRNA and nascent chains to the ER membrane.

Thus, ribophorins must either directly participate in targeting or be so close to the targeting site that the antibodies sterically blocked this early phase of the translocation process.

In the rough ER of higher eukaryotic cells, a complex molecular apparatus effects the signal sequence-mediated targeting, cotranslational translocation, and processing of nascent polypeptide chains that are synthesized on membrane bound ribosomes (for reviews see Rapaport, 1986; Walter and Lingappa, 1986; Sabatini and Adesnik, 1989). The targeting step begins in the cytosol when a large ribonucleoprotein complex, the signal recognition particle (SRP), binds to the ribosome and to the signal sequence in the emerging nascent polypeptide (Walter et al., 1981; Walter and Blobel, 1981). Subsequent steps take place in association with the ER membrane and, in recent years, several polypeptides characteristic of these membranes have been identified that appear to participate in the membrane insertion and processing of the nascent polypeptide. These include: (a) two subunit polypeptides of an SRP receptor or docking protein, that recognizes the SRP (Meyer and Dobberstein, 1980a, b; Lauffer et al., 1985; Tajima et al., 1986; Connolly and Gilmore, 1989); (b) a putative signal sequence receptor (SSR), which is a 35-kD integral membrane glycoprotein that can be cross-linked to the signal sequence and to other portions of the nascent chain during the course of transloca-

1. Abbreviations used in this paper: GH, growth hormone; RM, rough microsome; RI and RII, ribophorins I and II; SRP, signal recognition particle; SSR, signal sequence receptor.
nal domains and shorter cytoplasmically exposed carboxy terminal segments of 150 and 70 amino acids, respectively (Crimadu et al., 1987; Harnik-Ort et al., 1987). They, and a limited number of other membrane polypeptides, can be recovered with ribosomes when these are sedimented after the microsomal membranes are dissolved with certain nonionic detergents. In this case, a membrane remnant is obtained in which the residual proteins appear to form a two-dimensional network bearing ribosomes (Kreibich et al., 1978a). On this basis, it was proposed (Kreibich et al., 1978a, b) that the ribophorins play a structural role in the ER, providing a scaffolding within the membranes that restricts the ribosome binding sites and their associated translocation apparatus to the rough portions of the organelle. Ribophorins are sufficiently close to the membrane bound ribosomes that, in intact microsomes, can be cross-linked to them with bifunctional reagents (Kreibich et al., 1978b). It appears, however, that ribophorins are not directly involved in ribosome binding since the capacity of microsomal membranes stripped of ribosomes to rebind ribosomes in vitro, in an association that involves a salt sensitive linkage and is independent of the presence of a nascent chain (Borgese et al., 1974; Amar-Costescu et al., 1984), can be abolished by mild treatment of the membranes with proteases, which does not degrade the ribophorins (Hortsch et al., 1986). Moreover, liposomes containing microsomal phospholipids and only the nonglycoprotein components of rough microsomes have been shown to be capable of binding ribosomes under the same conditions (Yoshida et al., 1987).

In this paper, evidence is presented that the ribophorins are, indeed, functionally involved in the sequence of events that leads to protein translocation in the ER. It was found that incubation of rough microsomes with antibodies to either ribophorin I or II abolished the capacity of the membranes to effect the cotranslational translocation of an indicator protein, rat growth hormone (GH). Antibodies to sites in the cytoplasmic domain, but not antibodies to sites in the luminal domain of ribophorin I, were inhibitory. In addition, anti-ribophorin I antibodies were only effective when applied before translocation was initiated. This suggests that ribophorin I either plays a role in the initial stages of this process or is closely apposed to another component of the translocation apparatus that participates in the attachment of active ribosomes to the translocation site.

Materials and Methods

Preparation of Antibodies

Polyclonal antibodies were raised against SDS-PAGE-purified RI and RII (Marcantonio et al., 1984) or against synthetic peptides corresponding to specific RI sequences. These included RIL1 (aa 234–252), RIL2 (aa 351–364), RIC1 (aa 434–454), RIC2 (aa 455–476), RIC3 (aa 477–494), RIC4 (aa 511–531), RIC5 (aa 529–548), and RIC6 (aa 564–583). The synthetic peptides, coupled to keyhole limpet hemocyanin with glutaraldehyde, were used to immunize rabbits (for details, see Frey et al., 1985). Monoclonal antibodies were raised against SDS-denatured RI by standard procedures (de St. Groth and Scheidegger, 1980; Croze et al., 1989). Antibodies against ribophorins or cytotochrome P450 2c (Waxman et al., 1984) were affinity purified essentially as described by Wollner et al. (1986). Dog pancreas or rat liver microsomal proteins fractionated by preparative SDS-PAGE were electrophoretically transferred onto nitrocellulose paper. Strips corresponding to the desired antigens were cut out and used as an affinity matrix to purify specific antibodies. The eluted antibod-

Inhibition of Protein Translocation by Anti-RI and Anti-RII Antibodies

Dog pancreas rough microsomes and SRP (Walter and Blobel, 1982a, b), rat liver rough microsomes (Krump and Sabatini, 1977) and red blood cell ghosts (Steck and Kant, 1974) were prepared according to published procedures. Cell-free translocation using in vitro synthesized mRNA carried out as previously described (Harnik-Ort et al., 1987). A plasmid containing a rat GH cDNA insert was used to generate the mRNA template (Rizzolo et al., 1985). For cotranslational translocation, 30 µl of a wheat germ translation mixture was supplemented with rough microsomes (10 µg protein), 20 nM SRP and 0.002 % Nikkol and incubated for 1.5 h at 25°C. Translocation efficiency was assessed by digestion of the samples (1 h; 0°C) with a mixture of trypsin and chymotrypsin (100 µg/ml each). In most cases, the processed GH was not quantitatively protected from proteolysis; this may be due to the fragility of the microsomes or that the rather high concentrations of proteases required for complete digestion of preGH affected the integrity of the microsomal vesicles.

IgG fractions (preheated to 65°C for 5 min) with affinity-purified antibodies, or monovalent Fab fragments were all prepared in PBS (20 mM phosphate, 145 mM NaCl, pH 7.5) and, for antibody inhibition experiments, adjusted to concentrations that were all equally reactive in a Western blot test in which the immobilized antigens were present in large excess. For each antibody inhibition assay, the antibody preparation (&#8775;0.75 mg for the IgG fractions or Fab fragments, or 3 µg affinity-purified IgG) was mixed with 30 µl of a mixture of protease inhibitors (0.1% trypsin), and 0.1 µg/ml each of chymotrysin, leu-leu-leu, leupeptin, pepstatin, and antipain) in PBS and incubated at room temperature for 15 min. Dog pancreas (6 µg protein) or rat liver (10 µg protein) rough microsomes (RMs) were then added, and incubation was continued with gentle shaking at room temperature for 0.5 h followed by 0.5 h at 4°C. The microsomes, to a great extent stripped of ribosomes during the incubation with the PBS-containing incubation medium, were recovered by centrifugation for 0.5 h at 4°C in a microcentrifuge (model 235B; Fisher Scientific Co., Pittsburgh, PA), rinsed with washing buffer containing 20 mM Hepes (pH 7.4), 50 mM KAc and 1 mM MgAC2, and resuspended in 30 µl of a wheat germ cell-free translation mixture. When testing their capacity to neutralize the antibody inhibitory effect, the peptides were mixed with the antibodies and incubated for 0.5 h at 37°C (or 10 min at 65°C) in a water bath, and then for 0.5 h at room temperature before adding to the microsomes. The amount of peptide used (1.2 µg) was approximately in a 500-fold molar excess with respect to the amount of RI in the RM.

In control experiments, antibodies were preadsorbed with Sepharose beads, protein A-Sepharose beads, puromycin/high salt stripped rough microsomes, or red blood cell ghosts, and, after centrifugation, the flow-through fractions or supernatants were used to treat the microsomes. The intensities of the preGH and GH bands in the autoradiograms were determined using a scanning densitometer (GS 300; Hoefer Scientific Instruments, San Francisco, CA). The numbers corresponding to the intensities of the GH bands were multiplied by seven-sixths to correct for the loss of the Met residue in the signal sequence.

Assays for the Effect of the Antibody at Different Stages of Protein Translocation

Preincubation of RM with Antibodies. Dog pancreas RMs preincubated with or without affinity-purified antibodies were recovered by centrifugation and incubated at 25°C for 3 min in a GH mRNA-programmed wheat germ translation mixture containing 20 nM SRP and 0.002 % Nikkol. Translation was stopped by cooling on ice, and supernatant and RM pellet fractions were separated by centrifugation for 30 min in a microcentrifuge (Fisher Scientific Co.) at 4°C. To determine the extent to which translation initiation had taken place, the supernatant was divided into two equal parts. One was directly analyzed by SDS-PAGE, to confirm that no complete preGH and/or GH had been made, and the second was incubated for 1.5 h at 25°C for further elongation of initiated chains in the presence of 5 mM initiation inhibitor "MG53p before SDS-PAGE. The RM pellet fraction containing
ribosomes with initiated nascent polypeptides that attached during the 3-min incubation was rinsed twice and resuspended in a wheat germ translation mixture that contained the initiation inhibitor and no added mRNA. Elongation was then allowed to proceed during incubation for 1.5 h at 25°C.

**Incubation of RM with Antibodies after 3 min of Translation.** Dog pancreas RMs were added to a wheat germ translation mixture containing preGH mRNA, 20 nM SRP, 0.002% Nikkol, and incubation at 25°C was carried out for 3 min, before cooling on ice and centrifugation, as described above. The supernatant was used to assess the extent of initiation, as just described, and the sedimented RMs were resuspended and incubated in 30 μl PBS with or without the affinity-purified antibodies. The RMs were again recovered by centrifugation, rinsed, and resuspended in the wheat germ elongation mixture containing the initiation inhibitor in which they were incubated for 1.5 h at 25°C.

**Results**

**The Protein Translocation Capacity of RMs Is Inhibited by Pretreatment with Polyclonal Antibodies against RI or RII**

The capacity of RMs to effect cotranslational translocation was assessed in translation systems programmed with in vitro synthesized rat GH messenger RNA. GH serves as a convenient indicator protein to assess translocation since preGH, synthesized in the absence of RMs, is easily distinguished by its electrophoretic mobility (M₀ = 25 kD) from mature GH (M₀ = 22 kD) that has undergone removal of the signal (Fig. 1, compare lanes a and b or g and h). Translocation of the signal-cleaved product into the microsomal lumen can be easily assessed from its resistance to the attack of added proteases (Fig. 1, lanes d'–j'), which completely digests any untranslocated preGH molecules found in the same samples.

As shown in Fig. 1 (lanes c and e, c' and e'), preincubation with anti-RI or anti-RII IgGs inhibits the translocation competence of the microsomes by 67% (Fig. 1, lane c) and 78% (Fig. 1, lane e), respectively. On the other hand, the translocation capacity of RMs preincubated with IgG fractions from which antibodies had been removed by adsorption to protein A-Sepharose was unimpaired (<2%; Fig. 1, lanes d, f, d', and f'). This indicates that the inhibition of translocation was caused by antibodies and not by a contaminating agent in the immunoglobulin fractions. The inhibitory activity of the antibody preparations was also dramatically reduced when, before addition to the microsomes, the IgG samples were preincubated with excess amounts of RMs, which were then sedimented to remove the adsorbed antibodies (Fig. 1, lanes j and l, j' and l'). The preadsorbed anti-RI and anti-RII antibodies caused only a 14% and 3% reduction in translocation, respectively, as compared to the sample incubated with nonimmune IgG (Fig. 1, lane h). In contrast, the inhibitory effect of the IgG fractions was unimpaired when red blood cell ghosts were used in the preadsorption step (Fig. 1, lane i and k, i' and k'), still giving 93% (anti-RI) and 99% (anti-RII) inhibition. It can, therefore, be concluded that the inhibition of translocation observed was a specific effect caused by binding of antiribophorin antibodies to antigens exposed on the microsomal surface. It is noteworthy that only when the antibodies inhibited translocation did they cause a substantial reduction of growth hormone synthesis (preGH + GH). This is consistent with the possibility that the inhibition of translation is a consequence of the antibodies blocking the membrane-mediated relief of the arrest of polypeptide elongation caused by SRP.

![Figure 1. Antibodies against RI or against RII inhibit the translocation activity of RM. (A) In vitro synthesized rat GH mRNA was translated in a wheat germ cell-free system containing [35S]methionine, in the absence (lanes a and g) or presence (lanes b–f and h–l) of dog pancreas RMs (6 μg protein). The RMs had been preincubated in PBS with nonimmune (NI) IgG (lanes b and h), or with anti–RI IgG or anti–RII IgG that had been preincubated with Sepharose beads (S) (lanes c and e). As controls, anti–RI and anti–RII IgGs preincubated with protein A-Sepharose beads (SA) (lanes d and f), red blood cell membranes (RC) (lanes i and k) or dog pancreas RMs (lanes j and l) were used. After protein synthesis, duplicate samples (lanes a–f) were incubated with a mixture of trypsin/chymotrypsin (100 μg/ml each) for 1 h at 0°C. All samples were analyzed by SDS-PAGE (10–15% polyacrylamide) and autoradiography (12 h). The results shown in lanes a–f and g–l were obtained from two different experiments. (B) Quantitative analysis of the autoradiograms. The intensity of the preGH and GH bands in the autoradiograms in A were obtained by densitometry, as indicated in Materials and Methods. The numbers in each column are arbitrary numbers corresponding to intensities of the bands measured by densitometry:

1. GH

2. For each sample, this is expressed as GH

3. [translocation efficiency of each sample containing membranes) × 100; (translocation efficiency of the corresponding control)]

4. ([preGH + GH]sample/[preGH + GH]control) × 100.

**Antibodies That Recognize the Cytoplasmic Domain of RI, but Not Those That Recognize the Luminal Domain, Inhibit Protein Translocation**

To confirm that the inhibitory effect of the antiribophorin antibodies was due to their interaction with cytoplasmically exposed portions of the RI molecule, site-specific antibodies were prepared against synthetic peptides corresponding to segments within the cytoplasmic and luminal regions of RI.
Figure 2. Specificity of antibodies directed against epitopes in the cytoplasmic or luminal domains of ribophorin I. (A) Schematic representation of the primary structure of mature rat RI (583 aa). The numbers refer to amino acid residues, the N-glycosylation site (aa 275) is labeled with an asterisk, and the transmembrane domain (415-433 aa) by the cross-hatched vertical bar. The positions of the luminal (L1, L2) and cytoplasmic (C1-C6) sequences used to generate site-specific antibodies are indicated. Titers of antibodies against the peptides RICI, RIC2, and RIC3 were too low to obtain satisfactory Western blots. These antibodies were, therefore, not used for any of the subsequent experiments. (B) Dog pancreas RMs (0.5 mg protein/ml) were either digested with a mixture of trypsin and chymotrypsin (100 #g/ml each; 0°C, 1 h) (lanes a-f) to generate a protected fragment (RIe) corresponding to the luminal and transmembrane domains of RI, or kept undigested as controls (lanes a' to f'). The SDS solubilized microsomal protein samples (50 #g protein each) were then fractionated by SDS-PAGE and analyzed by immunoblotting with polyclonal anti-RI (lanes a and a'), anti-RIC4 (lanes b and b'), anti-RIC5 (lanes c and c'), anti-RIC6 (lanes d and d'), anti-RIL2 (lanes e and e'), or a monoclonal antibody against RI1 (MC) (lanes f and f').

(Fig. 2 A). The specificity of these antibodies was confirmed by Western blot analysis using RMs that were either intact or had been previously incubated with a mixture of trypsin and chymotrypsin to digest the cytoplasmic portion of RI. Antibodies to synthetic peptides corresponding to the cytoplasmic segments (RIC4, RIC5, and RIC6) detected only intact RI (Fig. 2 B, lanes b, b', c', and d, d'), while antibodies to a synthetic peptide corresponding to a luminal segment (RIL2) detected both the intact RI (Fig. 2, lane e') and the protected fragment (RIe) generated by proteolysis (Fig. 2, lane e). An mAb that was raised against purified RI also recognized both intact molecules (Fig. 2, lane f') and the fragments that lack the cytoplasmic domain (Fig. 2, lane f). Thus, this antibody must be directed to an epitope located in either the luminal or the transmembrane domain of RI. The polyclonal anti-RI antibody used for the experiments in Fig. 1 that blocked protein translocation recognized the protected fragment (Fig. 2, lane a) much less effectively than the intact molecule (Fig. 2, lane a'), which indicates that most of the antibody molecules in that preparation recognize cytoplasmic epitopes of RI.

When the different antibody preparations characterized by the immunoblot analysis of intact and proteolysed RMs were tested for their capacity to block translocation (Fig. 3), it was found that antibodies to synthetic peptides corresponding to different segments within the cytoplasmic domain of RI (RIC4, RIC5, RIC6; Fig. 3, lanes c-e, and c'-e'), showed similar inhibitory effects on translocation (62-97 % reduction) as the polyclonal antibody (83 % reduction) (Fig. 3, lanes b, b'). On the other hand, antibodies to the luminal domain of RI, such as anti-RIL2 and the mAb, had no significant inhibitory effect (Fig. 3, lanes f and g, f' and g'; 14 and 19 % inhibition, respectively). As expected, the inhibitory capacity of the affinity-purified antipeptide antibodies was neutralized when the corresponding synthetic peptides were added to block the antibody binding site (compare Fig. 3, lanes i, j, and k, and f, f', and k' for anti-RIC6). In this experiment, preincubation with anti-RIL2 caused a 93 % inhibition of translocation (Fig. 3, lane j) whereas the same antibody neutralized with the RIC6 peptide caused only
23% inhibition (Fig. 3, lane k). It should be noted that in this experiment, too, all the antibodies that inhibited translocation (and only these) also reduced overall protein synthesis to 38–55% of the control level. The polypeptide RIC₆ alone had no effect on the translocation capacity of the membrane (compare Fig. 3, lanes h and f). Moreover, neutralization of the anti-RIC₆ antibody with the RIC₆ peptide not only counteracted its ability to inhibit translocation but also its ability to inhibit translation.

**Monovalent Fab Fragments That Recognize a Cytoplasmic Segment of RI Inhibit Translocation**

The capacity of the various anti-RI antibodies to inhibit translocation could be the direct result of their blocking a functional site within the cytoplasmic domain of the protein, or a consequence of a redistribution of microsomal proteins involved in translocation that results from antibody mediated cross-linking of ribophorin molecules that can be displaced within the fluid microsomal membrane (Ojakian et al., 1977). To determine whether cross-linking of RI was necessary to cause an inhibition of translocation, the effect of monovalent Fab fragments of the anti-RIC₆ IgG was examined. As is shown in Fig. 4, the Fab fragments also caused a marked inhibition of translocation (Fig. 4, lanes b, b; 74% inhibition), and this effect was also abolished by preincubation with a large molar excess of the synthetic peptide, RIC₆ (Fig. 4, lanes c, c). As in the previous cases, the effect of the antibody in translocation paralleled that in translation.

To determine whether binding of antibodies to a microsomal membrane protein that is not involved in translocation could inhibit translocation, the effect of polyclonal antibodies against a constitutive form of rat liver cytochrome P₄₅₀ (P₄₅₀ 2c) (Waxman, 1984) was examined using rat liver microsomes. The concentration of this cytochrome in rat liver rough microsomes is at least as high as that of RI (~1%) (Dannan et al., 1983). Rat liver microsomes had to be used for this experiment since dog pancreas microsomes contain essentially no cytochrome P₄₅₀ and no antibody against a dog pancreas membrane protein that is not involved in translocation and present in sufficiently high concentration is known. As is the case with dog pancreas microsomes, anti-RIC₆ antibodies inhibited protein translocation by rat liver microsomes (compare Fig. 4, lanes d, d' with f, f; 89% inhibition), but the affinity-purified polyclonal anti-P₄₅₀ 2c antibodies had no effect (Fig. 4, compare lanes d, d', with e, e').

**RI Antibodies Inhibit Translocation Only When Added before the Active Ribosomes Become Associated with the ER Membrane**

To obtain an insight into which step in the translocation process is blocked by anti-RI antibodies, the in vitro translation-translocation of GH was experimentally dissected into two stages. In the first, assembly, targeting and attachment of ribosome-nascent chain-SRP complexes to translocation sites in dog pancreas microsomes was allowed to take place during a very brief (3-min) incubation period in the cell-free translation system. Protein synthesis was then halted by cooling on ice, and the microsomes, bearing ribosomes with initiated, but incomplete chains, were recovered by centrifugation. In the second stage, elongation of nascent chains that were initiated during the 3-min incubation period was allowed to take place in the presence of the inhibitor of initiation 7-methylguanosine-5-monophosphate (mG(5)P). It was demonstrated that, as expected, during the first brief incubation, no completed preGH or mature GH molecules were synthesized (Fig. 5, lane e). However, the synthesis of preGH molecules was initiated during this period and a substantial

![Figure 4](image-url)
Figure 5. Protein translocation is inhibited only when antibodies are added before translocation is initiated. Dog pancreas RMs preincubated with (lane b) or without (lane a) affinity-purified anti-RIC6 antibody were incubated in a translation mixture containing growth hormone mRNA for 3 min to allow initiation of preGH polypeptides and targeting of active ribosomes to the microsomal membranes. The microsomes were then recovered by centrifugation and resuspended in a translation mixture with no added mRNA, but containing the inhibitor of translational initiation \( \text{mG(5')p} \). After elongation was allowed to proceed for 90 min, the extent of synthesis and translocation was assessed by electrophoresis with \((a', b')\), or without \((a, b)\) previous incubation with proteases. In other samples \((c, d)\), untreated RMs were incubated for 3 min in the translation mixture to allow initiation and targeting, the microsomes were then recovered by centrifugation and resuspended in PBS alone (lane c) or PBS containing affinity-purified anti-RIC6 antibody (lane d). After the incubation, the RMs were once again recovered by centrifugation, washed, and incubated for 90 min to allow for elongation of nascent chains. The supernatants obtained after the 3-min incubation from both types of experiments were used to assess the extent of translation that takes place during that brief incubation and the amount of initiated preGH chains present in ribosomes that did not bind to the membranes. The supernatants were split into two aliquots, one of which was analyzed directly \((e, e')\), and the other after further incubation for 90 min in the presence of the inhibitor of initiation \((f, f')\). Since the results obtained from all four supernatants were indistinguishable, only one set of autoradiographs is shown. Duplicate samples were incubated with proteases \((a'-f')\) to assess the extent of translocation.

Discussion

The results just presented demonstrate that antibodies to either RI or RII abolished the capacity of rough microsomal membranes to effect the signal sequence-mediated cotranslational translocation of a polypeptide into the microsomal lumen. Using antibodies directed against specific sites within the RI primary sequence, it was shown that this effect is the direct result of the specific interaction of the antibodies with epitopes located within the cytoplasmic domain of the protein.

Ribophorins are known to be part of an extensive protein network within the ER membrane that links the ribosome binding sites to each other, and it has been shown that this network can undergo extensive displacement within the plane of the membrane (Ojakian et al., 1977). This is the case, for example, when the aggregation of bound ribosomes is induced by incubating the microsomes with low concentrations of detergents (Kreibich et al., 1982), with ribonuclease, or with antibodies to ribosomal proteins (Ojakian et
microsomal proteins are collected with the aggregated ribosomes in limited regions of the microsomal membrane. The ability of the ribophorins to form aggregates within the plane of the membrane is also manifested when microsomal membranes are solubilized with certain nonionic detergents. After this treatment, the ribosomes are recovered on cup-shaped membrane remnants that still contain ribophorins in amounts approximately equimolar with the bound ribosomes (Kreibich et al., 1978a). The possibility that the antibodies, by cross-linking ribophorin molecules, simply interfered with translocation by nonspecifically disturbing the spatial arrangement in the membrane of other proteins that participate directly in this process was, however, eliminated by the finding that monovalent Fab fragments of the antipeptide antibodies to RI were as effective as the intact antibody molecules in blocking translocation. Moreover, bivalent polyclonal antibodies to cytochrome P450, which are also capable of cross-linking microsomal proteins, had no effect on translocation.

Ribophorins constitute <1% of the total protein in the microsomal membrane (Marcantonio et al., 1984), and saturating amounts of anti-ribophorin Fab fragments, which are less than one-third the size of intact IgG molecules, could only cover a very small fraction of the microsomal surface. Hence, the efficacy of the Fab fragments in inhibiting translocation provides strong evidence for a close physical association of the ribophorins with the sites of translocation in the ER membrane, as would be expected if ribophorins participate directly in this process. The findings that the antibodies did not inhibit translocation when they were added as early as 3 min after the start of translational initiation, and that they, in fact, prevented the association of the SRP-nascent chain-ribosome-mRNA complexes with the membrane that normally takes place during that brief interval, would suggest that ribophorins are involved in steps within the targeting phase of translocation. This possibility is supported by the observation that the inhibition of translocation caused by the preincubation of RMs with antibodies was consistently accompanied by a reduction in the total translational yield, ranging from 30% to 60% and that antibodies that did not inhibit translocation, or had been blocked with competing antigen, did not inhibit total translation. This would be the expected outcome if the antibodies prevent the SRP receptor in the membrane from releasing the SRP-mediated elongation arrest of pre-GH. A similar observation has recently been made with an antibody directed against the putative signal sequence receptor (SSR) (Hartman et al., 1989).

The failure of the antibodies to inhibit translocation when added after the ribosome-membrane junction had been formed does not preclude that ribophorins actually play a role in posttargeting steps of translocation, such as the passage of the polypeptide through the membrane or its cotranslational modification. In fact, it is possible that the ribophorins only play a role in the late stages of translocation and that the antibodies to their cytoplasmic domains inhibited the targeting phase by sterically hindering the function of other proteins involved in targeting. The antibodies, however, may not be able to perturb the intrinsic late functions of the ribophorins because these involve directly the transmembrane or luminal domains of the proteins. Alternatively, after the ribosome-membrane junction is formed, the antibodies may no longer have access to the ribophorins if the cytoplasmic domains of these proteins, for example, become covered by the ribosome that attaches to the membrane.

Previous studies have shown that only a small fraction (<1%) of the total number of ribosome binding sites on the surface of rough microsomal membranes is actually capable of participating in the translocation of nascent polypeptides in in vitro systems (Walter and Blobel, 1980; Kreibich et al., 1981). In fact, although stripping of ribosomes from RMs markedly increases the capacity of the membranes to bind ribosomes (Borgese et al., 1974), it does not significantly increase the number of translocation sites (Kreibich et al., 1981; Walter and Blobel, 1983) that is reflected by the in vitro translocation capacity of the membranes. Our finding that essentially all the mRNA-ribosome-nascent chain complexes that after a brief initiation period become associated with the membranes successfully completed translocation in a second incubation indicates that, under the conditions of protein synthesis in which this association takes place, active ribosomes that contain mRNA only bind to those ribosome binding sites that are capable of translocation. This indicates that, physiologically, ribosome binding only takes place if signal-mediated targeting has occurred. Since the SRP receptor, which plays an essential role in targeting, is known to be present in quantities substantially below the number of ribosome binding sites (Tajima et al., 1986), the association of this receptor with a ribosome binding site may have been the factor that determined whether the site was functional in targeting during the first 3 min incubation and could, therefore, carry out the complete translocation process. Indeed, as suggested above, the effect of the antiribophorin antibodies in inhibiting targeting may have been due to their blocking the interaction between SRP and its receptor, a possibility that is now being tested experimentally.

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References

Amar-Costescu, A. A., J. A. Todd, and G. Kreibich. 1984. Segregation of the polypeptide translocation apparatus to regions of the endoplasmic reticulum containing ribophorins and ribosomes. I. Functional tests on rat liver microsomal subfractions. J. Cell Biol. 99:2247-2253.

Borgese, N., W. Mok, G. Kreibich, and D. D. Sabatini. 1974. Ribosomal-membrane interaction: in vitro binding of ribosomes to microsomal membranes. J. Mol. Biol. 88:559-580.

Connolly, T., and R. Gilmore. 1989. The signal recognition particle receptor mediates the GTP-dependent displacement of SRP from the signal sequence of the nascent polypeptide. Cell. 57:599-610.

Crimaudo, C., M. Hortsch, H. Gausepohl, and D. I. Meyer. 1987. Human ribophorins I and II: the primary structure and membrane topology of two highly conserved rough endoplasmic reticulum-specific glycoproteins. EMBO (Eur. Mol. Biol. Organ.) J. 6:75-82.

Croze, E. I. E. Ivanov, G. Kreibich, M. Adesnik, D. D. Sabatini, and M. G. Rosenberg. 1989. Endolyn-78, a membrane glycoprotein present in morphologically diverse components of the endosomal and lysosomal compartments: implication for lysosome biogenesis. J. Cell Biol. 108:1597-1613.

Dannan, G. A., F. P. Guengerich, L. S. Kaminisky, and S. D. Aust. 1983. Regulation of cytochrome P450. Immunochemical quantitation of eight isoforms in liver microsomes of rats treated with polybrominated diphenyl congeners. J. Biol. Chem. 258:1282-1288.
Meyer, D. I., and B. Dobberstein. 1980a. A membrane component essential for vectorial translocation of nascent proteins across the endoplasmic reticulum: requirements for its extraction and reassociation with the membrane. J. Cell Biol. 87:498-502.

Meyer, D. I., and B. Dobberstein. 1980b. Identification and characterization of a membrane component essential for the translocation of nascent proteins across the membrane of the endoplasmic reticulum. J. Cell Biol. 87:503-508.

Ojakian, K. G., G. Kreibich, and D. D. Sabatini. 1977. The mobility of ribosomes bound to microsomal membranes. A freeze-etch and thin-section study of the structure and fluidity of the rough endoplasmic reticulum. J. Cell Biol. 72:530-551.

Rapaport, T. A. 1986. Protein translocation across and integration into membranes. CRC Crit. Rev. Biochem. 20:73-137.

Rizzolo, L. J., A. Gonzalez, M. Arpin, I. E. Ivanov, M. Adesnik, and D. D. Sabatini. 1989. Biosynthesis and intracellular sorting of growth hormone: studies on endoplasmic reticulum membranes. J. Biol. Chem. 264:15253-15265.

Rizzolo, L. J., A. Gonzalez, M. Arpin, I. E. Ivanov, M. Adesnik, and D. D. Sabatini. 1989. Biosynthesis and intracellular sorting of growth hormone: envelope glycoprotein hybrids. J. Cell Biol. 99:1076-1082.

Sabatini, D. D., and M. Adesnik. 1989. The biogenesis of membranes and organelles. In The Metabolic Basis of the Inherited Disease, Vol. 1. C. R. Scriver, A. L. Baudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, Inc., New York. 177-223.

Steck, T. L., and J. A. Kant. 1974. Preparation of impermeable ghosts and inside-out vesicles from human erythrocyte membranes. Methods Enzymol. 31:172-180.

Tajiana, S., L. Lauffer, V. L. Rath, and P. Walter. 1986. The signal recognition particle receptor is a complex that contains two distinct polypeptide chains. J. Cell Biol. 103:1167-1178.

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.

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

Walter, P., and G. Blobel. 1983a. Preparation of microsomes for cotranslational protein translocation. Methods Enzymol. 96:84-93.

Walter, P., and G. Blobel. 1983b. Signal recognition particle: a ribonucleoprotein required for cotranslational translocation of proteins, isolation and properties. Methods Enzymol. 96:683-691.

Walter, P., and V. R. Lingappa. 1986. Mechanism of protein translation across the endoplasmic reticulum membrane. Annu. Rev. Cell Biol. 2:499-516.

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

Waxman, D. J. 1984. Rat hepatic cytochrome P-450 isozyme 2c: identification as a male-specific, developmentally-induced steroid 16 alpha-hydroxylase and comparison to a female specific isoenzyme. J. Biol. Chem. 259:15481-15490.

Wiesmann, M., T. V. Kurzchalia, E. Hartmann, and T. A. Rapoport. 1987. A signal sequence receptor in the endoplasmic reticulum membrane. Nature (Lond.) 328:830-833.

Wollner, D. A., and W. A. Catterall. 1986. Localization of sodium channels in axon hillocks and initial segments of retinal ganglion cells. Proc. Natl. Acad. Sci. USA. 83:4824-4828.

Yoshida, H., N. Tondokoro, Y. Asano, K. Mizusawa, R. Yamagishi, T. Horigome, and H. Sugano. 1987. Studies on membrane proteins involved in ribosome binding on the rough endoplasmic reticulum. Biochem. J. 245:811-819.