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A membrane component of the endoplasmic reticulum that may be essential for protein translocation

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We have purified a glycosylated, membrane-spanning protein of relative molecular mass ~34 000 (M_r ~34 K) from canine microsomes that appears to be essential for protein translocation across the endoplasmic reticulum (ER) as shown by the inhibitory action of antibodies directed against it and of monovalent F_{ab}-fragments produced from them. The ER membrane contains at least as many molecules of the 34 K membrane protein as bound ribosomes. The protein can be detected immunologically in tissues of various organisms, indicating an universal function.

Key words: endoplasmic reticulum/protein translocation/signal hypothesis/signal sequence receptor

Introduction

The process of protein translocation across the endoplasmic reticulum (ER) membrane is initiated in the cytoplasm. The signal sequence of a nascent polypeptide, upon emergence from the ribosome, is recognized by the signal recognition particle (SRP) (Walter and Blobel, 1981) through its 54 K polypeptide component (Krieg *et al.*, 1986; Kurzchalia *et al.*, 1986). In the absence of membranes, SRP can induce an arrest of elongation *in vitro* (Walter and Blobel, 1981). On interaction of the SRP with its cognate receptor in the ER membrane (docking protein) (Meyer *et al.*, 1982; Gilmore *et al.*, 1982a), it is released from both the ribosome (Gilmore and Blobel, 1983) and from the signal sequence (Wiedmann *et al.*, 1987a). The latter is then found in close vicinity of an integral, glycosylated membrane protein of the ER (M_r ~35 K), named signal sequence receptor (SSR) (Wiedmann *et al.*, 1987b).

The actual process of passage of the polypeptide across the phospholipid bilayer is still poorly understood. A ribosome receptor and a proteinaceous tunnel providing a hydrophilic environment for passage of the polypeptide chain have been postulated (Blobel and Dobberstein, 1975) but not yet identified. Such components must be abundant in rough microsomes since they are required in at least stoichiometric amounts compared with membrane-bound ribosomes. They may be assumed to consist of integral membrane proteins of the ER. Based on these assumptions we have purified such a protein that may be essential for translocation, and possibly identical with the SSR.

Results

Purification of a 34 K protein from canine microsomes

The procedure for the purification of the membrane protein was based on the assumption that it represents a major glycoprotein of the rough ER membrane. Rough microsomal membranes (RM) from dog pancreas served as starting material (Figure 1, lane 1). The membranes were first treated with alkali (lane 2). The residual pellet was dissolved in a SDS-containing buffer, diluted with a buffer containing Triton X-100 and incubated with concanavalin A (Con A)–Sepharose. After elution with α-methyl-mannoside (α-Man), one of the most prominent protein bands had a M_r of ~34 K (lane 4). Residual 34 K protein bound to the Con A–Sepharose could be released by SDS together with some other polypeptides (lane 5). The 34 K protein purified from preparative SDS–gels was electrophoretically pure (lane 6).

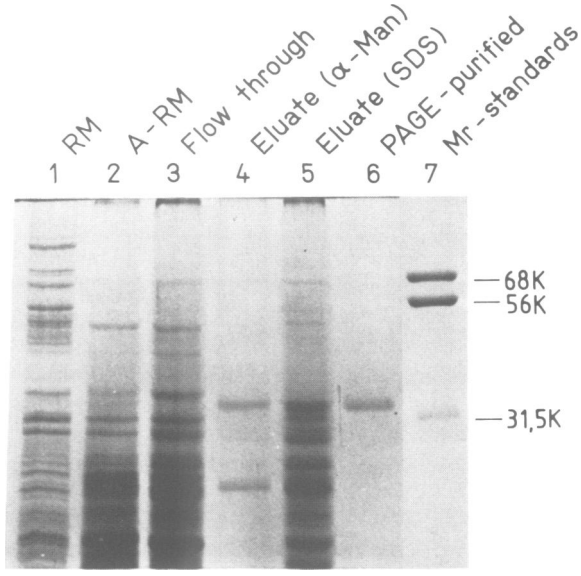

Fig. 1. Purification of the 34 K protein from canine microsomes. Shown is the protein pattern at different stages of the purification (Coomassie staining after SDS–PAGE). **Lane 1**, RM, rough microsomes (5 eq.); **lane 2**, A-RM, alkali-extracted rough microsomes (200 eq.); **lane 3**, flow through after binding to Con A–Sepharose (600 eq.); **lane 4**, eluate obtained from Con A–Sepharose by α-Man (2000 eq.); **lane 5**, eluate obtained by subsequent elution with SDS (2000 eq); **lane 6**, purified 34 K protein after preparative SDS–PAGE (2000 eq.); **lane 7**, M_r standards: bovine serum albumin, 68 K, catalase, 56 K, DNase I, 31.5 K. The quantities given in eq. (see Walter *et al.*, 1981) refer to the original membranes.

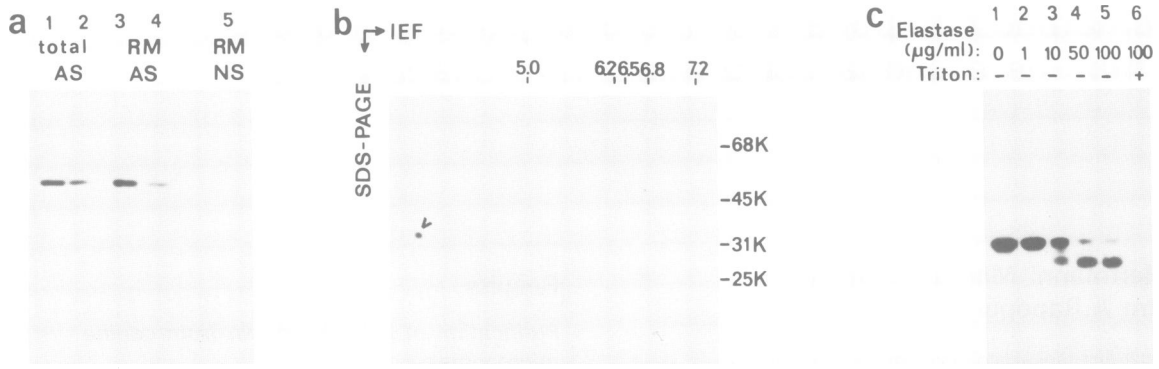

Fig. 2. Antibodies against the 34 K protein recognize a single, membrane-spanning ER protein. (**a**) Immunoblotting after one-dimensional separation of proteins from total pancreas (total) (**lane 1**, 50 μg; **lane 2**, 25 μg) and from rough microsomes (RM) (**lanes 3** and **5**, 2 eq.; **lane 4**, 0.5 eq.) (10% SDS–polyarylamide gel). (**b**) Immunoblotting with affinity-purified antibodies after two-dimensional O'Farrell electrophoresis of RM-protein. (**c**) Immunoblotting of RM-proteins after treatment of microsomes with elastase at different concentrations. AS, IgG from an antiserum against the 34 K protein; NS, IgG from a nonimmune serum; IEF, isoelectric focusing. M_r standards: bovine serum albumin, 68 K; ovalbumin, 45 K; carboanhydrase, 31 K; chymotrypsin, 25 K.

Antibodies against the 34 K protein recognize a single, membrane-spanning ER protein

Polyclonal antibodies against the purified protein were raised in rabbits. An immunoglobulin G (IgG) fraction obtained from an antiserum gave essentially only one band at the expected position (~34 K) in immunoblots with either microsomal membrane protein or total pancreatic protein (Figure 2a). This result was confirmed by immunoblotting after two-dimensional O'Farrell electrophoresis with antibodies affinity-purified on a Sepharose column to which the 34 K protein had been coupled (Figure 2b). The apparent isoelectric point of the 34 K protein was estimated to be around pH 4.1.

The results shown in Figure 2c indicate that the 34 K protein is partly exposed to the cytoplasm. Treatment of rough microsomes with elastase at high concentrations (>50 μg/ml) yielded an immunologially reactive fragment of ~28 K (lanes 4 and 5) which was degraded if detergent was present during proteolysis (lane 6). Similar results were obtained with trypsin and proteinase K (not shown). Since the 34 K protein is glycosylated, a portion of it must also be exposed to the lumen of the ER membrane, indicating that the protein spans the membrane at least once.

It should be noted that most of the 34 K protein remained undegraded after treatment of microsomes with 10 μg/ml elastase (lane 3), conditions in which the α-subunit of the SRP-receptor (docking protein) is completely cleaved (results not shown) (see Gilmore *et al.*, 1982a; Meyer and Dobberstein, 1980).

It was also demonstrated that some of the polyclonal antibodies react with the cytoplasmic domain of the 34 K protein in intact microsomes. Microsomes were incubated with the antibodies and, after sedimentation and washing, the amount of bound antibodies was determined with [^{125}I]protein A. Controls with unrelated antibodies and with proteolysed microsomes (100 μg/ml proteinase K) showed <15% binding (data not shown).

Antibodies to the 34 K protein inhibit protein translocation in vitro

The antibodies against the 34 K protein were tested for their inhibitory effect on protein translocation across canine microsomal membranes (Figure 3). Microsomal membranes from dog pancreas were extracted with high salt concentrations and EDTA to remove SRP and ribosomes. They were then preincubated with monovalent F_{ab} fragments produced from IgG-fractions of either preimmune serum or antiserum against the 34 K protein. After removal of the excess of the antibodies by repeated sedimentation, the microsomes were added to a translation system in which the synthesis of preprolactin had been arrested by SRP (Figure 3a, cf. lanes 1 and 2). Both the release of the translational arrest by the microsomes and cleavage of the signal peptide, which would be indicative for translocation, were determined. Globin was cosynthesized to rule out any unspecific inhibition of translation. It may be seen that control microsomes brought about both release of the translational arrest exerted by SRP (indicated by the increased radioactivity in the translation products) and translocation in a concentration-dependent manner (cf. lanes 3 and 8 with lane 2). F_{ab} fragments produced from the nonimmune serum had no effect (lanes 4 and 5 and 9 and 10). On the other hand, F_{ab} fragments produced from immunoglobulins directed against the 34 K protein clearly inhibited translocation of preprolactin and prevented the release of the translational arrest. The effects were dependent both on the amount of membranes present in the translation assay and on the ratio of antibodies to membranes in the preincubation mixture [0.3 equivalents (eq.) of membranes for lanes 6 and 7 and 1 eq. for lanes 11 and 12]. Intact immunoglobulins (Figure 3a, lane 13) and affinity-purified antibodies (Figure 3c, lane 2) had a similar effect as the F_{ab} fragments. However, the inhibition seen with F_{ab} fragments was not due to residual IgG molecules which were quantitatively removed by ion exchange chromatography (Figure 3b, lanes 1–4). Therefore it is likely that the inhibitory effect of the monovalent F_{ab} fragments is due to a specific binding to the 34 K protein rather than to gross changes in the membrane, e.g. patching, which may be conceivable for divalent antibodies.

The antibodies also inhibited the translocation of other secretory proteins. The translocation of pregrowth hormone was inhibited in the same experiments in which inhibition for preprolactin had been observed (seen on longer exposures of the X-ray films; not shown). Figure 3c demonstrates the inhibition of translocation of human placental prelactogen by affinity-purified immunoglobulins which were added directly to the SRP-arrested translation mixture (lanes 6 and 7). Unrelated immunoglobulins did not inhibit translocation

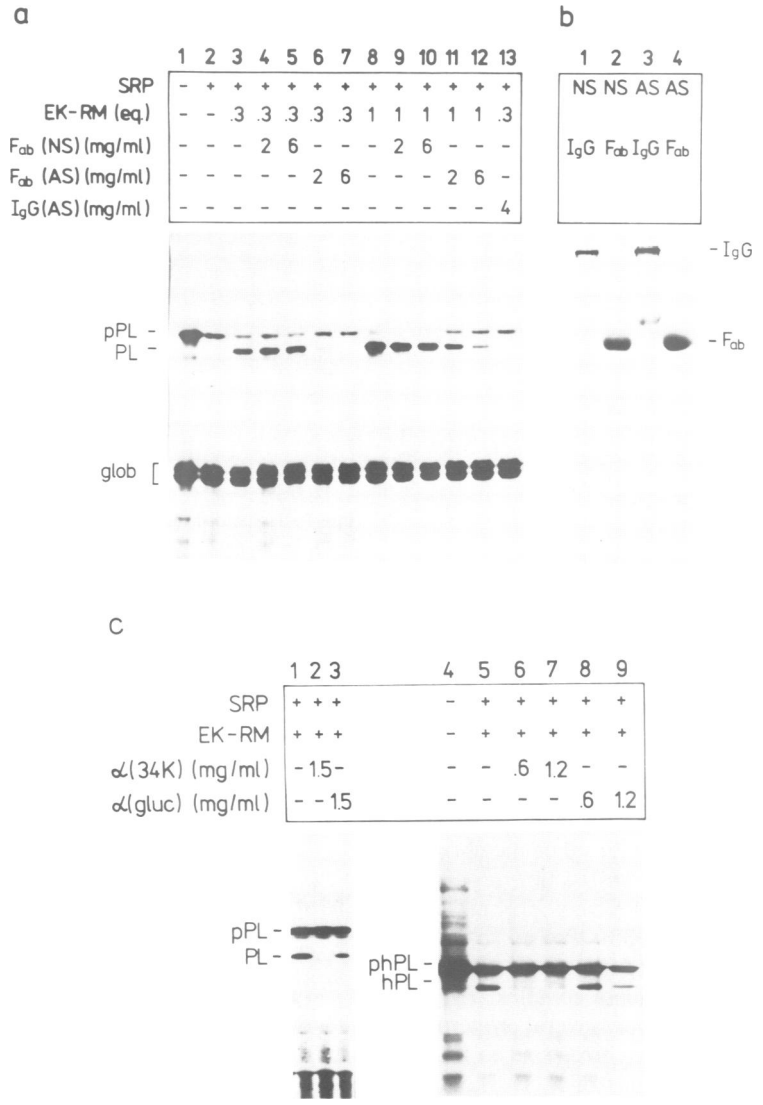

Fig. 3. Antibodies to the 34 K protein inhibit protein transloction *in vitro*. (**a**) Pretreatment of canine microsomes with F_{ab} fragments or with intact IgG directed against the 34 K protein inhibits translocation of preprolactin. (**b**) Electrophoresis of IgG and of F_{ab} fragments (Coomassie staining after SDS–PAGE). **Lane 1**, 5 μg protein; **lane 2**, 25 μg; **lane 3**, 7 μg; **lane 4**, 35 μg. (**c**) Translocation of preprolactin (**lanes 1–3**) or of human placental prelactogen (**lanes 5–9**) is inhibited by affinity-purified immunoglobulins directed against the 34 K protein. For **lanes 1–3**, microsomes were preincubated with antibodies, for **lanes 5–9** the antibodies were added directly to the translation mixture at the same time point as the EK-RM. EK-RM, RM extracted with high salt and EDTA; NS, nonimmune immunoglobulin; AS, immunoglobulin from an antiserum against the 34 K protein; F_{ab}, fragments of IgG produced by papain cleavage and purified by ion exchange chromatography; pPL, preprolactin; glob, globin; α(34 K), affinity-purified immunoglobulins directed against the 34 K protein; α(gluc), affinity-purified immunoglobulins directed against β-glucanase from *Bacillus amyloliquefaciens*; phPL, human placental prelactogen; hPL, human placental lactogen.

(lanes 8 and 9). The effect of the antibodies on the release of elongation arrest could not be studied in these experiments since the amounts of microsomes and antibodies had to be minimized to avoid inhibition of translation which occurred at higher concentrations of immunoglobulins (cf. lanes 8 and 9).

The assumption of a reversible blockade of translocation is further supported by the observation that freezing–thawing reactivated the translocation activity of the antibody-treated membranes (not shown). It should be noted that only a small percentage of the antibodies was actually bound to the membranes.

The 34 K protein is abundant

The quantity of the 34 K protein in microsomal membranes may allow inferences concerning its function in protein translocation. The amount of 34 K protein was determined by quantitative immunoblotting (Figure 4). It was estimated that 1.3 pmol of the 34 K protein are contained in 1 eq. of rough microsomes. This value is ~13 times higher than estimates of the amount of SRP receptor in the membranes (Tajima *et al.*, 1986) and at least equal to the amount of bound ribosomes [1 eq. of RM contain from 0.5 to 1 pmol bound ribosomes (Gilmore *et al.*, 1982b)]. By the same method it was estimated that the 34 K protein makes up ~0.15% of the total pancreatic protein.

The 34 K protein is ubiquitous

Figure 5 shows that the 34 K protein could be detected by immunological means in various tissues of different mammals and in the liver of chickens, in line with the assumption of its important general function. It could also be found in MDCK, CV-1 and vero cells (unpublished data).

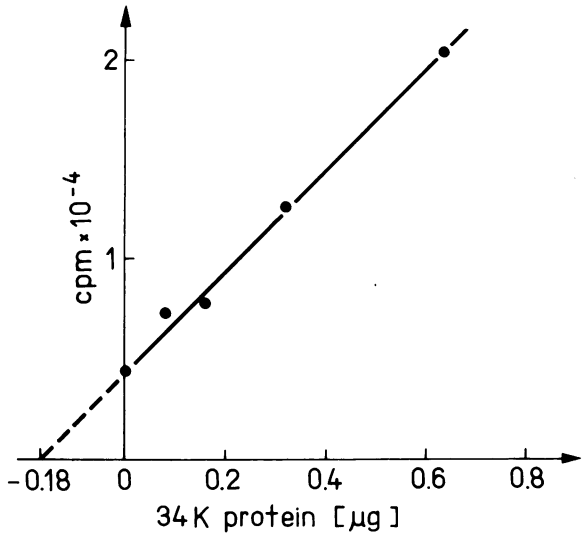

Fig. 4. Quantification of the 34 K protein in canine microsome by immunoblotting. An estimate for the amount of 34 K protein present in 4 eq. RM was obtained as described in Materials and methods. By plotting the radioactivity in the band versus the amount of 34 K protein added to the membranes, a value of 0.18 µg 34 K protein was obtained. From these data it is calculated that 1 eq. RM contains ~1.3 pmol 34 K protein (0.18 µg/4 eq. × 34 000 g/mol).

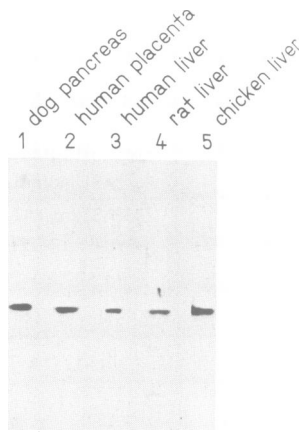

Fig. 5. Tissue distribution of the 34 K protein. RM were prepared from the various tissues and analysed by immunoblotting with antibodies to the 34 K protein and [^{125}I]protein A (4 eq. for dog pancreas, 100 eq. for chicken liver and 20 eq. for all other samples). An autoadiogram after exposure overnight is shown.

Discussion

The isolated protein is not identical with other membrane proteins hitherto implicated in the translocation process, e.g. the ribophorins (Kreibich *et al.*, 1978), a 30 K protein with affinity to SRP (Tajima *et al.*, 1986), the two subunits of the SRP receptor [69 K and 30 K (Tajima *et al.*, 1986)] or the polypeptides of the signal peptidase complex (Evans *et al.*, 1986) (data not shown). However, several properties of the isolated membrane component correspond to those of the SSR previously found by means of a crosslinking approach (Wiedmann *et al.*, 1987b). Both are glycosylated membrane proteins with a M_r of ~34–35 K which appear to span the phospholipid bilayer. Also, both are relatively insensitive to low concentrations of elastase.

The 34 K protein appears to play an essential role in protein translocation. It is unlikely that the inhibitory effects of the antibodies are indirectly caused by steric hindrance. We have estimated that no more than 1% of the total membrane surface area can be occupied by the antibodies (based on the amount of 34 K protein in microsomes, the size of F_{ab} fragments and that of microsomal vesicles). The fact that the antibodies not only inhibited translocation but also the release of translational arrest, which is solely a function of the SRP receptor (Gilmore *et al.*, 1982b), may be interpreted in two ways: either the 34 K protein and the SRP receptor are functionally linked or, perhaps more likely, blockade of a step subsequent to the function of the SRP receptor causes a backup of arrested translation complexes which cannot be removed by translocation.

The fact that the 34 K protein is present in at least equivalent amounts compared with ribosomes bound to the ER membrane suggests its direct involvement in the process of passage of the nascent polypeptide chain through the membrane. For example, it may be a constituent of a protein tunnel through which the polypeptide chain traverses the phospholipid bilayer (Blobel and Dobberstein, 1975; Rapoport, 1986). It would appear unlikely that the polypeptide alone forms the tunnel. However, preliminary results show that the 34 K protein is contained in the membrane in a complex with other protein(s) which together may represent the tunnel.

Materials and methods

Purification of the 34 K protein

Rough microsomes (RM) were prepared as described (Walter and Blobel, 1983a). RM (25 000 eq.) were extracted twice at 0°C for 15 min with 0.1 M Na_2CO_3 (pH 11–11.6). The pellet was dissolved in 5 ml buffer A [0.1 M Tris–HCl pH 7.5, 1% SDS, 50 mM dithiothreitol (DTT)] by incubation at 95°C. The sample was diluted with 35 ml Con A buffer (50 mM Hepes/KOH pH 7.5, 500 mM potassium acetate, 2.5 mM magnesium acetate, 1 mM $CaCl_2$, 1% Triton X-100, 0.5% Tween 20) and incubated for 4 h at room temperature with 2 ml prewashed Con A–Sepharose (Pharmacia) containing 10 mg Con A. After washing with Con A buffer, elution was performed with 0.5 M α-Man in Con A buffer overnight. Subsequent elution was carried out with buffer A at 95°C for 5 min. Final purification was achieved by preparative SDS–PAGE (10% polyacrylamide gel). The 34 K protein band was visualized by immersion of the gel in 0.5 M potassium acetate at 4°C, the band cut out and the material eluted by shaking overnight with 5 ml of 0.01 M Tris–HCl pH 7.5, 0.2% SDS, 50 mM DTT. The material was concentrated by centrifugation through dry Sephadex G25 (Pharmacia). The total yield of 34 K protein from 25 000 eq. RM was ~200 µg.

Immunological techniques

Rabbits (3 kg) were injected twice with ~100 µg 34 K protein in Freund's adjuvant. An immunoglobulin fraction was obtained from the serum by precipitation with $(NH_4)_2SO_4$. Affinity-purified immunoglobins were obtained as described (Walter and Blobel, 1983c) using CNBr-activated Sepharose to which ~50 µg of 34 K protein had been coupled. F_{ab} fragments were prepared from IgG by treatment with papain. They were purified by chromatography on CM-Sephadex (Malinowski and Manski, 1981).

Immunoblotting was carried out after electrophoretic separation of the proteins by electrotransfer onto nitrocellulose (Kyhse-Andersen, 1984) and subsequent incubations with antibodies followed by detection with [^{125}I]protein A and autoradiography (Burnett, 1981). Total immunoglobulins were used at ~6 µg/ml, affinity-purified immunoglobulins at ~1 µg/ml. [^{125}I]protein A was labelled to a specific radioactivity of ~60 TBq/mmol and used at ~75 Bq/ml. For immunological detection of the 34 K protein in various tissues, RMs were prepared according to the

procedure for canine microsomes (Walter and Blobel, 1983a). All membranes were suspended at 50 $A_{280\ nm}$ units/ml (1 eq./μl). Microsomes of chicken liver were extracted with alkali before electrophoresis.

Quantitative immunoblotting was carried out according to Tajima *et al.* (1986) with modifications. The concentration of the 34 K protein in a stock solution was determined by its absorption at 210 nm after size exclusion HPLC using bovine serum albumin as a standard. A Si 300 Polyol column (0.7 × 25 cm) was equilibrated with 50 mM sodium phosphate pH 7.0 containing 0.1% SDS. The estimate was in good agreement with determinations by standard methods (Lowry *et al.*, 1951; Schaffner and Weissmann, 1973). Known quantities of the 34 K protein were added to 4 eq. RM and the samples applied to SDS–PAGE. After transfer of the proteins to nitrocellulose, incubation with antiserum against the 34 K protein and with [^{251}I]protein A, the labelled bands were detected by autoradiography, cut out and counted in a gamma-counter.

Inhibition of translocation by antibodies

Translation was carried out essentially as described (Wiedmann *et al.*, 1984). The DTT present in the wheat germ extract was removed by centrifugation through Sephadex G25. The final assay contained in 12.5 μl: 2.5 μl wheat germ extract, 140 mM potassium acetate, 2.2 mM magnesium acetate, 0.8 mM spermidine, 0.3 mCi/ml [^{35}S]methionine, 400 U/ml placental RNase inhibitor, 0.1 μg/ml each of pepstatin, chymostatin, antipain and leupeptin, 10 U/ml trasylol. Poly(A)$^+$ RNA from rabbit reticulocytes (globin mRNA) and from bovine pituitaries (preprolactin mRNA) were added to 8 and 40 μg/ml respectively, total RNA from human placenta (placental lactogen) at 120 μg/ml. After translation for 5 min in the presence of SRP (25 nM), RM extracted with 0.5 M potassium acetate and 25 mM EDTA (EK-RM) (Walter and Blobel, 1983b) were added. In some experiments the antibodies were added at the same time point as the EK-RM. After further incubation for 25 min at 26°C, translation was stopped and the products were separated in a 12% polyacrylamide gel. Pretreatment of the microsomes with antibodies was carried out as follows: 10 eq. EK-RM were incubated with IgG, F_{ab}, affinity-purified immunoglobulin or without antibodies in 0.2 ml buffer M (50 mM Hepes/KOH pH 7.5, 80 mM potassium acetate, 5 mM magnesium acetate) for 1 h at 22°C and 1 h at 4°C. Protease inhibitors were present at concentrations twice as high as in the translation mixture and 1 mM PMSF was also added. The membranes were sedimented for 45 min in a microfuge (10 000 *g*), washed once with buffer M (without protease inhibitors) and finally resuspended in 10 μl one-tenth concentrated buffer M.

Miscellaneous techniques

SDS–PAGE was carried out according to Laemmli (1970). O'Farrell electrophoresis (O'Farrell, 1975) was carried out with isoelectric focusing (IEF) in the first dimension (ampholines Servalyte pH 4–9 and 5–9 mixed 1:1) and a 10% SDS–polyacrylamide gel in the second dimension. Proteins of Ehrlich ascites tumour cells of known isoelectric points served as markers (Benndorf *et al.*, 1988). Incubation of the microsomes with elastase was for 1 h at 0°C. Elastase was pretreated as described (Gilmore *et al.*, 1982a).

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