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Secretory protein translocation across membranes—the role of the ‘docking protein’

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On emergence of the signal sequence of nascent secretory proteins from the large ribosomal subunit, translation is stopped by a cytoplasmic protein complex. A specific membrane protein of the endoplasmic reticulum, the ‘docking protein’, releases this block and allows further synthesis to be directly coupled to transfer across the membrane.

NASCENT secretory proteins are extended at the N-terminus by 15–30 amino acids, the signal or leader sequence^{1–3}. This extension, or its functional equivalent⁴, is required for the vectorial transfer of the protein across the membrane of the endoplasmic reticulum (ER)^{1,5–9}. On emergence of the signal sequence from the large ribosomal subunit, the ribosomal complex specifically makes contact with the membrane, then the nascent protein is translocated across the ER membrane. As the translocation phenomenon has been reconstituted *in vitro* using isolated rough microsomes^{7,10}, the molecules which mediate this transfer must be present in, and dissectable from, these membranes^{11–16,17}.

The ability to transport nascent secretory proteins is lost when rough microsomes are exposed to high salt concentrations¹⁸. A protein complex of molecular weight (MW) 250,000 (250K) is removed from the membrane in these conditions, which results in the loss of translocation activity^{19,20}. Recently, it was determined that this 250K protein, referred to as the signal recognition protein (SRP), functions *in vitro* by selectively stopping the translation of nascent secretory proteins²¹. When salt-washed rough microsomes are added to the SRP-blocked system, translation continues and translocation of the nascent peptide occurs²¹. The question then arises as to which ER-specific membrane component is responsible for relieving the translation block. We have recently shown that a 60K peptide can be released from salt-washed rough microsomes by elastase and high salt treatment^{15,16}. This component is ER-specific, is required for vectorial transfer of secretory proteins, and is derived from a membrane-bound protein of molecular weight 72K (ref. 22). So far, the role of the 72K protein has remained unknown.

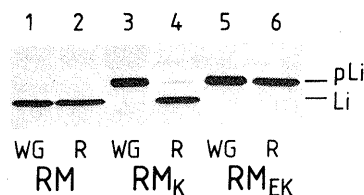


Fig. 1 Translocation activity of rough microsomes assayed in wheat germ and reticulocyte lysate. RM, whole rough microsomes; RM_K, KCl-washed RM; RM_{EK}, elastase-treated, KCl-washed RM; WG, wheat germ lysate; R, reticulocyte lysate system; pLi, pre-IgG light chain; Li, authentic IgG light chain. Membranes were prepared and *in vitro* translations were performed as described previously^{15,18}. Translocation activity is measured as the ability of rough microsomes to convert pLi to Li when added to a cell-free system primed with mRNA encoding mainly IgG light chain. The figure shows a fluorogram depicting the translation products of *in vitro* synthesis.

Table 1 Ability of protein components to restore translocation activity to microsomal membranes

Microsomes/components	Activity in:	
	Wheat germ	Reticulocyte lysate
RM	+	+
RM _K	–	+
RM _K +SRP	+	+
RM _K +DP _f	–	+
RM _{EK}	–	–
RM _{EK} +DP _f	–	+
RM _{EK} +SRP	–	–
RM _{EK} +DP _f +SRP	+	+

Rough microsomes (RM) in combination with isolated factors (DP_f or SRP) were tested for their ability to translocate nascent proteins in wheat germ and reticulocyte lysate systems. To exclude the possibility that reconstitution was being mediated by contaminating proteins in DP_f preparations, controls were done using a specific anti-DP_f antibody²². In these controls the antibody was used to specifically remove DP_f from the preparations. Such controls were performed in every case where DP_f was used in this report, and each time the preparation treated in this way was unable to reconstitute activity in inactive rough microsomes. RM_K, KCl-washed RM; RM_{EK}, elastase-treated and KCl-washed RM; SRP, signal recognition protein; DP_f, cytoplasmic fragment of docking protein released by elastase and high salt. Microsomes and factors were prepared and assays carried out as described previously^{15,16,20}.

We report here that this protein is indeed the molecule which relieves the SRP-induced block. Translation can then proceed when the initiated ribosomal complex has made contact with the correct membrane, that is, the one containing the 72K protein. In accordance with this function, we shall henceforth refer to this molecule as the ‘docking protein’ of the ER. We also report the presence in the cell cytoplasm of a functional equivalent of the microsomal signal recognition protein.

Signal recognition protein interacts with docking protein

Previously published data suggested that the source of the cell-free translation system affected greatly the translocation capability observed for salt-washed microsomes (compare refs 14, 15 with 19, 20). Whole rough microsomes (RM) were fully active in the wheat germ and reticulocyte lysate systems (Fig. 1, lanes 1 and 2). Salt-washed microsomes (RM_K), however, were only active in the reticulocyte lysate (compare Fig. 1, lane 3 with lane 4). Microsomes treated with protease and high salt (RM_{EK}) were inactive in either system (Fig. 1, lanes 5 and 6).

Salt washing exerts its effect by removing the 250K complex (SRP) from rough microsomes^{19,20}, whereas salt washing in conjunction with proteolysis removes from RM_K an active 60K cytoplasmic domain from the 72K docking protein (referred to as 'docking protein fragment', or DP_f)¹⁶. To define the requirements of translocation more precisely, we recombined the two factors with the various inactive rough microsomes and tested these in the two translation systems. The addition of the 60K fragment conferred activity on RM_{EK} in the reticulocyte lysate system alone; this combination is hence functionally equivalent to RM_K (see Table 1). It should then be possible to add SRP to $RM_{EK} + DP_f$ and restore activity in the wheat germ system. This was indeed the case, suggesting a functional identity between $RM_{EK} + DP_f + SRP$ and whole rough microsomes. We therefore conclude that two components are required to restore full activity to RM_{EK} : the 60K DP_f fragment and the 250K complex (SRP). It is clear also that reticulocyte lysate contributes the functional equivalent of the SRP.

The fact that the SRP and the DP_f could be removed sequentially from rough microsomes suggested a possible interaction between them. To test this, membranes (RM_{EK}) were incubated with either SRP or DP_f . After washing, the membranes were assayed in the wheat germ system in the presence of DP_f or SRP, respectively. In agreement with the results given above (Fig. 1), neither RM_{EK} (Fig. 2a, lane 1) nor RM_{EK} charged with

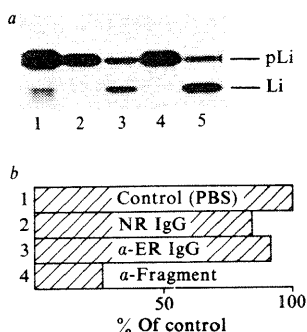


Fig. 2 Sequential reconstitution of translocation activity from isolated components. Inactive rough microsomes (RM_{EK}) were allowed to bind DP_f or SRP, and assayed in wheat germ in the presence of the component that was omitted from the preincubation. *a*, Lane 1, RM_{EK} ; lane 2, RM_{EK} charged with DP_f ; 3, RM_{EK} charged with DP_f assayed in the presence of SRP; 4, RM_{EK} charged with SRP assayed in the presence of DP_f ; 5, intact rough microsome control. RM , DP_f and SRP were prepared as described elsewhere^{15,16,20}. Activity is defined as described in Fig. 1 legend. *b*, RM_K were treated with anti- DP_f IgG, washed and assayed for translocation activity in the presence of SRP in a dithiothreitol-free wheat germ system. Lane 1, activity of RM_K pretreated with PBS; 2, activity of RM_K pretreated with normal rabbit (NR) IgG; 3, activity of RM_K pretreated with an affinity-purified anti-ER glycoprotein antibody specific for antigens on the surface of RM_K vesicles; 4, activity of RM_K pretreated with anti- DP_f IgG²². In the membrane-affinity-based translocation assay, 2.5 μ l rough microsomes ($A_{280} = 60 \text{ ml}^{-1}$) were incubated in the presence of the fraction being tested at 0 °C for 30 min in conditions where KCl concentration = 50 mM. The membranes were pelleted by centrifugation at 12,500 g for 20 min, then the rough microsome pellets were washed and suspended in the complete wheat germ cell-free translation system and assayed for translocation activity.

DP_f (lane 2) were active. When the $RM_{EK} - DP_f$ membranes were assayed in the presence of SRP (Fig. 2a, lane 3) the activity was restored to the level of whole rough microsomes (Fig. 2a, lane 5). No reconstitution was observed (Fig. 2a, lane 4) for RM_{EK} pre-incubated with SRP and assayed in the presence of DP_f . These data demonstrate that DP_f must be present on the membrane for SRP to interact functionally with rough microsomes.

Further evidence for the functional interaction of DP_f with SRP is provided by data obtained using a specific anti- DP_f antibody²². When RM_K were charged with this antibody (by the method described in Fig. 2 legend), the membranes were

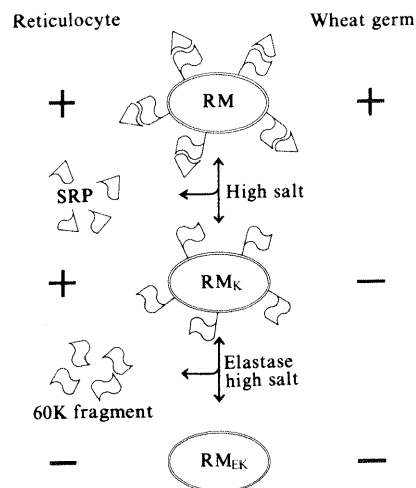


Fig. 3 Dissection and reconstitution of rough microsomes using protease and high salt. When rough microsomes are treated with 0.5 M KCl, translocation activity is lost in wheat germ (due to the salt-mediated removal of SRP) and RM_K are generated which are nonetheless active in reticulocyte lysate. Treatment of salt-washed microsomes (RM_K) with elastase and high salt yields RM_{EK} which are inactive in either system. This is due to the loss of a 60K peptide fragment from the docking protein (DP_f). Reconstitution can be achieved sequentially by re-addition of DP_f to RM_{EK} (thus restoring activity in reticulocyte lysate) followed by re-addition of SRP to $RM_{EK} - DP_f$ membranes. In this way full reconstitution is achieved in both cell-free systems.

no longer able to interact functionally with SRP (Fig. 2b). RM_K incubated with either pre-immune IgG or an ER-specific antibody directed against other antigens on the cytoplasmic surface of rough microsomes were still fully active when assayed in the wheat germ system in the presence of SRP.

A model summarizing these findings is shown in Fig. 3. High salt concentration removes the 250K complex (SRP), yielding RM_K which are inactive in the wheat germ system. Proteolysis and high salt remove the 60K portion of a larger membrane protein (DP), yielding RM_{EK} , which is inactive in either system. Reconstitution occurs in the reticulocyte lysate when DP_f is added to RM_{EK} and in the wheat germ system when DP_f and then SRP are added to RM_{EK} .

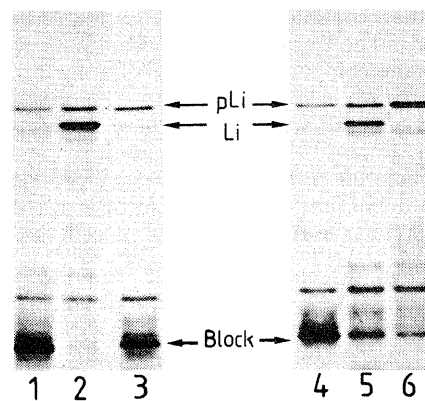


Fig. 4 SRP-induced block of translation of secretory proteins is removed by DP_f . Translations in the wheat germ system were blocked by the addition of SRP. We examined the ability of different membrane preparations to overcome this block. Lane 1, products of light chain mRNA-dependent cell-free translation (15 min) in wheat germ in the presence of SRP. Note the large accumulation of 8-9K material labelled 'block'. Lane 2, addition at 15 min of RM_K to a 'blocked' translation, observed at 40 min. 3, As in lane 2 except that RM_{EK} was added instead of RM_K . 4, Same as lane 1. 5, As in lane 2 except that $RM_{EK} + DP_f$ was added instead of RM_K . 6, As in lane 2 except that DP_f was added instead of RM_K (note the increase in pLi as opposed to Li due to the absence of membranes). Synchronous translation assays were carried out as described by Walter and Blobel²¹.

DP fragment releases SRP-induced translation block

Walter *et al.*²¹ have recently demonstrated that the SRP arrests the translation of preprolactin in the wheat germ cell-free system. This phenomenon is characterized by an accumulation of incomplete nascent peptides having a molecular weight of 7–8K. A similar result was obtained for IgG light chain (Fig. 4, lanes 1 and 4). This block is removed by the addition of RM_K (lane 2). Then the question arises: with which membrane component does SRP interact to result in the resumption of translation? DP_i , RM_{EK} or both could be required. As shown in Fig. 4, the block (lane 1) was removed by adding RM_K (lane 2), but not by addition of RM_{EK} (lane 3). When DP_i was added to the RM_{EK} , the block was removed (lane 5). The same effect was obtained when DP_i alone was added to the blocked system (lane 6). Thus we conclude that DP_i is necessary for the resumption of translation and translocation, while the residual membrane (RM_{EK}) is not.

Translation of secretory proteins blocked by soluble protein in reticulocyte lysate

The ability of RM_K to translocate nascent peptides in the reticulocyte lysate system, but not in wheat germ, suggested that a molecule equivalent to SRP must exist in the reticulocyte lysate. Such a component was characterized by making use of its ability to interact functionally with the membrane in low salt conditions. Lysate fractions were tested by allowing the component to bind to RM_K and assaying for reconstitution of translocation activity in the wheat germ system. The use of such an affinity-based assay was necessary, as direct addition of reticulocyte lysate inhibited the wheat germ cell-free system. Furthermore, it proved extremely sensitive. Several properties of the reticulocyte factor were determined; the results (Table 2) indicate that this factor has several of the basic features of SRP. It is an acidic protein of MW ~250,000, has a hydrophobic character, and blocks translation of nascent secretory proteins. The reticulocyte factor-induced block can be removed by adding RM_K , as was observed in the case of SRP. The presence of a block-inducing substance such as the reticulocyte factor in reticulocyte lysate may explain why the cytoplasmically-located nucleocapsid proteins of certain viral mRNAs were translated well and the membrane proteins only poorly²³. Due to the small quantity of the reticulocyte factor in reticulocyte lysate, it has not been possible to determine whether its subunit composition is the same as that of SRP.

The cytoplasmic location of reticulocyte factor suggested that SRP may also be only transiently associated with the membrane, and that it is present in the cell as a soluble, cytoplasmic factor. Using the membrane-affinity assay described in Fig. 2 legend, we found that post-microsomal supernatants of dog pancreas contained a substance capable of restoring activity to RM_K when assayed in the wheat germ system. It possessed all the

Table 2 Properties of the reticulocyte factor

Property	Method of determination
Proteinaceous	Proteolysis
Salt-linked to rough microsomes	Microassay
Acidic pI	DEAE binding*
Hydrophobic	ω -NH ₂ pentyl agarose*
MW = 250,000	Sucrose gradient*
Blocks translation of secretory proteins	Translation in wheat germ†

The ability to reconstitute translocation activity of RM_K in the wheat germ system was tested by adding various lysate-derived fractions using the method described in Fig. 2 legend. For proteolysis, the reticulocyte lysate was treated with trypsin ($20 \mu\text{g ml}^{-1}$) for 20 min at 25°C and then tested in the assay system described in Fig. 2 legend. In the microassay, the reticulocyte lysate was brought to 0.5 M in KCl and tested as above. * Ref. 20. † Ref. 21.

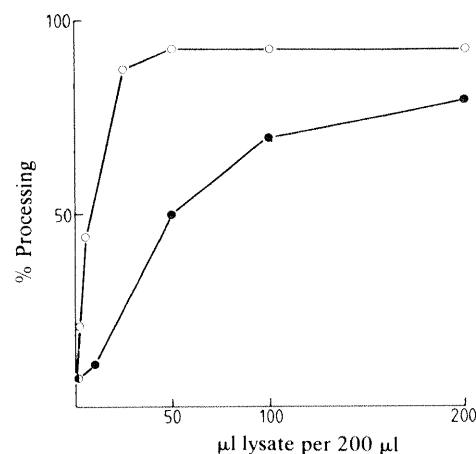


Fig. 5 SRP-like activity of reticulocyte lysate and dog pancreas post-microsomal supernatant. Reticulocyte lysate and dog pancreas supernatants (both depleted of ribosomes by centrifugation) were assayed for their ability to restore translocation activity to RM_K in the wheat germ system. $2.5 \mu\text{l}$ of RM_K ($A_{280} = 60 \text{ ml}^{-1}$) were incubated with reticulocyte lysate or pancreatic soluble phase (both corrected to 40% (v/v) homogenates) in amounts indicated on the abscissa. The membranes were washed and assayed for translocation activity in wheat germ lysate. Activity is expressed as the % conversion of pLi to Li. ●, Reticulocyte lysate; ○, dog pancreas supernatant.

characteristics of reticulocyte factor (see Table 2) and SRP. In addition, a fivefold higher level of SRP-like activity was found in dog pancreas supernatant when compared with reticulocyte lysate (Fig. 5). These findings are consistent with the functional role of SRP in translocation. The delay in translation of secretory proteins, that is, until contact with the proper membrane has been made, would be optimally localized in the cytoplasmic compartment.

Implications and conclusions

A model consistent with all the data reported here, and with data obtained previously^{14–16,18–22}, is shown in Fig. 6. The initial events in protein translocation can be described as follows. Synthesis of a secretory protein is initiated on free ribosomes. After 60–70 amino acids have been polymerized and the signal sequence has emerged from the large ribosomal subunit, further translation is interrupted by SRP²¹. This block persists until contact is made with the 72K docking protein of which the 60K fragment (DP_i) represents the cytoplasmic domain. At this point translation, coupled with translocation, proceeds.

Such a sequence of events ensures optimal co-translational processing of secretory proteins, even when synthesis commences on free ribosomes. The presence of a cytoplasmic form of

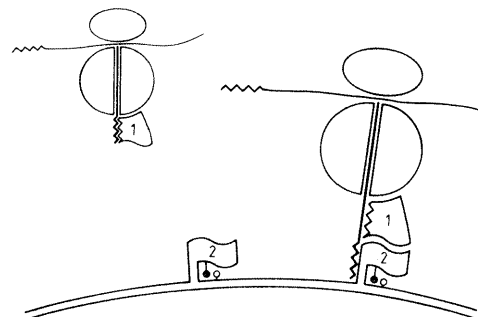


Fig. 6 Sequence of events in ER-specific translocation of secretory proteins. Initiation begins in the cytoplasm on free ribosomes. Translation is blocked by SRP (component 1) after 70–80 amino acids have been polymerized and the signal sequence emerges from the large ribosomal subunit (shown here in cross-section). This arrest of translation persists until contact is made with the 'docking' protein (component 2) which is a 72K, ER-specific membrane protein. Translation then resumes and translocation proceeds.

the signal recognition protein which blocks translation would guarantee that pre-secretory proteins are not completed in the cytoplasm. Efficient transfer of proteins can occur, therefore, only after the correct membrane has been contacted. Such a membrane can be operationally defined as one which contains the docking protein. This protein has been shown to be restricted to the endoplasmic reticulum²².

The 72K docking protein is the first site of interaction between nascent secretory proteins and the ER membrane. An understanding of the function of this protein and SRP explains

the specificity but not the mode of translocation. To determine the actual mechanism whereby proteins cross the membrane, further dissection of the membrane, including disassembly of the lipid bilayer, is necessary¹⁷. It is also conceivable that other soluble components provided by the cell-free translation systems function in this process. Their identification would require further fractionation of the lysates.

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Inducibility of human β -interferon gene in mouse L-cell clones

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Transfer of a 36-kilobase piece of human DNA containing the β -interferon (IFN- β) gene into mouse Ltk⁻ cells leads to transient expression of human interferon even without an exogenous inducer. A low level of human interferon expression is also found in most stable clones containing the transferred DNA. With double-stranded RNA or Newcastle disease virus (NDV) as inducer, human interferon expression is greatly increased. The induced transcript is identical to normal human IFN- β mRNA. Neighbouring genes contained on the transferred DNA are co-induced but are not essential for the production of human interferon in mouse L cells.

THE interferons (IFNs) are a class of proteins capable of inducing an antiviral state in many cell types against a wide range of viruses¹. Almost all cell types produce one or more interferons upon induction with virus or a synthetic inducer such as double-stranded RNA¹. FS4 cells, primary human fibroblasts, produce IFN- β (ref. 2) and several other proteins of unknown biological function³ in response to the inducer poly(rI)poly(rC). In these cells interferon is normally synthesized for a limited period of several hours⁴ during which the IFN- β mRNA accumulates and then rapidly disappears⁵. The shut-off occurs in the continuous presence of inducers and can be delayed by treatment with inhibitors of protein synthesis⁶. This treatment then allows the accumulation of IFN- β mRNA (superinduction)^{5,7}. It has been suggested that continuous protein synthesis supplies the cell with IFN- β mRNA degrading protein(s), thus involving the participation of other genes in IFN- β regulation⁶. We have analysed the induction of human IFN- β and interferon-associated genes in human fibroblast and lymphoblastoid cells^{8,9} and have previously reported the isolation and physical characterization of a 36-kilobase (Kb) region of the human genome including and surrounding an intact IFN- β gene¹⁰. The discovery that other poly(rI)poly(rC)-inducible genes are located in this cloned region presented the possibility that regulatory functions were acting through a common pathway on this family of genes.

We describe here the production of human interferon in mouse L-cell clones containing defined fragments from the

human genome surrounding and including the human IFN- β (HuIFN- β) gene. The human IFN- β gene is clearly inducible with a spectrum of induction protocols which reflect the responsiveness of the mouse cells' own interferon genes rather than those of human fibroblasts. Because a 1.9-kb DNA fragment carrying only the structural gene of HuIFN- β is sufficient to allow induction, the neighbouring genes located on the human chromosomal DNA are not essential for the human interferon induction in mouse L cells.

Isolation of mouse L cells containing HuIFN- β cosmid DNA

We have co-transferred¹¹ cosmid pCosIFN- β DNA containing the human β -interferon gene¹⁰ (Fig. 1) together with plasmid pHCT9 2cos/tk DNA containing the herpes simplex virus thymidine kinase gene¹² into mouse Ltk⁻ cells. More than 20 hypoxanthine-aminopterin-thymidine (HAT)-resistant clones were isolated. The single clones were maintained in culture for over 50 generations of HAT medium before further studies at the molecular level were carried out. During this period the properties of individual clones with respect to interferon production remained unaltered. High molecular weight DNA was prepared from these cell clones and compared with DNA from human diploid fibroblasts (FS4 cells), mouse Ltk⁻ DNA and cosmid clone pCosIFN- β DNA by Southern blotting¹³ after restriction enzyme digestion. Figure 2 shows the result for two