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MINI-REVIEW

Ribonucleoparticle-Independent Transport of Proteins into Mammalian Microsomes

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Abstract

There are at least two different mechanisms for the transport of secretory proteins into the mammalian endoplasmic reticulum. Both mechanisms depend on the presence of a signal peptide on the respective precursor protein and involve a signal peptide receptor on the cis-side and signal peptidase on the trans-side of the membrane. Furthermore, both mechanisms involve a membrane component with a cytoplasmically exposed sulfhydryl. The decisive feature of the precursor protein with respect to which of the two mechanisms is used is the chain length of the polypeptide. The critical size seems to be around 70 amino acid residues (including the signal peptide). The one mechanism is used by precursor proteins larger than about 70 amino acid residues and involves two cytosolic ribonucleoparticles and their receptors on the microsomal surface. The other one is used by small precursor proteins and relies on the mature part within the precursor molecule and a cytosolic ATPase.

Key Words: Mammalian endoplasmic reticulum; prepromelittin; prepropeptide GLa; preprocecropin A; M13 procoat protein; protein transport.

Introduction

Every polypeptide has a unique functional location, i.e., an intra- or extracellular location where it fulfills its function. The logic behind this subcellular compartmentalization is obvious; however, the understanding of the development and maintenance of such cellular compartments represents a

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central problem in modern cell biology. Two basic facts exist which complicate our attempts to understand this situation. These two facts are: (i) all proteins are synthesized in the cytosol (excluding mitochondrial and chloroplast protein synthesis); however, noncytosolic proteins must subsequently be directed to a variety of different subcellular locations, and (in the case of noncytosolic proteins) (ii) the sites of synthesis and of final functional location are separated by at least one biological membrane. Therefore, mechanisms must exist which ensure the specific transport of proteins across membranes and the assembly of proteins into membranes.

The relevant step in the biogenesis of most extracellular and many organellar proteins (e.g., resident proteins of the endoplasmic reticulum, plasma membrane proteins, proteins of the lysosomes and the Golgi complex) is their transport or assembly into the endoplasmic reticulum. In higher eukaryotes there appear to be at least four classes of precursor proteins with respect to their mechanism of transport and assembly, respectively, into microsomes. Two classes of precursor proteins depend on amino-terminal signal peptides and their putative receptor(s) on the microsomal surface (Robinson *et al.*, 1987; Wiedmann *et al.*, 1987a; Müller and Zimmermann, 1988a, Krieg *et al.*, 1989). One class consists of precursor proteins with a content of more than approximately 70 amino acid residues; another class consists of precursor proteins comprising less than 70 amino acid residues. The large precursor proteins typically involve the cytosolic ribonucleoparticles (ribosome and signal recognition particle) and their respective receptors on the microsomal surface (ribosome receptor and docking protein) (Müller and Zimmermann, 1987; Schlenstedt and Zimmermann, 1987); the small precursor proteins are not dependent on the ribonucleoparticles and their respective receptors (Watts *et al.*, 1983; Zimmermann and Mollay, 1986; Müller and Zimmermann, 1987; Schlenstedt and Zimmermann, 1987; Wiech *et al.*, 1987; Schlenstedt *et al.*, 1990). The other two classes of precursor proteins do not require amino-terminal signal peptides and ribonucleoparticles. So far, these two classes include resident membrane proteins of the endoplasmic reticulum only. One of these two classes is represented by cytochrome b_5, while the α subunit of the docking protein is an example of the other class. The cytochrome b_5-type proteins may be entirely receptor-independent and seem to involve a carboxy-terminal membrane insertion sequence (Bendzko *et al.*, 1982; Okada *et al.*, 1982; Anderson *et al.*, 1983); in contrast, the docking protein α subunit-type proteins are apparently receptor-dependent (Hortsch and Meyer, 1988; Andrews *et al.*, 1989).

Here we describe in detail the transport mechanisms of precursor proteins which contain signal peptides, i.e., the first two classes of precursor proteins which were mentioned above. We focus on presecretory proteins with special emphasis on precursors which contain less than 70 amino acid residues.

Materials and Methods

Our experimental system involves *in-vitro* systems for translation of native mRNAs or *in-vitro* transcripts and rough ER-derived vesicles (Zimmermann and Mollay, 1986; Wiech *et al.*, 1987; Schlenstedt *et al.*, 1990). We assay membrane insertion as removal of the signal peptide by signal peptidase on the luminal side of the microsomal membrane which results in the mature protein with a smaller apparent molecular weight on SDS-polyacrylamide gels (Zimmermann and Mollay, 1986; Müller and Zimmermann, 1988a; Schlenstedt *et al.*, 1990). Transport is assayed (i) as protection of the mature protein against externally added protease in the absence of detergent and sensitivity in the presence of detergent (Zimmermann and Mollay, 1986; Müller and Zimmermann, 1988a; Schlenstedt *et al.*, 1990), and (in the case of secretory proteins) (ii) as fractionation in the pellet after centrifugation at neutral pH values and fractionation in the supernatant at alkaline pH values (Müller and Zimmermann, 1987; Schlenstedt *et al.*, 1990).

Dog pancreas rough microsomes were prepared and stripped with micrococcal nuclease and EDTA as described (Watts *et al.*, 1983). Trypsin-pretreated microsomes were prepared as described (Schlenstedt *et al.*, 1990); specifically, microsomes were incubated with TPCK-trypsin, then soybean trypsin inhibitor and phenylmethylsulfonyl fluoride were added. Pretreatment of microsomes with sulfhydryl reagents was carried out as described (Zimmermann *et al.*, 1990); specifically, microsomes were incubated with *N*-ethylmaleimide, then dithiothreitol was added. For a mock pretreatment, microsomes were incubated with dithiothreitol plus *N*-ethylmaleimide.

Results

We have used the following presecretory proteins as tools for gaining insights into the molecular details of how proteins are transported into the mammalian endoplasmic reticulum: silkworm preprocecropin A (Boman *et al.*, Schlenstedt *et al.*, 1989; Schlenstedt *et al.*, 1990; Zimmermann *et al.*, 1990), honeybee prepromelittin (Zimmermann and Mollay, 1986; Müller and Zimmermann, 1987; Müller and Zimmermann, 1988a; Müller and Zimmermann, 1988b; Zimmermann *et al.*, 1988b), and frog prepropeptide GLa (Schlenstedt and Zimmermann, 1987). As an additional tool we have employed M13 procoat protein (Watts *et al.*, 1983; Wiech *et al.*, 1987; Sagstetter and Zimmermann, 1988; Zimmermann *et al.*, 1988a, 1990), the precursor of a bacterial plasma membrane protein. All four precursor proteins have cleavable signal peptides and contain about 70 amino acid residues (Fig. 1).

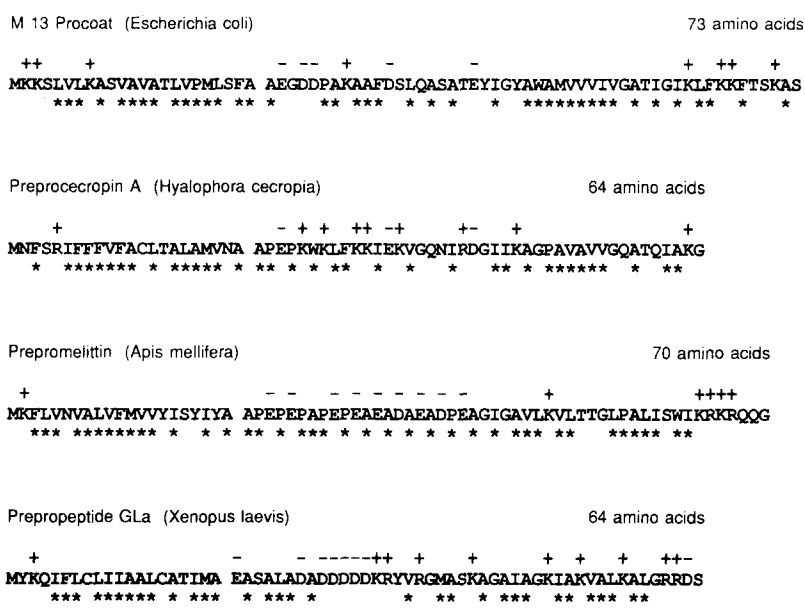

Fig. 1. Primary structure of procoat protein, preprocecropin A, prepromelittin and prepropeptide GLa. Amino acid sequences are given in the one-letter code. Positively (+) and negatively (−) charged amino acids are indicated as well as hydrophobic residues (*). Note that the hybrid protein ppmDHFR (257 amino acid residues) contains the complete sequence of ppm at the amino terminus and the complete sequence of mouse DHFR at the carboxy terminus, and that the hybrid protein ppcecDHFR (252 amino acid residues) contains the complete sequence of ppcec A at the amino terminus (it is linked by a threonine with the mouse DHFR sequence).

Small versus Large Presecretory Proteins

The transport of large precursor proteins involves the two ribonucleoparticles, ribosome (Perara *et al.*, 1986; Caulfield *et al.*, 1986; Chao *et al.*, 1987; Garcia and Walter, 1988; Roitsch and Lehle, 1988), and signal recognition particle (SRP) (Walter *et al.*, 1981; Walter and Blobel, 1981a, b), and their receptors on the microsomal surface, ribosome receptor (Hortsch *et al.*, 1986) and docking protein (Meyer and Dobberstein, 1980a, b; Meyer *et al.*, 1982). The transport of small precursor proteins does not involve the two ribonucleoparticles and their receptors (Zimmermann and Mollay, 1986; Müller and Zimmermann, 1987; Schlenstedt and Zimmermann, 1987; Schlenstedt *et al.*, 1990). The decisive feature of the precursor protein with respect to which of the two mechanisms is used is the chain length of the polypeptide. This conclusion was based on the observation that carboxy-terminal extension of a small precursor protein typically led to the phenotype of a large precursor protein (Müller and Zimmermann, 1987; Schlenstedt and Zimmermann, 1987). If one takes into account that approximately 40 amino

acid residues of a nascent polypeptide chain are burried in the ribosome (Maklin and Rich, 1967; Blobel and Sabatini, 1970; Bernabeu and Lake, 1982) and that a signal peptide contains 20–30 amino acid residues (von Heijne, 1981; Perlman and Halvorson, 1983; von Heijne, 1984) and, furthermore, that SRP can bind to signal peptides only as long as they are presented by a ribosome (Ainger and Myer, 1986; Wiedmann *et al.*, 1987b), one can imagine that precursor proteins with less than 60 to 70 amino acids cannot make use of the two ribonucleoparticles: they are released before SRP can bind to the signal peptide. Actually, this was observed when the ability of small precursor proteins to interact with SRP was assayed in an SRP arrest assay (Schlenstedt *et al.*, 1990).

Because of their apparent inability to efficiently use the SRP/ribosome system, the small precursors have apparently evolved with a special role of their mature part. The large precursors do not depend at all on their mature part whereas small precursor proteins rely on the structure of the mature part in order to be competent for transport. We carried out an extensive analysis of the role of the primary structure in the case of prepromelittin (Müller and Zimmermann, 1987; Müller and Zimmermann, 1988a, b; Zimmermann *et al.*, 1988b). The approach was to construct a hybrid protein between honeybee prepromelittin and mouse dihydrofolate reductase and, subsequently, to make internal deletions. Five classes of hybrid proteins were obtained which are different from each other with respect to their prepromelittin derived part. Within each class there were at least three different precursors differing in their size, i.e., the size of the part derived from dihydrofolate reductase. All precursors with more than 78 amino acid residues were competent for transort, irrespective of their prepromelittin content (Müller and Zimmermann, 1987).They were all behaving like large precursor proteins. Smaller precursors were either competent or incompetent depending on certain charged amino acid residues (Müller and Zimmermann, 1987). All competent forms behaved like prepromelittin. In prepromelittin and related precursor proteins a single (or cluster of) negatively charged amino acid residue(s) near the amino terminus of the mature part must be balanced by a single (or cluster of) positively charged amino acid residue(s) near the carboxy terminus (or charged amino acid residues must be absent from these positions altogether) in order to create a competent precursor protein. Competence in this case is a competence for binding and coincides with the ability of the mature part to form a hairpin loop which allows this compensation of charges. We have proved this by introducing an intramolecular disulfide bridge in such precursor proteins (Müller and Zimmermann, 1988a, b).

In general, transport of proteins into microsomes depends on nucleoside triphosphates. However, there are two systems to be discriminated between

in this respect: (i) There was a GTP requirement observed for the transport of ribonucleoparticle-dependent precursor proteins (Conolly and Gilmore, 1986, 1989). In this case, GTP could be replaced by nonhydrolyzable analogs. This GTP effect is related to the GTP-binding proteins SRP and docking protein (Conolly and Gilmore, 1989; Römisch *et al.*, 1989; Bernstein *et al.*, 1989). (ii) There was an ATP-requirement detected for the transport of ribonucleoparticle-independent precursor proteins (Schlenstedt and Zimmermann, 1987; Wiech *et al.*, 1987; Müller and Zimmermann, 1988; Schlenstedt *et al.*, 1990). Here, the ATP could not be replaced by nonhydrolyzable analogs. This ATP effect seems to have something to do with the competence of the precursor proteins for membrane insertion. Apparently, the small precursors have a mature part which allows a certain conformation of the precursors. On the other hand, an anti- or unfolding system seems to be involved in order to facilitate membrane insertion which does not allow a high degree of tertiary structure (Müller and Zimmermann, 1988a, b; Schlenstedt *et al.*, 1990). This system depends on the hydrolysis of ATP (Zimmermann *et al.*, 1988a). The ATP effect is related to components of the reticulocyte lysate (Wiech *et al.*, 1987). Heat-shock proteins of the hsp 70 family are part of this cytosolic ATPase, but at least one additional component from the lysate is involved (Zimmermann *et al.*, 1988a). The latter component, in contrast to hsp 70, is sensitive to alkylation with *N*-ethylmaleimide (H. Wiech, unpublished observation).

M13 Precoat Protein versus Small Presecretory Proteins

M13 procoat protein is known to insert into membranes in the absence of any membrane proteins, i.e., into liposomes which do not contain any protein (Geller and Wickner, 1985). Furthermore, it is known to insert into proteoliposomes which contain *E. coli* leader peptidase as the only proteinaceous component (Watts *et al.*, 1981; Ohno-Iwashita and Wickner, 1983; Ohno-Iwashita *et al.*, 1984). Under these conditions, procoat protein is processed to coat protein. We have shown previously that the insertion of procoat protein into such leader peptidase liposomes is stimulated by the cytosolic ATPase (Wiech *et al.*, 1987). Here we show that prepromelittin which is efficiently processed by *E. coli* leader peptidase in the presence of detergent (Cobet *et al.*, 1989) does not insert into leader peptidase liposomes, i.e., is not processed by shaved leader peptidase liposomes (Fig. 2).

Besides a putative signal peptide receptor, ribonucleoparticle-independent transport of presecretory proteins involves a membrane component which is sensitive to chemical alkylation with *N*-ethylmaleimide, i.e., which has an essential sulfhydryl (Zimmermann *et al.*, 1990). The sulfhydryl is cytoplasmically exposed (Zimmermann *et al.*, 1990) and is involved in membrane

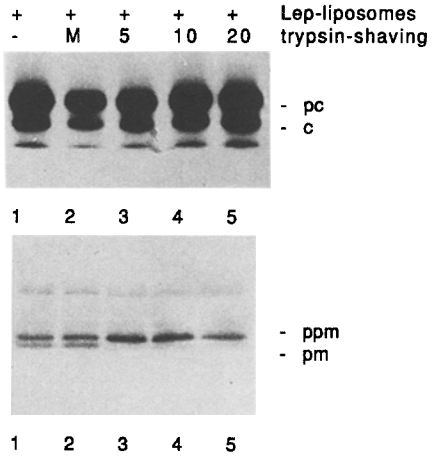

Fig. 2. Effect of trypsin pretreatment of leader peptidase liposomes on processing of M13 procoat protein and prepromelittin. Leader peptidase liposomes were prepared and pretreated with TPCK-trypsin as described (Zimmermann and Wickner, 1983) at the final concentrations of trypsin indicated. The trypsin-pretreated liposomes were characterized with respect to their abilities to process procoat protein and prepromelittin, respectively. Translation of procoat protein in the presence of [^{35}S]methionine and various liposomes was carried out in a bacterial extract for 60 min at 37°C (Wiech *et al.*, 1987). Translation of prepromelittin in the presence of [^{3}H]leucine and various liposomes was carried out in a rabbit reticulocyte lysate for 60 min at 37°C (Zimmermann and Mollay, 1986). The samples were analyzed by gel electrophoresis and fluorography.

insertion but not in membrane binding of the precursor proteins (M. Zimmermann, unpublished observation). This component may be identical to an *N*-ethylmaleimide-sensitive component which acts past docking protein and ribosome receptor in ribonucleoparticle-dependent transport (Hortsch *et al.*, 1986; Nicchitta and Blobel, 1989). The fact that M13 procoat protein, in contrast to the small secretory proteins, does not depend on the *N*-ethylmaleimide-sensitive membrane component rules out the possiblity that this component acts as a receptor for the cytosolic ATPase.

Small Presecretory Proteins versus Related Large Hybrid Proteins

The ribonucleoparticle-independent mechanism can also be used by a large precursor protein (Schlenstedt *et al.*, 1990). A synthetic hybrid between preprocecropin A and dihydrofolate reductase translocates posttranslationally (without the involvement of signal recognition particle and ribosome). This was directly demonstrated by adding methotrexate to the translocation reaction. Methotrexate and related drugs bind to ppcecDHFR after it is completed and released from the ribosome, stabilize the native conformation of the DHFR domain, and allow membrane insertion but block completion of translocation. Here we show two experiments supporting

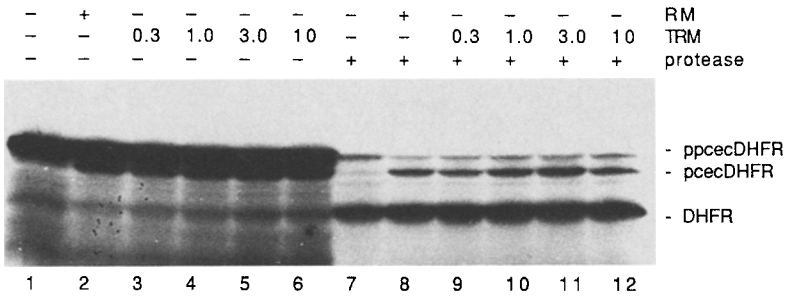

Fig. 3. Effect of trypsin pretreatment of microsomes on processing of ppcecDHFR and sequestration of pcecDHFR (posttranslational assay). Microsomes were pretreated with TPCK-trypsin as described (Schlenstedt *et al.*, 1990) at the final concentrations indicated. The trypsin-pretreated microsomes were characterized with respect to their abilities to process ppcecDHFR and import pcecDHFR (Schlenstedt *et al.*, 1990). Translation of ppcec DHFR in the presence of [^{35}S]methionine was carried out in the rabbit reticulocyte lysate for 15 min at 37°C. Translation was terminated by addition of cycloheximide and RNase A. Aliquots were further incubated for 30 min at 37°C in the presence of various microsomes at a concentration within the linear range. Each import reaction was divided into two halves and incubated further in the presence of absence of protease. The samples were analyzed by gel electrophoresis and fluorography.

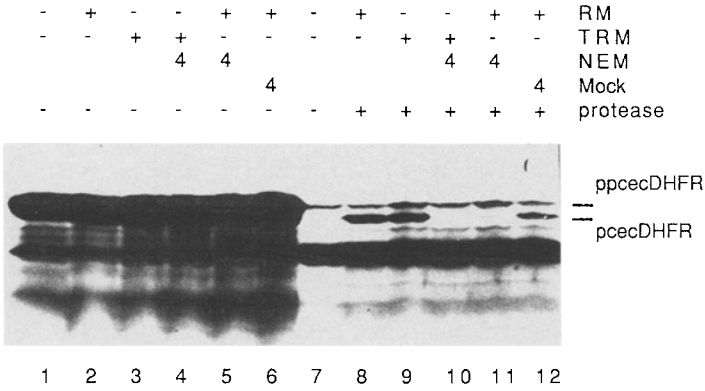

Fig. 4. Effect of trypsin pretreatment and subsequent *N*-ethylmaleimide-pretreatment of microsomes on processing of ppcecDHFR and sequestration of pcecDHFR (posttranslational assay). Microsomes were pretreated with *N*-ethylmaleimide as described (Zimmerman *et al.*, 1990) at the final concentrations indicated. Translation of ppcecDHFR in the presence of [^{35}S]methionine was performed in the rabbit reticulocyte lysate for 15 min at 37°C (Schlenstedt *et al.*, 1990). Protein synthesis was terminated by the addition of cycloheximide and RNase A. The samples were divided into six reactions, supplemented with RM-buffer (lanes 1, 7), untreated microsomes (lanes, 2, 8), microsomes pretreated with 30 μg trypsin/ml (TRM) (lanes 3, 9), microsomes pretreated with trypsin and subsequently with the indicated concentration of *N*-ethylmaleimide (NEM) (lanes 4, 10), microsomes pretreated with *N*-ethylmaleimide (lanes 5, 11), or mock-treated microsomes (lanes 6, 12) and incubated for 30 min at 37°C. Each reaction was divided into two halves and incubated further in the absence or presence of proteases as indicated. The samples were analyzed by gel electrophoresis and fluorography.

the notion that transport of ppecDHFR is qualitatively identical to the transport of the small parent protein ppcec A. Figure 3 shows that transport of ppcecDHFR under posttranslational conditions does not involve ribosome receptor and docking protein by shaving the microsomes under conditions known to eliminate both proteins. Figure 4, on the other hand, illustrates that transport of ppcecDHFR involves an *N*-ethylmaleimide-sensitive membrane component.

Discussion

The membrane which has to be traversed by most eukaryotic secretory proteins is the membrane of the endoplasmic reticulum. Secretory proteins are synthesized as precursors containing amino-terminal signal peptides (von Heijne, 1981; Perlman and Halvorsson, 1983; von Heijne, 1984). These signal peptides are essential for transport into the endoplasmic reticulum and are usually cleaved off during transport by signal peptidase which is located at the luminal face of the membrane (Jackson and Blobel, 1977; Baker and Lively, 1987; Shelness *et al:*., 1988: Greenburg *et al.*, 1989). There appear to be two classes of precursor proteins with respect to their mechanism of transport into mammalian microsomes. One class consists of precursor proteins with a content of more than approximately 70 amino acid residues, the other class consists of precursor proteins comprising less than 70 amino acid residues (Müller and Zimmermann, 1987; Schlenstedt and Zimmermann, 1987). The two mechanisms differ in several aspects from each other; these aspects, however, are related to each other (Fig. 5).

The large precursor proteins typically involve the ribosome and SRP and their respective receptors on the microsomal surface; the small precursor proteins do not depend on the ribosome or SRP nor the respective receptors (Watts *et al.*, 1983; Zimmermann and Mollay, 1986; Müller and Zimmermann, 1987; Schlenstedt and Zimmermann, 1987; Wiech *et al.*, 1987). Small precursor proteins can be transported in the absence of ongoing protein synthesis whereas large precursor proteins can only be transported under these conditions when they are artificially kept on the ribosome (Perara *et al.*, 1986). The explanation for these differences seems to come from the following facts: SRP typically binds to signal peptides of nascent polypeptides as soon as they emerge from the ribosome (Kurzchalia *et al.*, 1986; Krieg *et al.*, 1986). This interaction was proposed to lead to a subsequent SRP/ribosome interaction and to slow down or even block elongation (Walter *et al.*, 1981; Siegel and Walter, 1988; Bernstein *et al.*, 1989; Römische *et al.*, 1989); this effect on elongation is released by interaction of SRP with its receptor on the microsomal surface, the docking protein (dp in Fig. 5) (Meyer *et al.*, 1982;

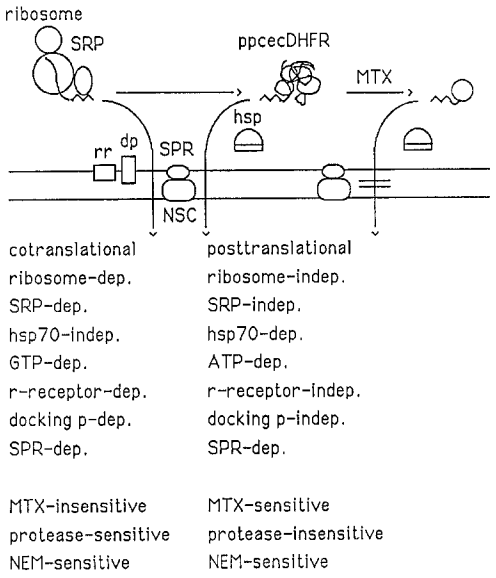

Fig. 5. A working model for the cotranslational and posttranslational modes of import of ppcecDHFR into mammalian microsomes. ATP: adenosine triphosphate; GTP: guanosine triphosphate; MTX: methotrexate; NEM: *N*-ethylmaleimide; NSC: NEM sensitive membrane component; SRP: signal recognition particle; SPR: signal peptide receptor; dep.: dependent; indep.: independent; docking p (dp): docking protein; hsp: heat shock protein; r-receptor (rr): ribosome receptor. See text for details.

Lauffer *et al.*, 1985; Tajima *et al.*, 1986; Connolly and Gilmore, 1989). At this point the signal peptide is believed to be handed over to a so-called signal sequence receptor (SPR in Fig. 5) (Wiedmann *et al.*, 1987a; Krieg *et al.*, 1989), and the ribosome is thought to bind to a putative ribosome receptor (rr in Fig. 5) (Hortsch *et al.*, 1986). Since the SRP/signal peptide interaction can occur only as long as the signal peptide is presented to SRP by ribosome (Ainger and Meyer, 1986; Wiedmann *et al.*, 1987b), the transport appears to be coupled to translation (Rapoport *et al.*, 1987). Since translation of a small precursor protein usually is terminated and the polypeptide is released from the ribosome before any of these interactions have occurred, the transport is not coupled to translation. Because the small precursor proteins cannot efficiently use this complex system, they apparently have evolved with constraints on the primary structure of their mature part and employ a cytosolic ATPase (hsp plus one additional component in Fig. 5). The sketch summarizes our current working model for the ribonucleoparticle-independent transport of proteins into mammalian microsomes. Small precursor proteins (and the hybrid protein ppcecDHFR) bind to the endoplasmic reticulum in a ribonucleoparticle-independent but receptor-dependent fashion (SPR in Fig. 5) (Müller and Zimmermann, 1988); however, a

cytosolic ATPase (Wiech *et al.*, 1987; Zimmermann *et al.*, 1988) and an *N*-ethylmaleimide-sensitive membrane component are involved (NSC in Fig. 5) (Zimmermann *et al.*, 1990). In order for insertion to occur, ATP hydrolysis is required for release of hsp 70.

The mechanism of transport of yeast prepro-α-factor (165 amino acid residues) into yeast microsomes appears to be similar to the mechanism of transport of small presecretory proteins into mammalian microsomes (Hansen *et al.*, 1986; Rothblatt and Meyer, 1986, Waters and Blobel, 1986). It also is ribosome-independent (Hansen *et al.*, 1986), depends on structural features of the precursor (Rothblatt *et al.*, 1987), and involves hsp 70, an additional component from the yeast lysate, and ATP (Chirico *et al.*, 1988; Deshaies *et al.*, 1988). In this case too the wheat germ lysate did not allow a similar type of transport (Rothblatt and Meyer, 1986; Waters *et al.*, 1986; Garcia and Walter, 1988). For unknown reasons it appears to be important to work with a homologous system (mammalian or yeast) in order to detect this type of transport. This may also be the reason why other authors made alternative interpretations of results concerning the transport of small precursor proteins into mammalian microsomes (Ibrahimi, 1987; Hull *et al.*, 1988).

Acknowledgments

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