A Large Presecretory Protein Translocates Both Cotranslationally, Using Signal Recognition Particle and Ribosome, and Post-translationally, without These Ribonucleoparticles, When Synthesized in the Presence of Mammalian Microsomes*

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Translocation of large presecretory proteins into the mammalian endoplasmic reticulum requires the ribonucleoparticles, signal recognition particle, and ribosome and is tightly coupled to ongoing protein synthesis. We have shown previously that small presecretory proteins can translocate post-translationally in a reaction that does not require these ribonucleoparticles. We now report that one large protein, a synthetic hybrid between preprocecropin A and dihydrofolate reductase, translocates both cotranslationally (with the aid of signal recognition particle and ribosome) and post-translationally (without the involvement of these ribonucleoparticles) during its in vitro synthesis in the presence of dog pancreas microsomes. The distinction between these two modes of translocation was made possible by adding methotrexate to the translocation reaction. Methotrexate can only form a tight complex with those preprocecropin A-dihydrofolate reductase hybrid chains that have completed their translation and folded, but in forming this tight complex, this drug prevents translocation of the dihydrofolate reductase domain across the membrane.

The only membrane that has to be traversed by a eukaryotic protein in the process of secretion is the membrane of the endoplasmic reticulum (for reviews, see Walter and Lingappa, 1986; Zimmermann and Meyer, 1986). Secretory proteins are typically synthesized as precursors containing amino-terminal signal peptides. These signal peptides are essential for import into the endoplasmic reticulum and are usually cleaved off during import by signal peptidase, which is located at the luminal face of the membrane (Jackson and Blobel, 1977). There appear to be two classes of precursor proteins with respect to their mechanism of import into mammalian microsomes. One class consists of precursor proteins with a content of more than approximately 75 amino acid residues, and the other class consists of precursor proteins comprising less than 75 amino acid residues (Müller and Zimmermann, 1987). This distinction was based on the SRP1 (signal recognition particle) and ribosome independence of naturally occurring small precursor proteins and the SRP and ribosome dependence of their artificial long derivatives (Müller and Zimmermann, 1987 and 1988a; Schlenstedt and Zimmermann, 1987) as well as on the fact that shortening of naturally occurring long precursor proteins to about 75 amino acid residues led to incompetent molecules (Ibrahimi et al., 1986; Siegel and Walter, 1988). Further support for this distinction came from the fact that the small precursor proteins showed constraints with respect to their primary structures whereas the large precursor proteins did not show any such constraints (Müller and Zimmermann, 1987, 1988a, 1988b). The two mechanisms differ in several aspects from each other: these aspects, however, are related to each other. 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 or 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 imported in the absence of ongoing protein synthesis whereas large precursor proteins can only be imported under these conditions when they are kept on the ribosome artificially (Porras et al., 1986; Mueckler and Lodish, 1986; Garcia and Walter, 1988; Roitsch and Lehle, 1988; Müller and Zimmermann, 1988a, 1988b; Zimmermann et al., 1988a). 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-riboseome interaction and to slow down or even block elongation (Walter et al., 1981); this effect on elongation is released by interaction of SRP with its receptor on the microsomal surface, the docking protein (Meyer et al., 1982; Gilmore et al., 1982). At this point the signal peptide is believed to be handed over to a so-called signal sequence receptor (Wiedmann et al., 1987a), and the ribosome is thought to bind to a putative ribosome receptor (Hortsch et al., 1986). Since the SRP-signal peptide interaction can occur only as long as the signal peptide is presented to SRP by the ribosome (Ainger and Meyer, 1986; Wiedmann et al., 1987b), the import appears to be coupled to translation.

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1 The abbreviations used are: SRP, signal recognition particle; DHFR, dihydrofolate reductase; pocc, preprocecropin; ppre, preprocecropin; TPCK, tosyl phenylalanyl chloromethyl ketone; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; SDS, sodium dodecyl sulfate; 7mG(5'ppp(5')G, RNA cap structure analogue.
It is important in this context that 30–40 amino acids of a nascent polypeptide are buried within the ribosome (Malkin and Rich, 1967; Blobel and Sabatini, 1970; Bernabeu and Lake, 1982) and that a signal peptide contains between 15 and 25 amino acids (von Heijne, 1984). Since translation of a small precursor protein usually is terminated and the polypeptide released from the ribosome before any of these interactions occur, the import is not coupled to translation. Because the small precursor proteins cannot make use of this complex system efficiently, they apparently have evolved with constraints on the primary structure of their mature part.

Here we describe the import of the small precursor protein preprocecropin A, the precursor of a secretary protein (Boman and Hultmark, 1987; Boman et al., 1989), and of a hybrid protein between the small precursor protein and the cytosolic protein dihydrofolate reductase (DHFR). Preprocecropin A (ppcec A, 64 amino acid residues) is processed and imported post-translationally by dog pancreas microsomes. Under these conditions import does not involve the ribonucleoparticles, ribosome, signal recognition particle, and their receptors on the microsomal surface but is energy dependent. Under cotranslational conditions, however, SRP and docking protein (SRP receptor) can mediate the import of this small precursor protein. All these characteristics are maintained by the hybrid protein (ppcDHFR, 252 amino acid residues) between ppcec A and DHFR (Fig. 1). This is demonstrated directly under the various conditions by employing folate analogues. 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 (Fig. 1). We conclude that the preprotein can use the SRP- and docking protein-independent import mechanism provided that it has the structural ability for ribosome-independent import. Therefore, we propose the term ribonucleoparticle-independent import for this mechanism that is not coupled to translation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cloning enzymes were purchased from Boehringer Mannheim as were RNase A, micrococcal nuclease, protein kinase K, TPCK-treated trypsin, soybean trypsin inhibitor, α1-antitrypsin, α2-macroglobulin, and the wheat germ lyase. The cap analogue 7mGTP(5′)ppp(3′)p, nucleoside triphosphates, and Sephadex G-20 were from Pharmacia LKB Biotechnology Inc. Plasmids pSP65 and pGEM8, SP6 polymerase, and human placental ribonuclease inhibitor (RNasin) were purchased from Promega Biotec. Rabbit globin mRNA was from Bethesda Research Laboratories. Cycloheximide, puromycin, penicillin, soy, amphotericin, trimethoprim, dihydrofolate, tetrahydrofolate, and 7-methylguanosine 5′-monophosphate were from Sigma. [35S]Methionine (100 Ci/mmol) from Pharmacia (100 Ci/mmol) were from Amersham Corp. X-ray films (X-Omat AR) were from Kodak. Phenylmethylsulfonyl fluoride and all other chemicals were purchased from Merck.

**Construction of Plasmids**—DNA manipulations were carried out according to Maniatis et al. (1982). Escherichia coli strain DH1 was routinely used and was transformed as described by Hanahan (1983). Plasmid pCA1, which contains the ppcec A coding region between its 3′ end and the SP6 promoter, was constructed by inserting an XbaI/PstI fragment of pCP9A1-2 (Liddilohm et al., 1987) into the polylinker of pSP65 (Melton et al., 1984). Plasmid pCA2 codes for the hybrid between the complete ppcec A and DHFR linked by a threonine. Plasmid pCP9A1-2 was treated with DdeI, which cleaves 4 base pairs in front of the termination codon of the ppcec A coding region and blunt ended with Klenow fragment of DNA polymerase. After cleavage with XbaI, a fragment was isolated and ligated into pGEM-DHFR, which had been cleaved previously with XbaI and Smal and dephosphorylated with calf intestine phosphatase. Plasmid pGEM-DHFR contains the SP6 promoter in front of the mouse DHFR coding region. It was constructed by inserting a BamHI/HindIII S1 nucleotidase-treated fragment of pGEM-DHFR (Stueber et al., 1984) into pGEM that was cleaved previously with SfiI and treated with S1 nuclease and calf intestine phosphatase. DNA sequencing was performed according to the chain termination method (Sanger et al., 1977).

**Preparation of Dog Pancreas Microsomes**—Dog pancreas microsomes were prepared and treated with micrococcal nuclease and EDTA as described (Watts et al., 1985); they were stored in RM buffer (20 mM Hepes [pH 7.5], 50 mM KC1, 2 mM magnesium acetate, 1 mM dithiothreitol, 200 mM sucrose) at an absorbance of 280 nm of 40, as measured in 2% SDS. Tryptsin-treated microsomes were prepared avoiding a resolation step (Zimmermann and Mollay, 1986); microsomes were incubated at different concentrations of TPCK-treated trypsin for 60 min at 0 °C, and then soybean trypsin inhibitor (35-fold molar excess with respect to trypsin) and phenylmethylsulfonyl fluoride (final concentration, 1 mM) were added, and the incubation was continued after each individual addition for 5 min. Finally, RM buffer was added to result in a 2-fold dilution of the trypsin-treated microsomes as compared with untreated microsomes. Salt-washed microsomes were prepared according to Walter and Blobel (1983) except that high salt buffer was used to wash the microsomes and that the microsomes were finally resuspended in RM-buffer.

**In Vitro Transcription and Translation**—Plasmids were linearized with appropriate restriction enzymes and transcribed and translated with SP6 polymerase as described (Watts et al., 1983; they were stored in RM buffer (20 mM Hepes [pH 7.5], 50 mM KC1, 2 mM magnesium acetate, 1 mM dithiothreitol, 200 mM sucrose) at an absorbance of 280 nm of 40, as measured in 2% SDS. Tryptsin-treated microsomes were prepared avoiding a resolation step (Zimmermann and Mollay, 1986); microsomes were incubated at different concentrations of TPCK-treated trypsin for 60 min at 0 °C, and then soybean trypsin inhibitor (35-fold molar excess with respect to trypsin) and phenylmethylsulfonyl fluoride (final concentration, 1 mM) were added, and the incubation was continued after each individual addition for 5 min. Finally, RM buffer was added to result in a 2-fold dilution of the trypsin-treated microsomes as compared with untreated microsomes. Salt-washed microsomes were prepared according to Walter and Blobel (1983) except that high salt buffer was used to wash the microsomes and that the microsomes were finally resuspended in RM-buffer.

**Post-translational Protein Import**—For post-translational import the translation was terminated after 15 min at 37 °C by the addition of cycloheximide (final concentration, 100 μg/ml) plus RNase A (final concentration, 50 μg/ml) or puromycin (final concentration, 80 μg/ml) and a further incubation for 5 min at 37 °C; then microsomes or RM buffer was added, and the samples were incubated for 10 or 30 min at 37 °C. Where indicated, folate analogues such as methotrexate (final concentration, 100 nM) were present during the import; when trypsin-treated microsomes were employed, α1-antitrypsin and α2-macroglobulin were added at final concentrations of 80 μg/ml. All samples of a particular experiment were adjusted to identical buffer and salt conditions.

**Analytical Procedures**—Sequestration assays were performed for 60 min at 0 °C in 80 mM sucrose and proteinase K at a final concentration of either 50 μg/ml (ppcemDHFR and preprolactin) or...
ppcec A and ppcecDHFR Are Imported into Mammalian Microsomes—The mechanisms of import of two related precursor proteins, preprocecropin A (ppcec A) and ppcecA dihydrofolate reductase hybrid (ppcecDHFR), into dog pancreas microsomes were characterized in cell-free systems derived from rabbit reticulocytes and wheat germ. The two precursor proteins are identical with respect to their signal peptide (22 amino acid residues) as well as their procecropin A domain (42 amino acid residues); they are, however, different in their size. ppcecDHFR in addition contains 1 threonine plus the full sequence of mouse dihydrofolate reductase (187 amino acid residues) on the carboxyl terminus of the ppcec A domain (Fig. 2).

Plasmids pCA1 and pCA2, which carry the cDNAs coding for ppcec A and ppcecDHFR, respectively, under the control of the strong bacteriophage SP6 promoter were transcribed in vitro. Translation in the different cell-free systems, programmed with these transcripts, yielded as major products with the expected molecular weights. Both precursor proteins synthesized in the rabbit reticulocyte lysate were efficiently processed by dog pancreas microsomes under cotranslational import conditions (Fig. 3A, lanes 1 versus 3 and 9; Fig. 4, A and C). The respective pro-forms fulfilled the accepted criteria for complete import into microsomes, i.e. they were inaccessible to proteases in the absence of detergent but degraded in the presence of detergent (Fig. 3A, lanes 3–5 and 9–11), and they were recovered with the microsomes upon subfractionation of import reactions at neutral pH values (Fig. 3B, lanes 1 versus 2) and with the soluble fraction at alkaline pH values (Fig. 3B, lanes 3 versus 4). The concentration of microsomes given as absorbance at 280 nm was 0.6 (lanes 3–8) or 1.2 (lanes 9–14). Translation reactions were incubated for 30 min at 37 °C, divided into aliquots that were incubated further as indicated without protease, with protease, or with protease in the presence of Triton X-100 as described under “Experimental Procedures.” The samples were analyzed by gel electrophoresis and fluorography. Note that after import the DHFR domain within ppcecDHFR folded and was able to bind methotrexate (lanes 12–14). B, translation of ppcec A in the presence of [3H]proline (lower panel) and ppcecDHFR in the presence of [35S]methionine (upper panel) was performed in the absence (lanes 1 and 2) or presence of dog pancreas microsomes (RM) (lanes 3–14). The concentration of microsomes given as absorbance at 280 nm was 0.6 (lanes 3–8) or 1.2 (lanes 9–14). Two reactions received methotrexate (MTX) (final concentration; 10 μM) (lanes 6–8 and 12–14). Translation reactions were incubated for 30 min at 37 °C, divided into aliquots that were incubated further as indicated without protease, with protease, or with protease in the presence of Triton X-100 as described under “Experimental Procedures.” The samples were analyzed by gel electrophoresis and fluorography. Note that after import the DHFR domain within ppcecDHFR folded and was able to bind methotrexate (lanes 12–14). B, translation of ppcec A in the presence of [3H]proline (lower panel) and ppcecDHFR in the presence of [35S]methionine (upper panel) was performed in the presence of dog pancreas microsomes (RM) for 30 min at 37 °C. Both samples were divided into three aliquots and subfractionated as described (Müller and Zimmermann, 1987). After two aliquots were adjusted to pH 11.5 by the addition of NaOH (final concentration, 50 mM), all six aliquots were incubated for 45 min at 0 °C. Then the pH 11.5-treated aliquots and two untreated aliquots (pH 7) were subjected to centrifugation for 30 and 5 min, respectively, at 30,000 g in a Beckman Airfuge. The resulting supernatants (S) and pellets (P) as well as the remaining unfractiobrated aliquots were analyzed by gel electrophoresis and fluorography.

ppcec A and ppcecDHFR Are Imported into Mammalian Microsomes in the Absence of Protein Synthesis—The import of mature cecropin A is underlined. Positively (+) and negatively (−) charged amino acids are indicated as well as hydrophobic residues (*). The hybrid protein ppcecDHFR (252 amino acids) contains the complete sequence of ppcec A at the amino terminus. It is linked by a threonine with the mouse DHFR sequence (Nunberg et al., 1980; Crouse et al., 1982). For plasmid construction, see “Experimental Procedures.” The sequence of the fusion region between ppcec A and DHFR was verified by DNA sequencing of plasmid pCA2.

Fig. 2. Primary structure of ppcec A and ppcecDHFR.

Amino acid sequences of ppcec A and ppcecDHFR are given in the one-letter code. The sequence of ppcec A (64 amino acids) is taken from Lichtenfels et al. (1987). The cleavage sites of signal peptides (arrow) was determined recently (Boman et al., 1989). The sequence of mature cecropin A is underlined. Positively (+) and negatively (−) charged amino acids are indicated as well as hydrophobic residues (*). The hybrid protein ppcecDHFR (252 amino acids) contains the complete sequence of ppcec A at the amino terminus. It is linked by a threonine with the mouse DHFR sequence (Nunberg et al., 1980; Crouse et al., 1982). For plasmid construction, see “Experimental Procedures.” The sequence of the fusion region between ppcec A and DHFR was verified by DNA sequencing of plasmid pCA2.
of both ppcec A and ppcecDHFR occurred under post-translational conditions also (Fig. 4, B and D; Fig. 5, lanes 2 versus 6). The efficiency of import under these conditions, however, was different for the two precursor proteins (Fig. 4, B versus D; Fig. 5, lanes 6). Import of ppcec A under post-translational conditions was very efficient (Fig. 4B); post-translational import of ppcecDHFR was less efficient but nevertheless significant (Fig. 4D) (see below). Furthermore, assays for processing of ppcecDHFR, on the one hand, and for sequestration of ppcecDHFR, on the other hand, gave different results under post-translational conditions; only about 50% of ppcecDHFR became protease resistant (Fig. 4D; Fig. 5, lanes 6) (see below). These data support our earlier conclusions with respect to the ability of mammalian microsomes to import small precursors under post-translational conditions. Furthermore, we report here on the first full-length large precursor protein that is imported by mammalian microsomes under identical conditions (data not shown).

According to our criteria, post-translational import of ppcecDHFR into mammalian microsomes should show three key features (Fig. 1). It should be ribosome independent but energy dependent, and it should be sensitive to methotrexate, a drug that is known to stabilize the native conformation of DHFR even within hybrid proteins (Eilers and Schatz, 1986). We have tested all these predictions in our in vitro system.

**Post-translational Import of ppcec A and ppcecDHFR Is Inhibited by ATP Depletion**—In order to study the energy requirements of the import of the two precursor proteins, post-translational import of both precursor proteins was tested after depletion of the translation reactions of nucleoside triphosphates either by treatment with apyrase (Fig. 6, lanes 3 and 4 versus 5 and 6) or by desalting with gel filtration chromatography. In both cases not even membrane insertion, as judged by removal of the signal peptide, was observed after ATP depletion; adding back ATP after gel filtration restored processing and sequestration (not shown).

**Post-translational Sequestration of ppcecDHFR Is Inhibited by Folate Analogues**—The post-translational import of a precursor protein, containing dihydrofolate reductase, allowed us to study the effect of methotrexate, a competitive inhibitor of the enzyme activity, on the import of this particular precursor protein (Fig. 1); ppcec A served as a control for this type of experiment since it does not contain the DHFR moiety and therefore should not be influenced by this drug. To rule out further any unspecified effects, other inhibitors (aminopterin and trimethoprim) as well as natural ligands (dihydrofolate and tetrahydrofolate) of DHFR were also tested. When ppcecDHFR was subjected to protease treatment, as it was usually employed to assay for sequestration, the highly protease-resistant DHFR domain was generated. Strikingly, in the presence of methotrexate, complete protease resistance of the DHFR domain was observed whereas in its absence, the protection of the DHFR domain was partial but nevertheless significant and reproducible (Fig. 5, lanes 3 versus 5; DHFR is shown in Fig. 5, lane 1, as a reference). All tested folate analogues gave similar results at different concentrations.

**Fig. 4. Efficiency of processing and sequestration of ppcec A and ppcecDHFR in cotranslational and post-translational assays in the rabbit reticulocyte lysate.** A and C, translation of ppcec A in the presence of [3H]proline (A) and ppcecDHFR in the presence of [35S]methionine (C) was performed in the absence of presence of dog pancreas microsomes (RM) for 30 min at 37 °C. B and D, translation of ppcec A in the presence of [3H]proline (B) and ppcecDHFR in the presence of [35S]methionine (D) was carried out for 15 min at 37 °C. Translation was terminated by the addition of cycloheximide and RNase A. Aliquots were incubated further for 30 min at 37 °C. The samples were analyzed by gel electrophoresis and fluorography.

**Fig. 5. Effect of methotrexate on processing and sequestration of ppcec A and ppcecDHFR by untreated and trypsin-pretreated microsomes in a post-translational assay.** Translational of ppcec A in the presence of [3H]proline (lower panel) and ppcecDHFR in the presence of [35S]methionine (upper panel) was performed in the rabbit reticulocyte lysate for 15 min at 37 °C. Protein synthesis was terminated by the presence of cycloheximide and RNase A during the following incubation for 5 min at 37 °C. The samples were divided into six reactions, supplemented with RM buffer (lanes 2–5), microsomes (lanes 6–9), microsomes pretreated with 3 μg of trypsin/ml (TRM) (lanes 4, 5, 8, 9, 12, and 13) and incubated for 30 min at 37 °C. Each reaction was divided into two halves and incubated further in the absence or presence of protease as indicated. The samples were analyzed by gel electrophoresis and fluorography. Upper panel, lane 1, shows an independent translation programmed with transcript of the plasmid pGEM-DHFR encoding mouse DHFR.
ppcEcDHFR in the absence of ATP or ribosomes (post-translational assay). Translation of ppcEc in the presence of \[^{3}H\]methylated methionine (upper panel) was carried out in the rabbit reticulocyte lysate for 16 min at 37 °C. Translation was terminated by addition of cycloheximide (CHI) and RNase A (lanes 1–6 and 11–14) or puromycin (lanes 7–10). The ATPase potato apyrase (10 units/ml) was added to one reaction (lanes 5 and 6). Furthermore, one reaction received apyrase that was denatured by pretreatment at 95 °C for 5 min (lanes 3 and 4). Following incubation for 5 min at 37 °C, samples were chilled on ice. One reaction was centrifuged for 15 min at 2 °C and 14,000 X g. Lanes 1–14 represent the postribosomal supernatant. Aliquots were incubated further for 15 min at 37 °C in the absence (lanes 1, 2, 7, 8, 11, 12) or presence of microsomes (RM) (lanes 3–6, 9, 10, 13, 14). Each aliquot was divided into two halves and incubated further in the presence or absence of protease as indicated. The samples were analyzed by gel electrophoresis and fluorography.

**Table I**

Inhibition of ppcEcDHFR sequestration and stabilization of the native DHFR conformation within ppcEcDHFR occur at different concentrations of various folate analogues

ppcEcDHFR was synthesized in the rabbit reticulocyte lysate in the presence of \[^{3}S\]methionine (upper panel) was carried out in the rabbit reticulocyte lysate for 16 min at 37 °C. Translation was terminated by addition of cycloheximide and RNase A (lanes 1–6 and 11–14) or puromycin (lanes 7–10). The ATPase potato apyrase (10 units/ml) was added to one reaction (lanes 5 and 6). Furthermore, one reaction received apyrase that was denatured by pretreatment at 95 °C for 5 min (lanes 3 and 4). Following incubation for 5 min at 37 °C, samples were chilled on ice. One reaction was centrifuged for 15 min at 2 °C and 14,000 X g. Lanes 1–14 represent the postribosomal supernatant. Aliquots were incubated further for 15 min at 37 °C in the absence (lanes 1, 2, 7, 8, 11, 12) or presence of microsomes (RM) (lanes 3–6, 9, 10, 13, 14). Each aliquot was divided into two halves and incubated further in the presence or absence of protease as indicated. The samples were analyzed by gel electrophoresis and fluorography.

**Table I**

<table>
<thead>
<tr>
<th>Folate analogue</th>
<th>Concentration of folate analogue resulting in half-maximal effect on ppcEcDHFR</th>
<th>DHFR</th>
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<tr>
<td>Methotrexate</td>
<td>12 nM</td>
<td>13 nM</td>
</tr>
<tr>
<td>Aminopterin</td>
<td>13 nM</td>
<td>13 nM</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>5 μM</td>
<td>4 μM</td>
</tr>
<tr>
<td>Dihydrofolate</td>
<td>130 μM</td>
<td>190 μM</td>
</tr>
<tr>
<td>Tetrahydrofolate</td>
<td>180 μM</td>
<td>190 μM</td>
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(DHFR in Table I). When methotrexate was present during the import of ppcEcDHFR into microsomes under post-translational conditions, there was no significant effect on the processing efficiency (Fig. 5, lanes 6 versus 8); the sequestration, however, was completely prevented (Fig. 5, lanes 8 versus 9). The inhibitory effect of methotrexate was relieved when an excess of isolated DHFR was added after the drug but prior to the microsomes (data not shown). The effect of methotrexate on the protease resistance of the DHFR domain served as an internal control for the action of the drug (Fig. 5, lanes 7 and 9). The other folate analogues gave similar results at different concentrations (ppcEcDHFR in Table I). No effect of methotrexate was observed for ppcEc A (Fig. 5, lanes 8–9). In every case, a clear correlation was observed between the increase of the protease resistance of the DHFR domain and the decrease of the efficiency of ppcEcDHFR sequestration as a result of increasing concentrations of the respective reagent (Table I). Furthermore, the active concentration of each reagent in both these respects correlated reasonably with the affinities of the respective reagent toward DHFR. Thus, several observations support the conclusion that compact folding of the DHFR domain within ppcEcDHFR is inhibiting import and that this occurs even in the absence of methotrexate to a certain extent (Fig. 1): (i) the high efficiency of ppcEc A sequestration under any import condition (Fig. 4, A and B); (ii) the low efficiency of ppcEcDHFR sequestration under post-translational import conditions in the absence of methotrexate (Fig. 4, C versus D) and the complete lack of sequestration in its presence (Fig. 5, lane 9); and (iii) the partial protease resistance of the DHFR domain in the absence of methotrexate (Fig. 5, lanes 3 and 7) and the complete resistance in its presence (Fig. 5, lanes 5 and 9).

After subfractionation of import reactions at neutral pH values, the processed but protease-sensitive ppcEcDHFR that had been generated in the presence of methotrexate was partially recovered in the soluble fraction (typically between 50 and 80%). Therefore, we conclude that only a transient membrane intermediate of ppcEcDHFR was formed under these conditions. Further support for this notion comes from (i) the lack of any chase of the microsome-associated ppcEcDHFR from a protease accessible to a protease-protected location under conditions in which methotrexate was removed (as monitored by an increase of the protease sensitivity of the DHFR domain), and (ii) the observation that the microsome-associated material behaved like a membrane protein (e.g. docking protein) in subfractionation employing alkaline pH values or urea (data not shown).

Post-translational Import of ppcEc A and ppcEcDHFR Does Not Involve the SRP-Docking Protein and Ribosome-Ribosome Receptor System—The effect of methotrexate on the import of ppcEcDHFR into microsomes indicated that the post-translational import does not involve ribosomes (Fig. 1). To substantiate this point further, we studied the ribosome independence of import of ppcEc A and ppcEcDHFR, respectively, into mammalian microsomes with two alternative approaches: independence of ribosomes was tested by the addition of puromycin instead of cycloheximide and RNase A (Fig. 6, lanes 9 and 10) and after recovering the precursors in a postribosomal supernatant (Fig. 6, lanes 13 and 14). From the fact that import occurred under both conditions we conclude that ribosomes are not involved in the post-translational import of both precursor proteins.

The post-translational import of both precursors does not depend on docking protein and the ribosome receptor, either. This conclusion is based on the observation that trypan
pretreatment of microsomes, known to eliminate ribosome receptor (Hortsch et al., 1986) and docking protein (Meyer and Dobberstein, 1980) (Fig. 7A), did not to any significant extent affect the post-translational import of ppcec A and ppcecDHFR (Fig. 5, lanes 6 and 7 versus 10 and 11; Fig. 7D). Furthermore, we did not detect a significant difference in the kinetics of import of both precursors into the various microsomes (data not shown).

Import of ppcec A and ppcecDHFR Can Be Mediated by SRP and Docking Protein under Cotranslational Assay Conditions—We have observed previously that the import of hybrid proteins, derived from the small precursor proteins preproelutri or prepropeptide GLA, into microsomes depends on SRP and docking protein (Müller and Zimmermann, 1987; Schlenstedt and Zimmermann, 1987). In order to study the effect of docking protein on import under cotranslational conditions, the two precursor proteins ppcec A and ppcecDHFR were synthesized in the rabbit reticulocyte lysate in the presence of mammalian microsomes that had been pretreated at different concentrations of trypsin (Fig. 7C). The import experiments were carried out under conditions in which microsomes were limiting (Fig. 4, A and C). The microsomes that had been treated with trypsin at a final concentration of 3 µg/ml or more did not contain any residual docking protein (Fig. 7A) and were completely inactivated with respect to their ability to import bovine preprolactin (Fig. 7B) or yeast prepro-a-factor (data not shown). On the other hand, these trypsin treated microsomes were not damaged to any significant extent, as was deduced from their residual signal peptidase activities (Fig. 7A) and their content of a luminal marker protein (i.e. immunoglobulin heavy chain binding protein; data not shown) as well as from their ability to process M13 procoat protein and sequester coat protein (data not shown). The hybrid protein derived from ppcec A represents the largest precursor protein for which import into mammalian microsomes in the absence of docking protein was observed. The fact that methotrexate had a slight inhibitory effect on the import of ppcecDHFR and not of ppcec A under cotranslational import conditions (Fig. 3A, lanes 6–8 and 12–14) implies that there was a ribosome-independent import taking place even in the presence of ongoing protein synthesis. The fact that the majority of ppcecDHFR was imported in a methotrexate-resistant manner, on the other hand, supports the idea that there was mainly translocation of nascent chains taking place under these conditions (Fig. 1).

In order to study the effect of SRP on elongation as well as on import of the two precursor proteins ppcec A and ppcecDHFR, synthesis was carried out in the wheat germ lysate. The effect of mammalian SRP on elongation of two precursors containing an identical signal peptide but differing in their size was tested in a synchronized system under conditions in which mRNA levels were both constant and limiting, i.e. ribosomes were in excess (data not shown), and in which only the concentration of SRP was varied. The concentrations of the two precursor proteins, synthesized during the incubation, were equimolar; synthesis of the cytoplasmic protein globin served as an internal control in both translation reactions (Fig. 8). The effect of SRP on import of the respective precursor protein into mammalian microsomes was evaluated on the basis of import efficiencies into limiting amounts of salt-washed (i.e. SRP-depleted) microsomes in the absence and presence of SRP (Fig. 9, lanes 3–5 versus 6–8). The salt-washed microsomes had been inactivated previously by titrating untreated microsomes; see Fig. 4). (ii) Translation of ppcec A (D) in the presence of [3H]proline and ppcecDHFR (C), bovine preprolactin (B) and M13 procoat protein (B) in the presence of [35S]methionine was performed in the rabbit reticulocyte lysate in the presence of various microsomes at two different concentrations within the linear range (for each precursor the linear range for processing and sequestration had been determined previously by titrating untreated microsomes; see Fig. 4). (iii) Translation of ppcec A (D) in the presence of [3H]proline and ppcecDHFR (D) in the presence of [35S]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 incubated further 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 or absence of protease. The samples were analyzed by gel electrophoresis and fluorography. The efficiencies of processing and sequestration, respectively, were quantified by laser densitometry of the autoradiographs and plotted against the concentration of trypsin. The two assays gave identical results except for procoat protein (Wiech et al., 1981) and ppcecDHFR (see Fig. 4D); the sequestration efficiencies are shown.

![Fig. 7](image-url). Effect of trypsin pretreatment of microsomes on docking protein content, signal peptidase activity, and import of four different precursor proteins. Microsomes were pretreated with TPCK-treated trypsin as described under "Experimental Procedures" at final concentrations, which are indicated. The trypsin-pretreated microsomes were analyzed with respect to their docking protein content by Western blotting and decoration with antibodies directed against docking protein and ['H]protein A (A). Furthermore, these microsomes were characterized with respect to their abilities to process different precursor proteins under different conditions. (i) Signal peptidase assays were carried out using ppcecDHFR as a substrate in 0.25% Triton X-100; titration curves were obtained for the various microsomes; the slope of the curves served as a basis for the calculation of signal peptidase activities (A). (ii) Translation of ppcec A (C) in the presence of ['H]proline and ppcecDHFR (C), bovine preprolactin (B) and M13 procoat protein (B) in the presence of [35S]methionine was performed in the rabbit reticulocyte lysate in the presence of various microsomes at two different concentrations within the linear range (for each precursor the linear range for processing and sequestration had been determined previously by titrating untreated microsomes; see Fig. 4). (iii) Translation of ppcec A (D) in the presence of [3H]proline and ppcecDHFR (D) in the presence of [35S]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 incubated further 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 or absence of protease. The samples were analyzed by gel electrophoresis and fluorography. The efficiencies of processing and sequestration, respectively, were quantified by laser densitometry of the autoradiographs and plotted against the concentration of trypsin. The two assays gave identical results except for procoat protein (Wiech et al., 1981) and ppcecDHFR (see Fig. 4D); the sequestration efficiencies are shown.

![Fig. 8](image-url). The effect of SRP on length and import of the two precursor proteins ppcec A and ppcecDHFR, synthesis was carried out in the wheat germ lysate. The effect of mammalian SRP on elongation of two precursors containing an identical signal peptide but differing in their size was tested in a synchronized system under conditions in which mRNA levels were both constant and limiting, i.e. ribosomes were in excess (data not shown), and in which only the concentration of SRP was varied. The concentrations of the two precursor proteins, synthesized during the incubation, were equimolar; synthesis of the cytoplasmic protein globin served as an internal control in both translation reactions (Fig. 8). The effect of SRP on import of the respective precursor protein into mammalian microsomes was evaluated on the basis of import efficiencies into limiting amounts of salt-washed (i.e. SRP-depleted) microsomes in the absence and presence of SRP (Fig. 9, lanes 3–5 versus 6–8). The salt-washed microsomes had been inactivated previously by titrating untreated microsomes; see Fig. 4). (ii) Translation of ppcec A (D) in the presence of ['H]proline and ppcecDHFR (D) in the presence of [35S]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 incubated further 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 or absence of protease. The samples were analyzed by gel electrophoresis and fluorography. The efficiencies of processing and sequestration, respectively, were quantified by laser densitometry of the autoradiographs and plotted against the concentration of trypsin. The two assays gave identical results except for procoat protein (Wiech et al., 1981) and ppcecDHFR (see Fig. 4D); the sequestration efficiencies are shown.
**Fig. 8.** Effect of canine SRP on synthesis of ppcec A, ppcecDHFR, and rabbit globin in the wheat germ lysate. Limiting amounts of synthetic transcripts of either plasmid pCA1 or pCA2 were translated together with rabbit globin poly(A)+ RNA in the presence of [35S]methionine. Each 25-μl translation reaction contained the indicated amounts of purified canine SRP. After incubation for 90 s at 30 °C, the translations were synchronized by the addition of 7-methylguanosine 5′-monophosphate (final concentration, 6 mM) and incubated further for 30 min at 30 °C. The samples were analyzed by gel electrophoresis and fluorography. The amount of completed protein synthesized was quantified by laser densitometry of the autoradiograph and plotted against the concentration of SRP.

**Fig. 9.** Processing and sequestration of ppcec A and ppcecDHFR in the wheat germ lysate (cotranslational assay). Translation of ppcec A in the presence of [3H]proline (lower panel) and ppcecDHFR in the presence of [35S]methionine (upper panel) was performed in the presence of RM buffer (lanes 1 and 2), salt-washed microsomes (KRM) (lanes 3–10), microsomes pretreated with 3 μg of trypsin/ml (TRM) (lanes 11–14), canine SRP (2 μl in 25-μl reaction) (lanes 6–8), or methotrexate (MTX, 10 μM) (lanes 9, 10, 13, and 14). Following incubation for 60 min at 30 °C, translation reactions were divided into aliquots that were further incubated as indicated without protease, with protease, or with protease in the presence of 0.5% Triton X-100 as described under “Experimental Procedures.” The samples were analyzed by gel electrophoresis and fluorography.

**Protein Import into the Endoplasmic Reticulum**

employed here is the smallest precursor protein for which an SRP arrest has been observed so far. On the other hand, the hybrid protein derived from ppcec A is the largest precursor protein to date for which import into mammalian microsomes was observed in the absence of SRP. This import was not limited to small precursor proteins but also ribosomes and ribosome receptors. In all these respects ppcec A behaves like the other small precursor proteins that we have studied previously (Watts et al., 1983; Zimmermann and Mollay, 1986; Müller and Zimmermann, 1987; Schlenstedt and Zimmermann, 1987; Wiech et al., 1987). Furthermore, we have shown that a hybrid protein between ppcec A and the cytosolic protein dihydrofolate reductase behaves like a small precursor protein when import is carried out under post-translational conditions, in contrast to a pre-promelittin-DHFR hybrid protein (Müller and Zimmermann, 1987, 1988a, 1988b). The direct evidence for this type of import comes from the fact that binding of folate analogues such as methotrexate to the hybrid protein ppcecDHFR inhibits import. The reason for this unique ability of ppcec-DHFR must reside in the ability of the hybrid protein to fold in a way that allows the two domains, ppcec A and DHFR, to act independently of each other. This is apparently not the case for a pre-promelittin-DHFR hybrid protein and should be related to the important role of the carboxyl terminus of prepromelittin in import competence (Müller and Zimmermann, 1987). The differences in the efficiencies of ppcec A and ppcecDHFR for this type of import may be attributed to differences in the folding of the ppcec A domain in the absence versus the presence of the DHFR domain.

Since methotrexate binds only to completed and folded polypeptide chains (Eilers and Schatz, 1986; Verner and Schatz, 1987), it was possible to detect import of completed ppcecDHFR chains under cotranslational import conditions by observing an inhibitory effect of the drug, again, in contrast to a pre-promelittin-DHFR hybrid protein (Zimmermann et al., 1988a). Therefore, we conclude that ribosome-independent import of ppcecDHFR occurs even under cotranslational import conditions and that this does not involve SRP and docking protein (Fig. 1).

A Single Precursor Protein Can Be Imported into Mammalian Microsomes in a Ribonucleoparticle-dependent and -independent Fashion—On the other hand we have shown here that ppcec A and ppcecDHFR can interact productively with SRP and that import under cotranslational conditions is more efficient in the presence of SRP and docking protein. Nevertheless, both import mechanisms can occur simultaneously. One can conclude on the basis of these observations that a small size does not necessarily prevent a precursor from interacting with SRP. Currently, we can only speculate as to why ppcec A synthesis is inhibited by SRP more strongly as compared with other small precursor proteins (Ibrahim, 1987; Hull et al., 1988). (i) The ppcec A has a very hydrophobic signal peptide that may have a particularly high affinity for SRP. (ii) It has a carboxyl-terminal region with a number of glycine residues (3 within the last 21 amino acids) that are coded for by a rare codon (GGC); this could result in a particularly slow elongation and termination rate, i.e. ribosome pausing at these positions (Wolin and Walter, 1988), and in an “SRP window” unusually long for the size of the precursor (Rapoport et al., 1987).

**DISCUSSION**

Ribonucleoparticle-independent Import of Proteins into Mammalian Microsomes Is Not Limited to Small Precursor Proteins—Here we have shown that preprocopropcin A can be imported efficiently into dog pancreas microsomes in the absence of not only signal recognition particle and docking protein but also ribosomes and ribosome receptors. In all these respects ppcec A behaves like the other small precursor proteins that we have studied previously (Watts et al., 1983; Zimmermann and Mollay, 1986; Müller and Zimmermann, 1987; Schlenstedt and Zimmermann, 1987; Wiech et al., 1987). However, demonstrates that a ribosome-independent import occurred even in this system and that this type of import corresponded to the SRP- and docking protein-independent import (Fig. 1).
Yeast Microsomes Do Not Have a Unique Ability to Import Full-length Precursor Proteins—We described here for a small and a large precursor protein an ATP effect that we attribute to the action of 70-kilodalton heat shock proteins and the additional component(s) from the reticulocyte lysate (Wiech et al., 1987; Zimmermann et al., 1988b) (Fig. 1). A similar situation seems to exist for the import of yeast prepro-a-factor (165 amino acid residues) into yeast microsomes (Hansen et al., 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986; Wiedmann et al., 1988). This is also 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 import (Rothblatt and Meyer, 1986; Waters et al., 1986; Garcia and Walter, 1988). For some reason it appears to be important that the wheat germ lysate did not allow a similar type of import (Rothblatt and Meyer, 1986; Waters et al., 1986; Garcia and Walter, 1988). For some reason it appears to be important to work with a homologous system (mammalian or yeast) in order to detect this type of import. This may also be the reason why other authors made alternative interpretations of results concerning the import of small precursor proteins into mammalian microsomes (Ibrahimi, 1987; Hull et al., 1988).

Binding of Folate Analogues Inhibits Import of a Preproecerin A-Dihydrofolate Reductase Hybrid Protein into Mammalian Microsomes—We have shown here that stabilization of the DHFR domain within ppcecDHFR by natural ligands as well as inhibitors of DHFR is compatible with membrane insertion of ppcecDHFR, i.e. allows processing by signal peptidase. Furthermore, we have shown that the same agents prevent sequestration, i.e. do not allow completion of translocation (Fig. 1). This allows us to conclude that a stabilized conformation of a certain domain within a precursor protein blocks membrane transport. A similar conclusion has been reached previously for mitochondrial protein import (Vestweber and Schatz, 1988; Rassow et al., 1989). Another analogy between protein import into microsomes and mitochondria has to be considered in this context: insertion of a pre-coxIV-DHFR hybrid protein into mitochondrial contact sites is inhibited by stabilization of the DHFR domain (Eilers and Schatz, 1986); moreover, insertion of a small precursor protein into mammalian microsomes is prevented under conditions in which the mature part was stabilized by an intramolecular disulfide bridge (Müller and Zimmermann, 1988a). The distance between the prepeptide and the domain that was stabilized seems in both systems to be the crucial feature in determining whether the initial membrane insertion or some later step during translocation was inhibited.

Finally, despite the observed membrane insertion of ppcecDHFR and inhibition of sequestration of ppcecDHFR in the presence of methotrexate, this did not allow us to detect a stable transmembrane intermediate. On the contrary, the methotrexate-stabilized ppcecDHFR seemed to have either one of two fates: (i) One population came out of the membrane and was recovered in the soluble fraction, a similar behavior has been observed previously for hepatitis B virus core protein (Garcia et al., 1988). (ii) The other population was recovered with the membranes but did not fulfill the criteria that have to be attributed to a transport intermediate, i.e. it was not to be chased to a protected species, and it behaved like a membrane protein upon subfractionation of the microsomes. The most likely interpretation of these observations is that the block of completion of translocation through the action of methotrexate leads to an intermediate that is released quickly from any membrane component(s) involved in the translocation. Because of the amphiphatic character of the mature ecropin domain, this may result either in a lateral diffusion, i.e. a membrane protein, or in a release from the membrane, i.e. a soluble protein in the equivalent of the cytoplasm. Both types of behavior may be relevant to protein import into microsomes. The membrane-integrated population of ppcecDHFR may point to the possibility that in the case of integration of a membrane protein into the microsomal membrane folding of cytoplasmic domains may have an important role in the membrane assembly; the soluble population of ppcecDHFR may be seen as support for the long held assumption that folding of a protein on the luminal side of the membrane may be a force in completion of translocation.

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