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Import of honeybee prepromelittin into the endoplasmic reticulum: structural basis for independence of SRP and docking protein

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Honeybee prepromelittin is correctly processed and imported by dog pancreas microsomes. Insertion of prepromelittin into microsomal membranes, as assayed by signal sequence removal, does not depend on signal recognition particle (SRP) and docking protein. We addressed the question as to how prepromelittin bypasses the SRP/docking protein system. Hybrid proteins between prepromelittin, or carboxy-terminally truncated derivatives, and the cytoplasmic protein dihydrofolate reductase from mouse were constructed. These hybrid proteins were analysed for membrane insertion and sequestration into microsomes. The results suggest the following: (i) The signal sequence of prepromelittin is capable of interacting with the SRP/docking protein system, but this interaction is not mandatory for membrane insertion; this is related to the small size of prepromelittin. (ii) In prepromelittin a cluster of negatively charged amino acids must be balanced by a cluster of positively charged amino acids in order to allow membrane insertion. (iii) In general, a signal sequence can be sufficient to mediate membrane insertion independently of SRP and docking protein in the case of short precursor proteins; however, the presence and distribution of charged amino acids within the mature part of these precursors can play distinct roles.

Key words: endoplasmic reticulum/signal recognition particle/docking protein/prepromelittin/protein import

Introduction

The transport of eucaryotic secretory proteins across membranes related to the endoplasmic reticulum (ER) can be subdivided into the following stages: (i) specific association of the precursor with the membrane; (ii) membrane insertion and removal of the signal sequence by signal peptidase; and (iii) complete transfer of the mature protein across the membrane (for review see Hortsch and Meyer, 1984; Wickner and Lodish, 1985). In general, precursors of secretory or membrane proteins with cleaved (Meyer *et al.*, 1982) or uncleaved signal sequences (Rottier *et al.*, 1985) require signal recognition particle (SRP) and docking protein for transport.

Honeybee prepromelittin is a precursor protein consisting of 70 amino acids and containing a cleavable signal sequence (Suchanek *et al.*, 1978). In contrast to the situation described above, it can be inserted into dog pancreas microsomal membranes (as assayed by correct removal of the signal peptide) in the absence of SRP and docking protein (Zimmermann and Mollay, 1986). Furthermore, a protease-sensitive component of the microsomal membrane (component PS) was found to be required for completion of membrane transfer of promelittin.

The primary structure of prepromelittin shows the following interesting features (Vlasak *et al.*, 1983): (i) small size; (ii) a typical signal sequence (Perlman and Halvorson, 1983); (iii) a negatively charged prosequence; (iv) a hydrophobic domain within the mature melittin; and (v) a cluster of four positively charged amino acids near the carboxy terminus. In this report we addressed the question as to how prepromelittin bypasses the SRP/docking protein system. Specifically, we asked which structural characteristics of prepromelittin are responsible for its independence of SRP and docking protein and its dependence on component PS. Furthermore, we wanted to investigate how general this type of behaviour is.

We systematically altered the characteristic features of prepromelittin by constructing hybrid proteins between prepromelittin and mouse dihydrofolate reductase. We studied the transport of the various precursors into microsomes in a coupled transcription/translation/translocation system (Lingappa *et al.*, 1984; Stueber *et al.*, 1984). Transcription was programmed by plasmids, with an SP6 promoter placed in front of the cDNA, encoding prepromelittin or its derivatives (Krieg and Melton, 1984). Translation and transport were carried out in reticulocyte lysates supplemented with dog pancreas microsomes (Blobel and Dobberstein, 1975). Trypsin-treated microsomes served as an assay for SRP/docking protein independence (Meyer and Dobberstein, 1980) as well as for dependence on component PS (Zimmermann and Mollay, 1986). Membrane insertion was assayed as removal of the signal peptide, i.e. processing by signal peptidase (Jackson and Blobel, 1977; Gilmore and Blobel, 1985). Transfer of the proteins across the microsomal membrane and into the lumen of the vesicles was assayed as resistance of the mature forms to digestion by externally added proteases in the absence of detergent, but sensitivity in the presence of detergent.

We demonstrate that the size of prepromelittin is critical for SRP/docking protein independence. An import mechanism not depending on SRP and docking protein does exist for any precursor provided that it fulfills the following two structural criteria: (i) the length of the polypeptide chain must not exceed ~80 amino acids; and (ii) charged amino acid(s) at the amino terminus of the mature part of the protein have to be compensated for by amino acid(s) with the opposite charge at the carboxy terminus.

Results

Construction of hybrid proteins between honeybee prepromelittin and mouse dihydrofolate reductase

The cDNAs coding for honeybee prepromelittin and mouse dihydrofolate reductase, respectively, were cloned into plasmids containing the SP6 promoter (Figure 1A). Fusion of prepromelittin cDNA, or various truncated derivatives, with dihydrofolate reductase cDNA was accomplished by exonuclease digestion and subsequent religation. Amino acid deletions and substitutions at the 3′-coding end of the resulting hybrid proteins were introduced by removal of appropriate restriction fragments from the 3′-coding region of dihydrofolate reductase cDNA or by inser-

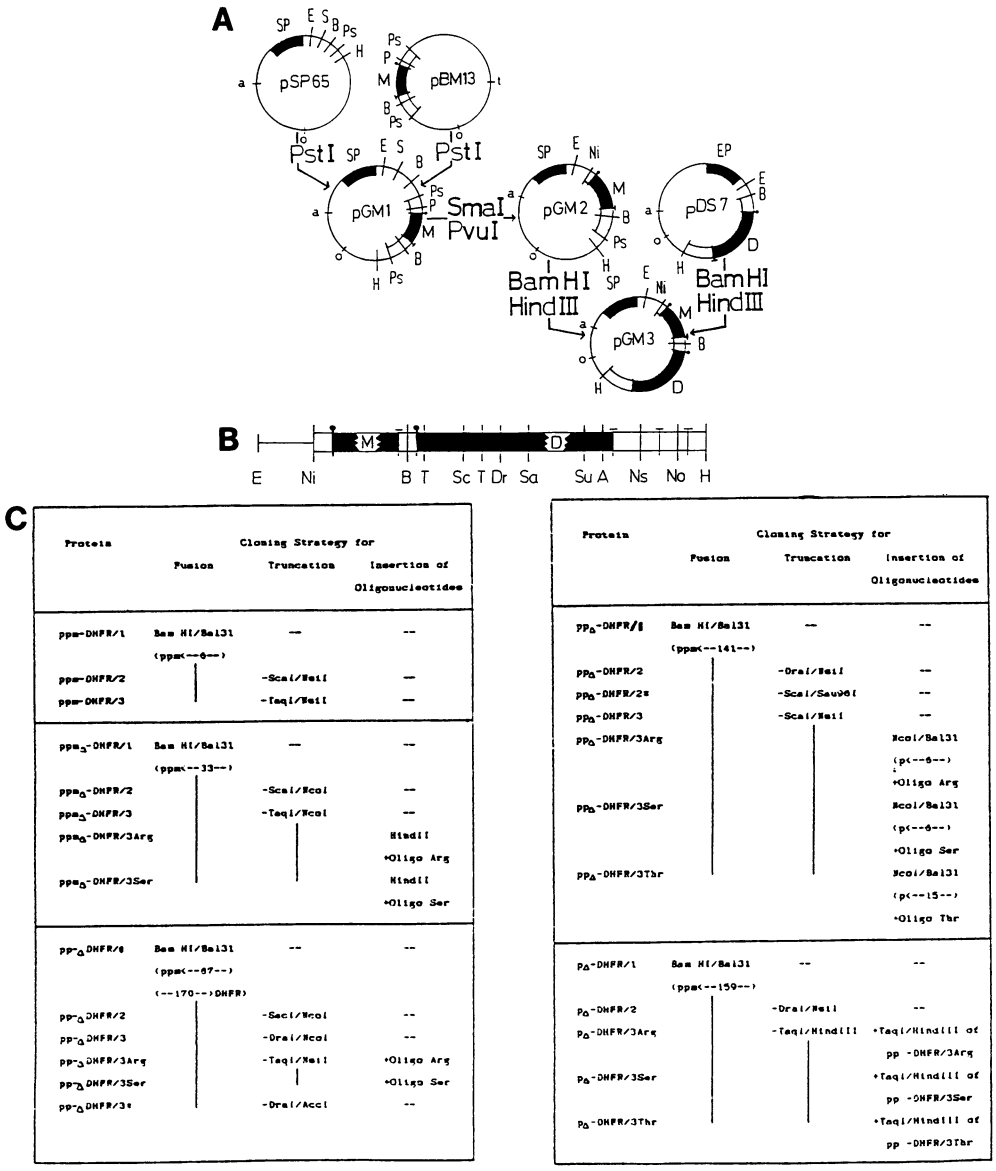

Fig. 1. Construction of plasmids. (**A**) Honeybee prepromelittin cDNA was excised from plasmid pBM13 (Vlasak *et al.*, 1983) and the *Pst*I fragment was cloned into the *Pst*I site of the polylinker of plasmid pSP65 (Krieg and Melton, 1984). The clones were screened for plasmids containing inserts in the right orientation with respect to the upstream located SP6 promoter (pGM1). The 5′ non-coding region of the cDNA was removed by cleavage of plasmid pGM1 with *Sma*I and *Pvu*I, a subsequent fill-in reaction of the 5′ overhanging ends with Klenow fragment of DNA polymerase I, purification of the plasmid in agarose gels and subsequent religation (pGM2) using T4 DNA ligase. Mouse dihydrofolate reductase cDNA was excised from plasmid pDS7 (Hurt *et al.*, 1984) as a *Bam*HI/*Hin*dIII fragment and cloned into plasmid pGM2 cleaved at these sites. The resulting plasmid pGM3 served as a starting vector for all subsequent constructions of hybrid genes between prepromelittin and dihydrofolate reductase and derivatives thereof. (**B, C**) For fusion of cDNAs coding for prepromelittin and dihydrofolate reductase, plasmid pGM3 was cleaved at the unique *Bam*HI site and digested with nuclease *Bal*31 under controlled conditions. After purification by agarose gel electrophoresis, the vector was ligated directly yielding a hybrid DNA lacking a 3′ region of the ppm cDNA and a 5′ region of the DHFR cDNA. Alternatively, ligation was carried out with a cDNA coding for full-length DHFR as *Bam*HI/*Hin*dIII fragment after removal of the partially digested DHFR cDNA by cleavage with *Hin*dIII. This procedure resulted in a library of plasmids with hybrid DNAs between 3′ truncated versions of ppm cDNA and full-length DHFR cDNA. For truncation of the hybrid genes appropriate restriction fragments were excised from DHFR cDNA and the plasmids were religated after isolation from agarose gels. Stop codons were derived from the 3′ non-coding region of the DHFR cDNA if the authentic one could not be used. For introduction of two complementary synthetic oligonucleotides d(3′AATGCTGCTGCTGCTATT5′) and d(5′TTACGACGACGACGATAA3′), coding for four arginines or four serines and a stop codon, plasmids with truncated versions of the hybrid genes were either cleaved at restriction sites just before the stop codon or just after it with subsequent removal of the stop codon by limited *Bal*31 digestion. After isolation from agarose gels the plasmids were ligated in the presence of an excess of phosphorylated and annealed oligonucleotides. Alternatively, restriction fragments of the 3′ end of truncated hybrid genes with oligonucleotides, introduced by the method described above, were cloned into plasmids with hybrid genes differing only at the 5′ coding region, from which the corresponding restriction fragments at the 3′ coding region had been removed. During all three cloning steps the cleaved restriction sites at the fusion points within coding sequences were made blunt-ended prior to ligation by treatment with S1 nuclease, Klenow fragment of DNA polymerase I or T4 DNA polymerase or a combination of them. The identity of the products with respect to the correct reading frame was confirmed by restriction and sequence analysis of the genes and analysis of their *in vitro* transcription/translation products. SP, SP6 promoter; EP, *E. coli* promoter; a, ampicillin resistance; t, tetracycline resistance; o, origin of replication; M, prepromelittin; D, mouse dihydrofolate reductase; E, *Eco*RI; S, *Sma*I; B, *Bam*HI; Ps, *Pst*I; H, *Hin*dIII; P, *Pvu*I, Ni, *Nci*I; T, *Taq*I; Sc, *Sca*I; Dr, *Dra*I; Sa, *Sac*I; Su, *Sau*96I; A, *Acc*I; Ns, *Nsi*I; No, *Nco*I; ♀, start codon; T, stop codon; single lines represent sequences derived from plasmid pSP65; open bars indicate non-coding sequences; black filled bars indicate coding sequences. For names of the proteins refer to Figure 2; the arrow within the brackets indicates the direction of the *Bal*31 deletion, the number refers to the nucleotides digested starting from the *Bam*HI site; (−) indicates a removal of a restriction fragment; (+) indicates introduction of a restriction fragment or two complementary oligonucleotides coding for four arginines and serines respectively.

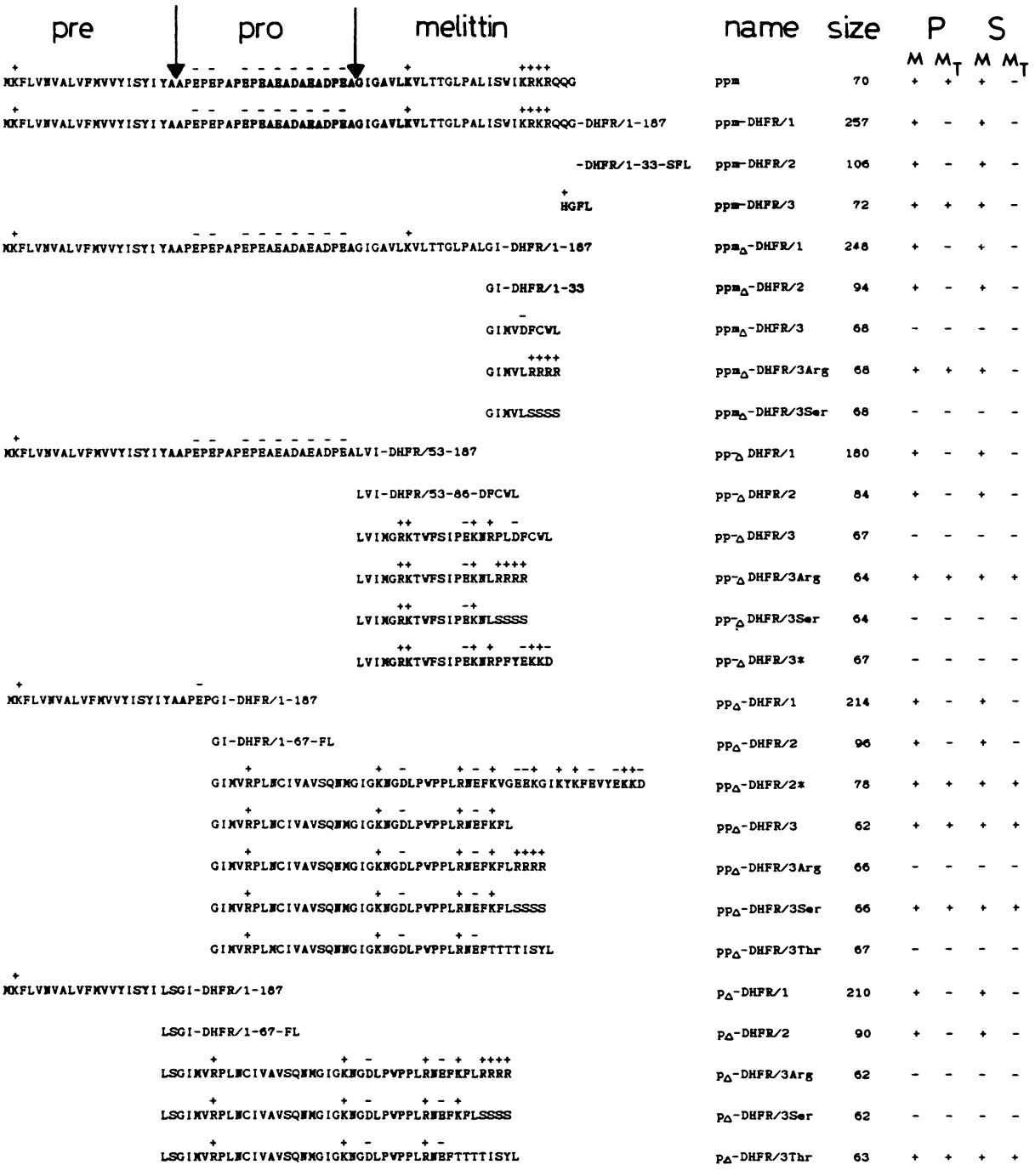

Fig. 2. Amino acid sequences of hybrid proteins between prepromelittin and dihydrofolate reductase. Amino acid sequences, given in the single-letter code, are derived from DNA sequencing of the corresponding plasmids using the chain termination method with dideoxynucleotides. Boundaries between the pre-, pro- and mature region of prepromelittin and between the presequences and mature parts of all its derivatives are indicated by arrows. The proposed cleavage site of the precursor proteins, from which the original cleavage site has been removed, follows the predicted rules, described by von Heijne (von Heijne, 1983, 1986). The numbers given as 'DHFR/X-X' refer to the amino acid sequence of mouse dihydrofolate reductase (Nunberg *et al.*, 1980). The number, included in the name of the various proteins, refers to the approximate mol. wt (1: >20 kd, 2/2*: 10 kd, 3/3*: 8 kd). We refer to a protein as mature if it fulfills the following prerequisites: (i) appearance dependent on the presence of microsomes; (ii) protease resistance in the absence of detergent but protease sensitivity in the presence of detergent; (iii) cofractionation with microsomes upon sedimentation analysis; and (iv) extraction from microsomes at pH 11.5 (Mostov *et al.*, 1981). ppm, prepromelittin; pp, preprosequence of prepromelittin; p, presequence of prepromelittin; DHFR, mouse dihydrofolate reductase; the triangles placed after or before these symbols indicate carboxy- or amino-terminal deletions respectively; P indicates processing observed and S indicates sequestration observed for dog pancreas microsomes (M) and microsomes pretreated with trypsin (M_r).

tion of oligonucleotides into restriction sites near the stop codon of the dihydrofolate reductase cDNA (Figure 1B, C). In all cases, the result of these DNA manipulations was monitored by sequencing of the corresponding cDNAs (Figure 2).

Five classes of hybrid proteins between prepromelittin and dihydrofolate reductase were obtained which are different from each other with respect to their prepromelittin derived part. Within each class there are at least three different cases differing in the size of the part derived from dihydrofolate reductase (i.e. their size).

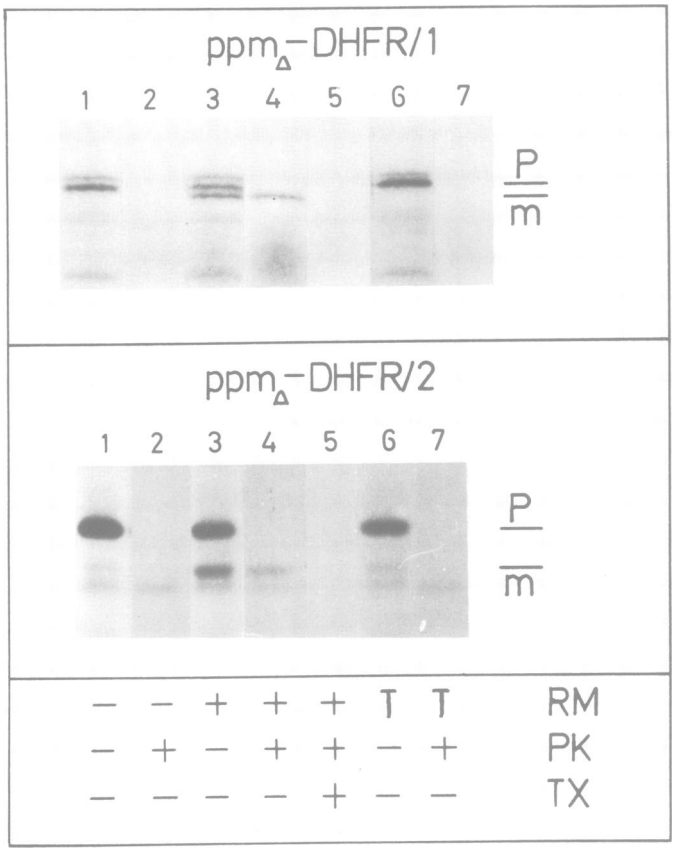

Fig. 3. Docking protein-dependent processing of hybrid proteins between prepromelittin and dihydrofolate reductase. Precursor proteins were synthesized in rabbit reticulocyte lysates in the absence of membranes (**lanes 1** and **2**) or in the presence of dog pancreas microsomes which were either not treated with trypsin prior to their inclusion in the translation reactions (**lanes 3–5**) or pretreated with trypsin (**lanes 6** and **7**), following transcription of the corresponding plasmids with SP6 polymerase for 45 min at 40°C. After translation for 30 min at 37°C the reaction mixtures were divided into three aliquots. One aliquot served as a control (**lanes 1, 3** and **6**) and was incubated in the absence of Proteinase K. The other two aliquots were incubated with Proteinase K in the absence (**lanes 2, 4** and **7**) or presence of Triton X-100 (**lane 5**). After incubation for 60 min at 0°C all samples were made 1 mM in PMSF, diluted 2-fold with double-strength sample buffer and analysed by gel electrophoresis. The relevant portions of the fluorographs are shown. p, precursor; m, mature form; RM, microsomes; PK, Proteinase K; TX, Triton X-100; T, microsomes pretreated with trypsin.

Increase of the molecular weight of prepromelittin results in a precursor protein that is docking protein–dependent

A hybrid protein between honeybee prepromelittin and mouse dihydrofolate reductase (ppm-DHFR/1) was processed and sequestered by dog pancreas microsomes, but only in the presence of active docking protein (Figure 2). Internal deletion of the carboxy terminus of the melittin domain (ppmΔ-DHFR/1), or of the complete melittin domain (pp-ΔDHFR/1), or of most of the promelittin domain (ppΔ-DHFR/1) from this hybrid protein did not alter this import behaviour [i.e. typical secretory proteins, with respect to processing of the precursors as well as sequestration of the processed forms, were produced (Figures 2 and 3)]. Reconstitution of trypsinized microsomes with an elastase extract from microsomes restored the processing and sequestration activity of these microsomes for these precursors (Figure 4). Internal deletion of the complete promelittin domain together with the authentic cleavage site for signal peptidase resulted in a precursor protein (pΔ-DHFR/1) which was processed and sequestered as efficiently as the hybrid proteins with the authentic cleavage site (Figure 2). Again, the import of this precursor protein (pΔ-DHFR/1) was strictly dependent on the presence of functional docking protein (Figure 2).

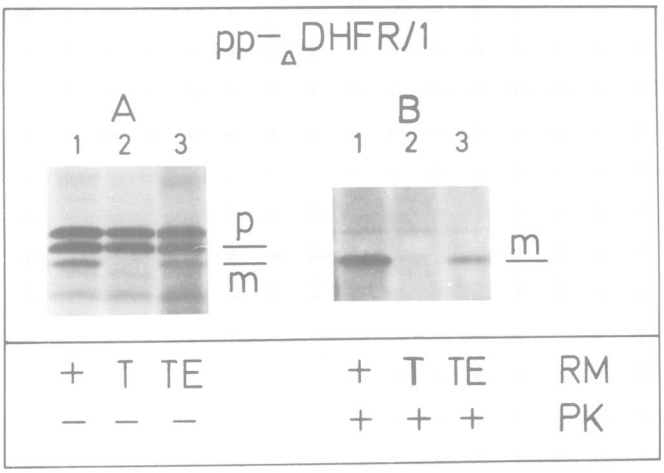

Fig. 4. Effect of trypsin treatment of microsomes and reconstitution with docking protein fragment on sequestration of docking protein-dependent hybrid proteins. Precursor proteins were synthesized in rabbit reticulocyte lysates in the presence of dog pancreas microsomes which were either not treated with trypsin prior to their inclusion in the translation reactions (**lanes 1**) or were treated with trypsin (**lanes 2**) or were treated with trypsin and supplemented with an elastase extract from microsomes (**lanes 3**). After *in vitro* translation for 30 min at 37°C, each sample was divided into two aliquots. One aliquot was analysed without protease treatment (**A**), the other aliquot after protease treatment (**B**) by gel electrophoresis. The relevant portions of the fluorographs are shown. p, precursor; m, mature form; RM, microsomes; PK, Proteinase K; T, trypsin-treated microsomes; TE, trypsin-treated microsomes supplemented with an elastase extract from microsomes.

Truncation of these precursors by carboxyterminal deletions down to a mol. wt of 9 kd did not alter the requirements of membrane insertion and sequestration of the respective precursors (Figures 2 and 3). This was also true for sequestration with trypsinized microsomes which had been reconstituted with an elastase extract (data not shown). The smallest precursor of this type contains 84 amino acids (pp-ΔDHFR/2).

We conclude that the signal sequence of prepromelittin can interact with SRP/docking protein and that it actually does so in the case of prepromelittin related precursors with a mol. wt of ≥9 kd. These precursors require an active docking protein for membrane insertion, at least when synthesized in the presence of SRP. Apparently, there are no limitations on the structure of the mature part for docking protein-dependent precursors. Furthermore, these data indicate that the small size of prepromelittin is the reason for SRP- and docking protein–independence.

A small size is crucial but not sufficient for docking protein–independent membrane insertion of precursor proteins

Further truncation of the hybrid proteins (described above) down to a mol. wt of 8.5 kd resulted in two types of precursor proteins.

(i) The first type contained either the complete prepromelittin domain (ppm-DHFR/3) or the presequence and a small amino terminal part of the promelittin domain (ppΔ-DHFR/3) or only a large amino-terminal portion of the authentic presequence of prepromelittin and an altered cleavage site (pΔ-DHFR/3Thr). These precursors were processed in the presence as well as in the absence of active docking protein (Figures 2 and 5). The largest precursor protein with SRP- and docking protein–independent membrane insertion activity consisted of 78 amino acids, comprising the complete presequence, a short

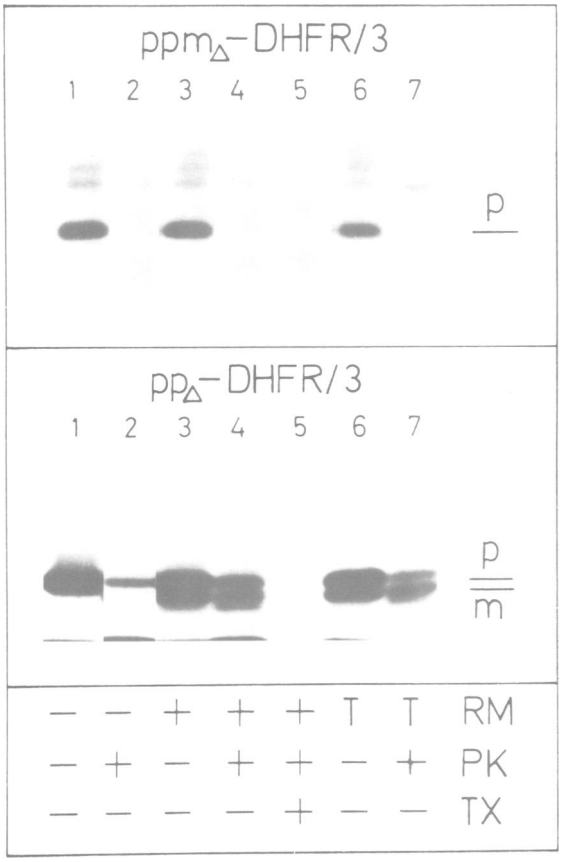

Fig. 5. Docking protein-independent processing of hybrid proteins between prepromelittin and dihydrofolate reductase. Precursor proteins were synthesized in rabbit reticulocyte lysates in the absence of membranes (**lanes 1** and **2**) or in the presence of dog pancreas microsomes which were either not treated with trypsin prior to their inclusion in the translation reactions (**lanes 3–5**) or treated with trypsin (**lanes 6** and **7**). After translation for 30 min at 37°C the reaction mixtures were divided into three aliquots. One aliquot served as a control and was incubated in the absence of Proteinase K (**lanes 1, 3** and **6**). The other two aliquots were incubated with Proteinase K in the absence (**lanes 2, 4** and **7**) or presence of Triton X-100 (**lane 5**). After incubation for 60 min at 0°C all samples were made 1 mM in PMSF and analysed by gel electrophoresis. The relevant portion of the fluorographs are shown. p, precursor; m, mature form; RM, microsomes; PK, Proteinase K; TX, Triton X-100; T, trypsin-treated microsomes.

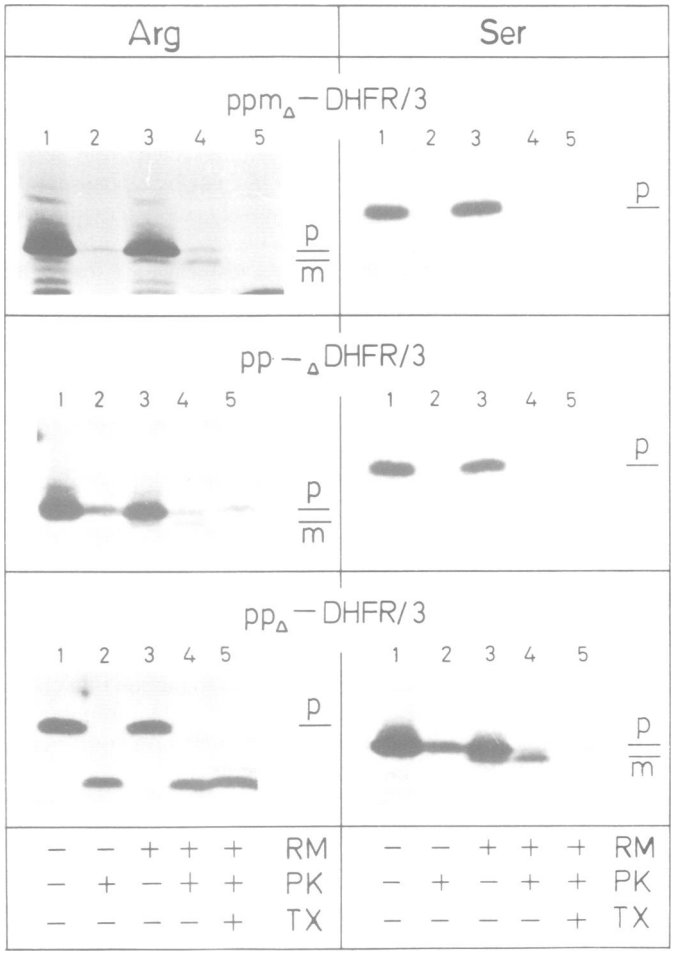

Fig. 6. Effect of clusters of charged amino acids at the carboxy termini of docking protein-independent hybrid proteins on processing and sequestration by microsomes. Prepromelittin derivatives were synthesized in rabbit reticulocyte lysates in the presence (**lanes 1** and **2**) or absence of dog pancreas microsomes (**lanes 3–5**). After 30 min at 37°C the translation reactions were divided into three aliquots. One aliquot served as a control and was incubated in the absence of Proteinase K (**lanes 1** and **3**). The other aliquots were incubated with Proteinase K in the absence of Triton X-100 (**lanes 2** and **4**) or in the presence of Triton X-100 (**lanes 5**). After incubation for 60 min at 0°C all samples were made 1 mM in PMSF and analysed by gel electrophoresis. Only the relevant portions of the fluorographs are shown. p, precursor; m, mature form; RM, microsomes; PK, Proteinase K; TX, Triton X-100.

amino-terminal part of the prosequence and dihydrofolate reductase with a large internal deletion (ppΔ-DHFR/2*).

(ii) The second type of precursor proteins contained either the presequence and the complete prosequence (pp-ΔDHFR/3) or the presequence and the prosequence together with the hydrophobic domain of melittin (ppmΔ-DHFR/3). These precursors were neither processed nor imported by dog pancreas microsomes (Figures 2 and 5).

These data support the view that the size of prepromelittin is responsible for SRP/docking protein-independence, whereas the hydrophobic domain of mature melittin does not seem to play a role in this respect. Furthermore, they suggest that a signal sequence can be sufficient to mediate membrane insertion in the case of small precursors. They also indicate, however, that there are certain limitations on the structure of the mature part for some docking protein-independent precursors.

The hybrid protein containing the complete prepromelittin domain with a few additional amino acids attached to the carboxy terminus (ppm-DHFR/3) was not sequestered by trypsin-treated microsomes, in contrast to the hybrid proteins lacking most of the promelittin domain (ppΔ-DHFR/3 and pΔ-DHFR/3Thr) (Figure 2). We conclude that the promelittin domain does not play a role in docking protein-independence of prepromelittin. Furthermore, component PS seems to be necessary only for completion of membrane transfer of authentic promelittin or of mature hybrid proteins containing a certain part of promelittin (see below).

Role of the mature part of prepromelittin in membrane insertion

The prepromelittin derivatives lacking the positively charged carboxy terminus but still containing the negatively charged prosequence (ppmΔ-DHFR/3 and pp-ΔDHFR/3) were not processed at all. After insertion of four arginines into the carboxy terminus of these constructs, however, competence for insertion and sequestration of the resulting precursors (ppmΔ-DHFR/3Arg and pp-ΔDHFR/3Arg) was restored (Figures 2 and 6). The complete translocation of these hybrid proteins into the lumen of the

microsomal vesicles was confirmed by the fractionation behaviour of their mature forms at different pH values. At pH 7 the processed hybrid proteins co-fractionated with microsomes upon centrifugation whereas at pH 11.5 they were recovered as soluble proteins in the supernatant (Figure 7). The positively charged amino acid residues could not be substituted for by four serines (ppmΔ-DHFR/3Ser and pp-ΔDHFR/3Ser) or by a polar domain containing two positively and two negatively charged amino acids (pp-ΔDHFR/3*) (Figures 2 and 6). This emphasizes the importance of the positively charged amino acids near the carboxy terminus of prepromelittin.

On the other hand, substitution of most of the promelittin, except for the extreme amino terminus, resulted in a precursor protein whose insertion and sequestration can occur without the aid of docking protein (ppΔ-DHFR/3) and apparently did not require a cluster of positively charged amino acids. Furthermore, insertion of four serines into the carboxy terminus did not interfere with the docking protein-independent membrane insertion and sequestration of the resulting precursor (ppΔ-DHFR/3Ser) (Figures 2 and 6). Strikingly, however, reconstitution of the positively charged carboxy terminus (ppΔ-DHFR/3Arg) completely inactivated the precursor with respect to membrane insertion and sequestration (Figures 2 and 6). In the absence of the negatively charged prosequence, the introduction of a cluster of positive charges into the carboxy terminus of an import competent precursor apparently abolishes the insertion competence.

Taken together, we conclude that the two clusters of charged amino acids within promelittin have to balance each other in order to keep prepromelittin competent for membrane insertion.

There are additional ways to eliminate the SRP and docking protein-independent insertion competence of the precursor protein ppΔ-DHFR/3Ser besides the introduction of a cluster of positively charged amino acids at the extreme carboxy terminus (ppΔ-DHFR/3Arg).

(i) Substitution of the four carboxy-terminal serine residues by a cluster of four threonine residues with concomitant removal of a single positively charged amino acid nearby the carboxy terminus also inactivated the resulting precursor protein (ppΔ-DHFR/3Thr) with respect to processing and sequestration in the presence as well as in the absence of functional docking protein (Figures 2 and 8). We suggest that this is not caused by the exchange of a cluster of four serine residues by one of four threonine residues. It seems to be more likely that the loss of insertion competence of ppΔ-DHFR/3Thr is due to the loss of the single lysine residue located proximal to the carboxy-terminal cluster of serine residues in the active precursor protein ppΔ-DHFR/3Ser.

(ii) Incompetence for insertion as well as sequestration was observed after substitution of a few amino acids around the original cleavage site of ppΔ-DHFR/3. The lack of processing may be due to the amino acid deletions at the amino-terminal end of the prosequence, including a glutamic acid. In this case it did not matter whether there were four arginine or four serine residues following the single lysine residue located at the carboxy terminus of these insertion-incompetent precursor proteins (pΔ-DHFR/3Arg and pΔ-DHFR/3Ser) (Figures 2 and 8). We assume that the failure of processing of these preproteins with an altered cleavage site reflects their inability to insert into microsomal membranes. This altered signal peptide can be recognized by SRP and signal peptidase if present in larger precursor proteins (pΔ-DHFR/1 and pΔ-DHFR/2) (Figure 2).

An SRP/docking protein-independent precursor was obtained by combining the amino terminus of the inactive precursor pΔ-DHFR/3Ser with the carboxy terminus of the inactive precursor

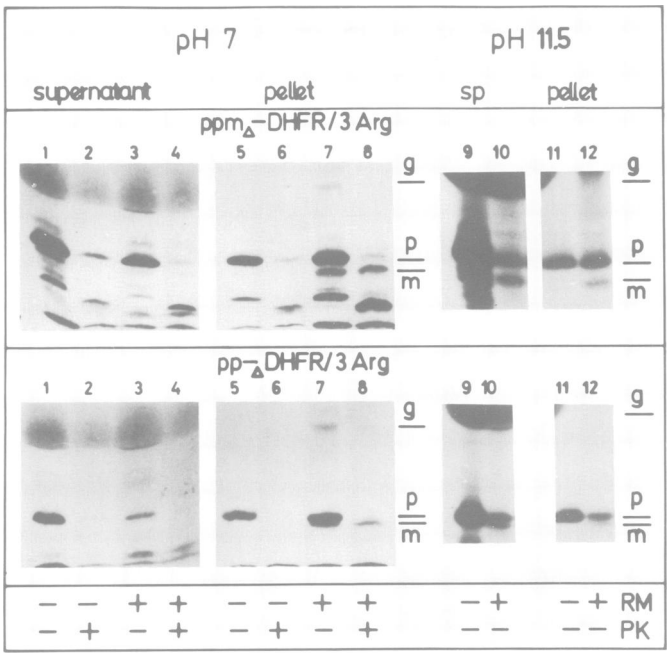

Fig. 7. Fractionation behaviour of docking protein-independent hybrid proteins at different pH values. Precursor proteins were synthesized in rabbit reticulocyte lysates in the absence (**lanes 1, 2, 5, 6, 9, 11**) or in the presence of dog pancreas microsomes (**lanes 3, 4, 7, 8, 10, 12**). After translation for 30 min at 37°C the reaction mixtures were divided into two aliquots. One aliquot (pH 7) was again divided into two halves. One half received Proteinase K (final concentration 50 μg/ml) (**lanes 2, 4, 6, 8**), the other half did not (**lanes 1, 3, 5, 7**). The other aliquot was adjusted to pH 11.5 by addition of NaOH (final concentration 50 mM) (Mostov *et al.*, 1981). (**lanes 9–12**). All samples were incubated for 45 min at 0°C and then centrifuged for 30 min at 30 p.s.i. in a Beckman airfuge. The samples containing Proteinase K were made 1 mM in PMSF prior to centrifugation. The supernatant was removed and an equal volume of double-strength sample buffer was added (**lanes 1–4, 9, 10**). The pellet was dissolved in sample buffer (**lanes 5–8, 11, 12**). The supernatant and pellet fractions were analysed by gel electrophoresis. g, globin; p, precursor; m, mature protein; RM, microsomes, PK, Proteinase K; sp, supernatant.

ppΔ-DHFR/3Thr. This resulted in a precursor protein (pΔ-DHFR/3Thr) which has lost the glutamic acid near the amino terminus as well as the lysine residue at the carboxy terminus of its mature part (Figures 2 and 8).

Thus, the insertion competence of the precursor proteins ppΔ-DHFR/3 and ppΔ-DHFR/3Ser appears to rely on a charge compensation between the glutamic acid near the amino terminus and the lysine residue near the carboxy terminus. A number of amino acid substitutions occur upon construction of insertion-competent precursors (ppΔ-DHFR/3Ser and pΔ-DHFR/3Thr) from inactive precursors (ppΔ-DHFR/3Thr and pΔ-DHFR/3Ser). However, we assume these substitutions do not play a significant role but that a balance of charges has to exist between single charged amino acids located at the amino and carboxy terminus in the mature part of small precursor proteins; alternatively, charged residues have to be absent from both these positions at the same time in order to allow SRP- and docking protein-independent membrane insertion.

Role of component PS in completion of membrane transfer of prepromelittin

Experiments, shown above, demonstrated that component PS apparently acts exclusively on membrane transfer of those hybrid proteins which contain at least a certain part of the promelittin domain. Hybrid proteins missing certain structural features of

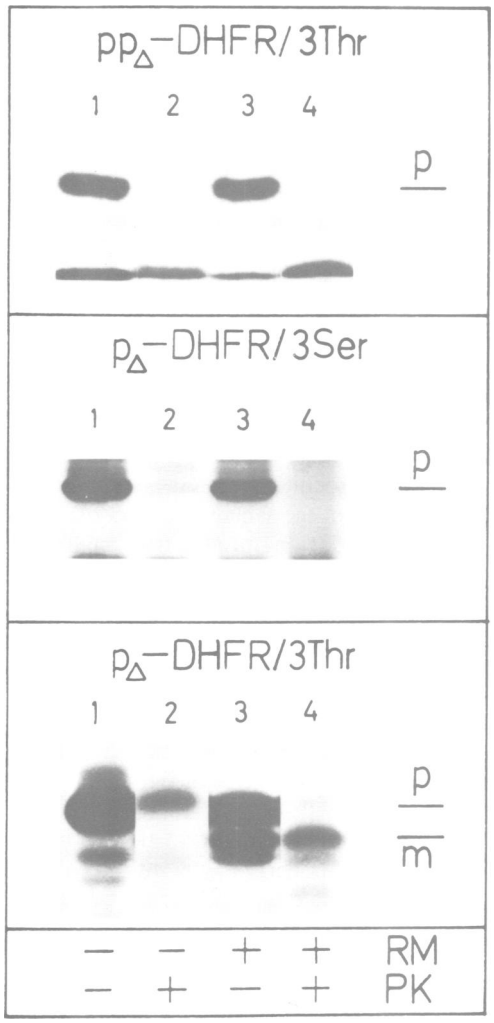

Fig. 8. Effect of single charged amino acids at the carboxy termini of docking protein-independent hybrid proteins on processing and sequestration by microsomes. Precursor proteins were synthesized in rabbit reticulocyte lysates in the absence of membranes (**lanes 1** and **2**) or in the presence of dog pancreas microsomes (**lanes 3** and **4**). After translation for 30 min at 37°C the reaction mixtures were divided into two aliquots. One aliquot served as a control and was incubated in the absence of Proteinase K (**lanes 1** and **3**). The other aliquot was incubated with Proteinase K (**lanes 2** and **4**). After incubation for 60 min at 0°C all samples were made 1 mM in PMSF and analysed by gel electrophoresis. Only the relevant portions of the fluorographs are shown. p, precursor; m, mature form; RM, microsomes; PK, Proteinase K.

promelittin, therefore, should allow a correlation between a certain structure within prepromelittin and the participation of component PS in completion of membrane transfer.

The prepromelittin derivative with the original carboxy terminus replaced by four arginines (ppmΔ-DHFR/3Arg) behaved exactly like authentic prepromelittin with respect to the requirement of component PS for complete translocation across the membrane since microsomes pretreated with trypsin were not able to sequester the mature form of this hybrid protein (Figures 2 and 9). However, the corresponding prepromelittin derivative lacking the hydrophobic domain of promelittin (ppΔ-DHFR/3Arg) was sequestered by microsomes with inactivated component PS (Figure 9). Furthermore, this translocation behaviour was shared by all other SRP/docking protein-independent hybrid proteins, lacking the hydrophobic domain (Figures 2 and 5).

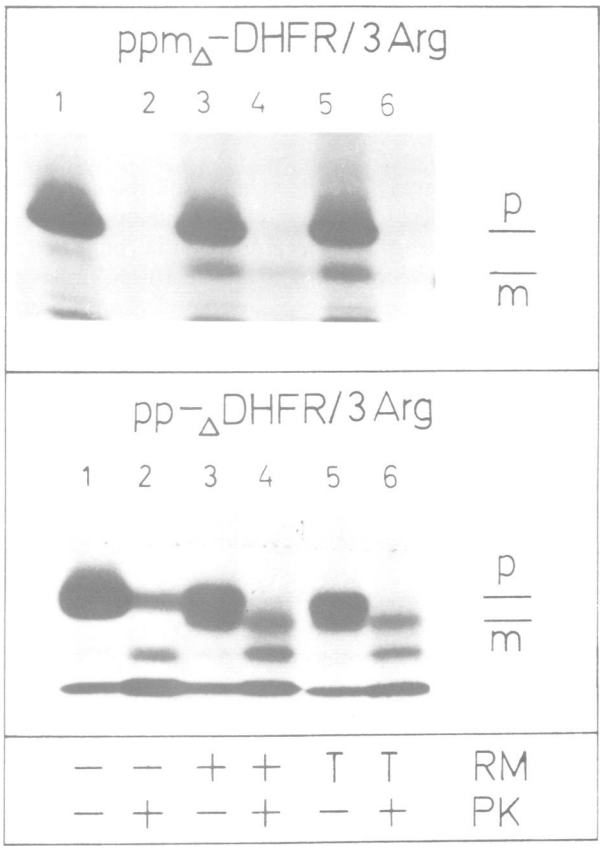

Fig. 9. Effect of trypsin treatment of microsomes on sequestration of docking protein-independent hybrid proteins. The precursor proteins were synthesized in rabbit reticulocyte lysates in the absence (**lanes 1** and **2**) or presence of dog pancreas microsomes (**lanes 3–6**). The microsomes had been either not treated prior to their inclusion in the translation reaction (**lanes 3** and **4**) or had been treated with typsin (**lanes 5** and **6**). After incubation for 30 min at 37°C each reaction mixture was divided into two aliquots. One aliquot was incubated in the absence of Proteinase K (**lanes 1, 3** and **5**), the other aliquot was incubated with Proteinase K (**lanes 2, 4** and **6**) for 60 min at 0°C. The samples were made 1 mM in PMSF and the microsomes were re-isolated by centrifugation in a Beckman airfuge (5 min, 30 p.s.i.). The resulting pellets were resuspended in sample buffer and analysed by gel electrophoresis and fluorography. p, precursor; m, mature form; RM, microsomes; PK, Proteinase K; T, trypsin-treated microsomes.

We conclude that the component PS, which is necessary for completion of membrane transfer of promelittin, seems to be involved in release of the hydrophobic part of mature melittin from the membrane into the lumen of the microsomes.

Discussion

Five classes of hybrid proteins between prepromelittin and dihydrofolate reductase were analysed to understand the basis for SRP- and docking protein-independence of prepromelittin. All hybrid proteins have the cleavable signal sequence from prepromelittin; four classes contain the authentic prepromelittin signal sequence and one class contains a derivative with a slightly altered cleavage site. Both signal sequences can direct dihydrofolate reductase into microsomal vesicles with the aid of SRP and docking protein provided that the precursors are large enough. Apparently, the prepromelittin signal peptide can interact with SRP *per se*. Both signal sequences are functional in smaller precursor proteins which do not require SRP and docking protein for membrane insertion, even if the mature parts are not related to prepromelittin. From these data we conclude that, in general,

a signal sequence can be sufficient to trigger SRP- and docking protein-independent membrane insertion of precursor proteins. The transition from SRP/docking protein-independent to SRP/docking protein-dependent membrane insertion occurs at an increase of the apparent mol. wt from 8 to 9 kd. This seems to contradict a recent report that a truncated form of the secretory protein prelysozyme with 74 amino acids still requires SRP for insertion into the microsomal membrane (Ibrahimi *et al.*, 1986). However, in this case peptidyl-tRNAs, and not completed polypeptide chains, were assayed. The attached tRNA may cause competence for SRP/docking protein-dependent membrane insertion of an otherwise incompetent precursor protein.

It is unclear at present whether SRP can interact with the signal sequences of the larger but not of our smaller SRP/docking protein-independent precursors. Probably, however, this is the case, as the presence of functional docking protein does not increase the efficiency of membrane insertion of these small preproteins. Interaction of SRP with precursors can occur late during their synthesis but only as long as they are not completed (Ainger and Meyer, 1986). Fully synthesized preproteins may fail to interact with SRP, perhaps because the ribosome is somehow involved in this step. In the case of small precursor proteins like prepromelittin, the polypeptide chain should be completed and released from the ribosome before the signal sequence emerges far enough from the ribosome to interact with SRP. It is assumed that 40−50 amino acids of a polypeptide chain are buried within the large subunit of the eucaryotic ribosome (Blobel and Sabatini, 1970). Indeed a chain length of ~70 amino acids seems to be required before the interaction between SRP and the nascent chain can occur (Walter and Blobel, 1981). Further translocation experiments employing wheat germ lysates supplemented with salt-washed microsomes should decide as to whether the activity of SRP and docking protein is an obligate prerequisite for membrane insertion of fusion proteins with mol. wts >8.5 kd whose truncated versions can be inserted into microsomal membranes independent of SRP and docking protein.

In addition to the size of a precursor protein, the presence (or absence) and balance of charged amino acids in the amino and carboxy termini of the mature part of a precursor protein appear to be critical for SRP/docking protein-independent membrane insertion. Either both ends of the mature part of a precursor protein have to be devoid of charged amino acids or charged amino acids at one end have to be compensated by amino acids with the opposite polarity at the other end. In the case of prepromelittin, a cluster of negatively charged amino acids at the amino terminus of promelittin has to be balanced by a cluster of positively charged residues at the carboxy terminus in order to keep prepromelittin competent for membrane insertion. We propose that the insertion competence of small SRP/docking protein-independent precursor proteins requires the following structural motifs: (i) a membrane-spanning region, represented by the hydrophobic core of the signal sequence; and (ii) a loop formed by the mature part of the precursor protein causing close proximity of the amino and carboxy termini which may be held together by ionic interactions between the oppositely charged amino acids. The initial interaction of the precursor with the membrane could involve this structure. A role for the mature part of a precursor protein in membrane insertion has recently been demonstrated for M13 procoat protein. Interestingly prepromelittin shows striking similarity in size and structure to M13 procoat protein. The precursor of M13 coat protein does not depend on complex proteinaceous components for assembly into bacterial plasma membranes or microsomal membranes *in vitro* (Wickner, 1980; Watts *et al.*, 1983) as well as *in vivo* (Wolfe *et al.*, 1985). The insertion of M13 procoat into the inner membrane of *Escherichia coli* does not only require a positively charged carboxy terminus (Kuhn *et al.*, 1986b) but, in contrast to prepromelittin, is also strictly dependent on the presence of the hydrophobic domain within the mature part (Kuhn *et al.*, 1986a).

If small precursor proteins do not require SRP and docking protein, why are these components necessary for the insertion of larger eucaryotic secretory proteins? Signal sequence and the amino-terminal portion of the mature part may not be sufficiently exposed in larger precursors and may not allow folding in such a way that penetrating into the lipid bilayer is possible. SRP may help to stabilize a domain in larger precursors which triggers membrane insertion. This may occur by formation of a hairpin loop between the signal sequence and a part of the mature protein (Engelman and Steitz, 1981). With these larger SRP/docking protein-dependent secretory proteins, structural restrictions within the mature part of the sequence do not exist; the interaction between SRP and docking protein may mediate, in concert with the signal sequence, the insertion of any amino-terminal sequence of the mature part.

Our studies indicate the existence of two mechanisms conferring insertion competence to precursors of secretory proteins: one mechanism depending on SRP and docking protein and being independent of the mature part of the precursor, and a second one which is independent of SRP and docking protein but relies on certain structural features of the mature part. Precursor proteins with a mol. wt ≤8.5 kd seem to be capable of maintaining an insertion-competent conformation without the aid of SRP and docking protein. This view is supported by our observation that two other naturally occurring precursor proteins behave in the same way as prepromelittin: namely, bacteriophage M13 procoat protein (Watts *et al.*, 1983) and frog skin prepropeptide GLa (Schlenstedt and Zimmermann, 1987). Therefore we suggest a function of SRP and docking protein in maintaining insertion competence of larger precursor proteins. In this respect the SRP/docking protein system may be analogous to the recently defined factor in the cytoplasm of *E. coli* (Randall and Hardy, 1986).

Materials and methods

Materials

[^{35}S]dATP (1500 Ci/mmol) was purchased from Dupont New England Nuclear, [^{3}H]proline (100 Ci/mmol), [^{3}H]leucine (100 Ci/mmol), and [^{35}S]methionine (1000 Ci/mmol) were purchased from Amersham Corp. Trypsin, soybean trypsin inhibitor, Proteinase K, restriction endonucleases, calf intestinal phosphatase, T4 DNA polymerase, Klenow fragment of DNA polymerase I, exonuclease *Bal*31, ribonuclease A and SP6 RNA polymerase were from Boehringer Mannheim. T4 DNA ligase, T4 polynucleotide kinase and nuclease S1 were from New England Biolabs. Dideoxy-, deoxy- and ribonucleotides and the cap analogue 7mG(5′)ppp(5′)G were from Pharmacia P-L Biochemicals. Human placental ribonuclease inhibitor (RNasin) was from Promega Biotec. All other chemicals were from Merck unless otherwise indicated.

Construction of plasmids

DNA manipulations were carried out as described by Maniatis *et al.* (1982) with the following exceptions: DNA fragments were isolated from polyacrylamide gels by electroelution into dialysis bags (McDonnel *et al.*, 1977) and from agarose gels (Dretzen *et al.*, 1981) by electrophoretic transfer onto DEAE paper (Schleicher and Schüll). Oligonucleotides were eluted directly from denaturing polyacrylamide gels with high-salt buffer and passed through a small column of glass wool (Smith, 1980).

Plasmids were prepared on large or small scales by an alkaline lysis procedure (Birnboim and Doly, 1979), followed by CsCl/ethidium bromide equilibrium centrifugation (Radloff *et al.*, 1967), from bacteria grown in LB-medium (Kedes *et al.*, 1975) (yeast extract and bacto tryptone were purchased from Difco) supplemented with 100 μg/ml ampicillin or 25 μg/ml tetracycline (Serva).

All cloning procedures were routinely performed in *E. coli* strain Hb 101

(Mandel and Higa, 1970). Transformations were carried out following the method described by Hanahan (1983).

DNA sequence analysis was performed according to the chain termination method using dideoxynucleotides and [^{35}S]dATP (Sanger *et al.*, 1977) and employing 12% SDS–polyacrylamide gels containing 8 M urea (Garoff and Ansorge, 1981). DNA fragments coding for hybrid proteins or derivatives of prepromelittin were cloned into plasmid pUC 19 (Vieira and Messing, 1982) and each strand was sequenced after alkaline denaturation of the plasmids (Guo *et al.*, 1983) using appropriate primers.

Oligonucleotides were phosphorylated by T4 polynucleotide kinase in the presence of 12% (w/v) PEG 8000 (Pharmacia) (Harrison and Zimmerman, 1986).

In vitro transcription and translation

Plasmids (10 μg) with SP6 promoter located before the cDNA (Krieg and Melton, 1984) were transcribed with SP6 RNA polymerase in a reaction volume of 25 μl in 24 mM Hepes pH 7.4, 3.6 mM Mg acetate, 1.2 mM spermidine (Sigma), 60 μg/ml BSA (Bethesda Research Laboratories), 6 mM DTT (Boehringer Mannheim), 0.3 mM ATP, CTP, UTP, 0.06 mM GTP, 0.25 mM 7mGpppG, 25 units RNasin, and 10 units SP6 RNA polymerase for 45 min at 40°C (Galili *et al.*, 1986). The plasmids were used in either supercoiled or linearized form, after cleavage at a unique restriction site, with subsequent purification and precipitation. For translation in reticulocyte lysates, the transcription mixture was used directly or after concentration of the RNA by ethanol precipitation.

Translation in nuclease-treated rabbit reticulocyte lysates (Pelham and Jackson, 1976; Zimmermann *et al.*, 1979) was performed at reticulocyte concentrations of 40% (v/v) in total volumes of 12.5 μl for 30 min at 37°C in the presence of *in vitro* transcripts and either [^{3}H]leucine (final concentration 1.35 mCi/ml) and [^{3}H]proline (final concentration 1.35 mCi/ml) – in the case of prepromelittin and its derivative ppm-DHFR/3 – or [^{35}S]methionine (final concentration 1.4 mCi/ml).

Preparation of microsomes

Dog pancreas microsomes were prepared and treated with nuclease and EDTA as described previously (Watts *et al.*, 1983). The amount of microsomes used in a particular translation reaction corresponded to an absorbance at 280 nm of 1.2, as measured in 2% SDS, which would be due to microsomes alone. Treatment of microsomes with trypsin was performed following the procedure described recently (Zimmermann and Mollay, 1986). Experiments were performed to demonstrate the following experimental prerequisites: (i) treatment of microsomes with trypsin completely destroyed docking protein and inhibited the processing of pre-κ-light chain by microsomes in a reticulocyte lysate (Meyer *et al.*, 1982); (ii) reconstitution of trypsin-treated microsomes with docking protein fragment restored processing of pre-κ-light chain and sequestration of κ-light chain in a reticulocyte lysate (Meyer and Dobberstein, 1980); and (iii) trypsin-treated microsomes were able to process prepromelittin but failed to sequester promelittin (Zimmermann and Mollay, 1986).

Analytical procedures

Sequestration of the processed form of precursor proteins by dog pancreas microsomes was analysed by incubation of the complete translation reaction with Proteinase K in the presence or absence of Triton X-100 (Zimmermann and Mollay, 1986).

Samples containing *in vitro* synthesized proteins with a mol. wt above or below the mol. wt of globin were diluted with an equal volume of double-strength sample buffer (Laemmli, 1970) and analysed directly by polyacrylamide gel electrophoresis. Alternatively, samples containing *in vitro* synthesized proteins with a mol. wt close to the mol. wt of globin were supplemented with $(NH_4)_2SO_4$ [final concentration 66% (w/v)] and centrifuged (5 min, 10 000 *g*) (Hoober *et al.*, 1982). The pellets were washed once with 5% TCA, two times with acetone, then dried briefly under vacuum and dissolved in sample buffer. Polyacrylamide gel electrophoresis, using 19% (acrylamide, w/v) separating gels, was carried out in the presence of SDS and urea (Ito *et al.*, 1980). After soaking the gels in sodium salicylate (Chamberlain, 1979) they were dried under vacuum and exposed to X-ray films (Kodak X-Omat AR) for 12–24 h at −80°C.

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