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Membrane translocation and insertion of NH_2-terminally anchored γ-glutamyl transpeptidase require a signal recognition particle

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The two subunits of the renal brush border enzyme, γ-glutamyl transpeptidase (EC 2.3.2.2), are derived from a single-chain propeptide. The membrane-spanning domain consists of a hydrophobic sequence near its NH_2-terminus and the protein is oriented with its NH_2-terminus on the cytoplasmic side. The enzyme is synthesized without a cleavable signal sequence. Translocation and insertion of this enzyme have been shown to be dependent on the signal recognition particle and presumably require the same translocation machinery that other secretory and membrane proteins use for these processes.

γ-Glutamyl transpeptidase; Brush border enzyme; Membrane insertion; Signal sequence; Signal recognition particle

1. INTRODUCTION

The rat kidney enzyme, γ-glutamyl transpeptidase, is a heterodimeric glycoprotein located on the lumenal surface of the proximal tubule brush border membranes [1,2]. The membrane-binding domain of the enzyme is located near the NH_2-terminus of the larger subunit and consists of a 22-residue long (positions 5–26) sequence of hydrophobic and neutral amino acids [3,4]. Asymmetric labeling experiments indicate that the NH_2-terminus of the enzyme is exposed on the cytoplasmic side of the brush border membranes, the bulk of the protein extending out from the lumenal surface [5]. Such membrane orientation is a characteristic common to several other brush border membrane enzymes, both kidney and intestinal [6,7].

Tissue labeling and in vitro translation of mRNA provided evidence that the two subunits (M_r ~52000 and 25000, respectively) of transpeptidase are synthesized as a common single-chain propeptide [8–10]. Molecular cloning and the nucleotide sequence of the cDNA complementary to rat kidney transpeptidase mRNA have confirmed this mode of biosynthesis [4]. In vitro translation of the transpeptidase mRNA shows that the membrane insertion and glycosylation of the propeptide are cotranslational events, and that the translocation of the primary translation product across the microsomal membranes is not associated with the cleavage of an NH_2-terminal signal sequence [9]. We proposed that the hydrophobic sequence near the NH_2-terminus of the propeptide functions as a cotranslational translocation signal sequence which remains uncleaved to serve as the membrane-spanning domain for the enzyme.

Much is known about the biosynthesis and mechanism of membrane insertion of transmembrane proteins which are oriented with their COOH-terminus exposed on the cytoplasmic side of the membrane. Like the secretory proteins, such transmembrane proteins are synthesized with tran-

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sient, hydrophobic, NH_2-terminal signal sequences which direct their translocation across the microsomal membranes [11–13]. A signal recognition particle (SRP) binds to the signal sequence and allows the protein to be cotranslationally translocated through the membrane [14,15]. Signal peptidase cleaves the signal sequence from the nascent protein producing a new NH_2-terminus located in the lumen of the endoplasmic reticulum. The transmembrane proteins remain bound to the membrane because a second stretch of hydrophobic amino acids in the nascent protein acts as a stop transfer sequence and eventually serves as the membrane-spanning domain. The secretory proteins are translocated completely across the membrane. Relatively little is known about the mechanism of membrane translocation and insertion of the NH_2-terminally anchored brush border membrane proteins. Thus, although the primary structure of the uncleaved NH_2-terminal membrane-spanning domain of γ-glutamyl transpeptidase [4] resembles the amino acid sequences of the signal for cotranslational translocation of various secretory and membrane proteins across the endoplasmic reticulum of eukaryotic cells [13,16], it is not known whether the membrane translocation and insertion of this protein, and other brush border membrane proteins, are mediated by SRP or some other factor. We now present evidence that translocation across and insertion of transpeptidase into microsomal membranes require SRP and presumably its receptor.

2. MATERIALS AND METHODS

Rabbit reticulocyte lysate and wheat germ in vitro translation kits were purchased from Bethesda Research Laboratories. Dog pancreas microsomes, salt-washed microsomes and SRP were generous gifts from Dr Reid Gilmore, The Rockefeller University, New York, NY (present address: University of Massachusetts, Worcester, MA). Rabbit antibodies (γ-globulin fraction) against the purified rat kidney γ-glutamyl transpeptidase and the rat kidney poly(A^+) RNA were prepared as described [9]. In vitro translation of the RNA in the rabbit reticulocyte lysate (90 min at 30°C) and wheat germ (60 min at 25°C) systems using 4 μg RNA/60 μl reaction mixture, immunoprecipitation with anti-transpeptidase IgG, and analysis of the products on SDS-PAGE followed by autoradiography were performed as in [9]. [^{35}S]Methionine (100 μCi/reaction mixture) was used to label the products. Where noted, microsomal membranes were added to the translation reactions. Post-translational incubation with pronase (1 h at 0°C) was carried out as described [9].

3. RESULTS AND DISCUSSION

Our previous studies have shown that translation of rat kidney poly(A^+) RNA in vitro in a rabbit reticulocyte lysate system results in the synthesis of a 63 kDa nonglycosylated polypeptide which contains domains of both the subunits of γ-glutamyl transpeptidase [9]. When the translation was carried out in the presence of dog pancreas microsomes, a 78 kDa core-glycosylated species is synthesized. The increase in molecular mass upon glycosylation is consistent with the fact that there are five *N*-glycosylation sites in transpeptidase [4] since each high-mannose group adds about 3 kDa to the molecular mass of a protein [17]. The transpeptidase-related species synthesized in the presence and absence of dog pancreas microsomes in the reticulocyte lysate system are shown for reference in fig.1 (lanes 1,2). We showed previously that translocation, glycosylation, and membrane integration of the 78 kDa species are cotranslational events [9].

One method of demonstrating that SRP is involved in the initial biosynthetic steps of a protein depends on the finding that SRP inhibits translation of secretory and COOH-terminally anchored transmembrane proteins [15,18]. This translation arrest can be released if salt-washed microsomes, which are devoid of SRP but contain the SRP receptor, are added back to the translation system. Using a wheat germ translation system (which, in contrast to the rabbit reticulocyte lysate system, is devoid of endogenous SRP [14]), we show that SRP, indeed, strongly inhibits the translation of transpeptidase mRNA (fig.1, lanes 3,4). In the same system, in a control experiment, it can be demonstrated that the synthesis of rabbit globin, a cytosolic protein, is not affected by SRP. The globin mRNA was obtained as part of the rabbit reticulocyte lysate kit.

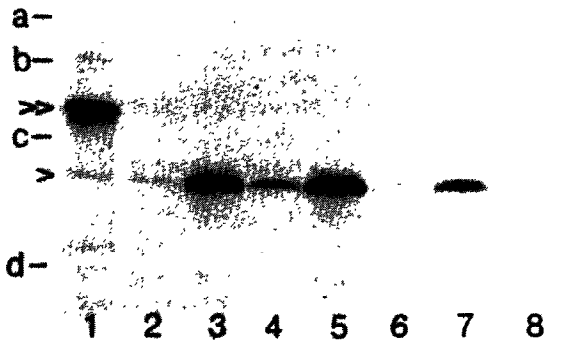

Fig.1. SRP-dependent insertion of γ-glutamyl transpeptidase propeptide into microsomal membranes. Rat kidney poly(A)$^+$ RNA was translated in a rabbit reticulocyte lysate system either in presence (lane 1) or absence (lane 2) of dog pancreas microsomes as described [9]. The immunoprecipitates (using anti-transpeptidase IgG) were analyzed on SDS-PAGE followed by autoradiography. The 63 kDa non-glycosylated propeptide is marked with a single arrowhead and the 78 kDa core-glycosylated species is marked with a double arrowhead. Results of translations in presence of a wheat germ system are shown in lanes 3–8. Lane 3 shows the 63 kDa primary translation product. Lane 4 shows the effect of adding SRP (20 U/reaction mixture) on the synthesis of transpeptidase propeptide. Lanes 5 and 6 show that addition of salt-washed microsomes relieves the inhibition of synthesis of the 63 kDa species. After completion of translation, the microsomes were separated from the soluble proteins by centrifuging the reaction mixture in a Beckman airfuge at 150000 × *g* over a 0.2 M sucrose cushion containing 0.09 M potassium acetate (pH 11.5) and 1 mM magnesium acetate. The pellet and supernatant were separately immunoprecipitated (lanes 5 and 6, respectively). Lanes 7 and 8 show the effect of pronase treatment in the absence (lane 7) and in the presence (lane 8) of 0.5% Triton X-100. Translation was carried out in the presence of SRP and salt-washed microsomes as in lane 5 and the reaction was then divided into two equal aliquots for treatment with pronase. The position of protein standards (from Sigma) are shown on the left (in kDa): a, β-galactosidase (116); b, phosphorylase (94); c, bovine serum albumin (68); d, ovalbumin (45).

The translation arrest of transpeptidase synthesis by SRP in wheat germ system was relieved when salt-washed microsomes are added to the translation reaction (fig.1, lane 5). Furthermore, this experiment shows that the 63 kDa species is inserted into the microsomes since it remains associated with the membranes when the translation reaction was subjected to centrifugation at 150000 × *g* over a sucrose cushion to separate the microsomes from soluble proteins (lanes 5,6). That the 63 kDa species synthesized in the presence of SRP and salt-washed microsomes has been translocated and sequestered within the cisternae of the microsomal membranes is evident from results shown in lanes 7,8. The membrane-associated polypeptide is protected from the action of pronase whereas it becomes susceptible to proteolysis when the membranes are solubilized with Triton X-100. When translation is carried out in the wheat germ system in the presence of salt-washed membranes, but with no added SRP, a result comparable to that seen in lane 3 is obtained; however, the 63 kDa species is not integrated into the membranes since it is readily degraded by pronase in the absence of detergent (not shown).

It should be noted that when translation was carried out in the presence of salt-washed microsomes and SRP, only the 63 kDa species corresponding to the nonglycosylated transpeptidase propeptide was synthesized (fig.1, lanes 3,5), indicating that the salt-washed microsomes had lost glycosylating ability. It is known that microsomes which have been salt-extracted to remove SRP glycosylate poorly [19]. Indeed, other preparations of salt-washed microsomes (prepared by us as described by Walter and Blobel [15]) have been shown to synthesize varying but low amounts (compared to results in lane 1, fig.1) of glycosylated transpeptidase propeptide (not shown). We have chosen to present the data shown in fig.1 since they clearly demonstrate that glycosylation per se is not essential for membrane integration and translocation of this brush border membrane enzyme.

The low efficiency of translation of transpeptidase mRNA in rabbit reticulocyte lysate system (cf. lanes 2,3 in fig.1) is presumably related to the translation arrest by the endogenous SRP in the lysate [14]. Thus, the marked increase in the synthesis of transpeptidase propeptide in the presence of dog pancreas microsomes (fig.1, lane 1) may be explained by release of this inhibition.

Our results thus clearly demonstrate that SRP mediates the cotranslational translocation across the microsomal membranes and membrane insertion of γ-glutamyl transpeptidase, a brush border

membrane protein that is synthesized without a cleavable signal sequence and is anchored to the membranes via an NH_2-terminal hydrophobic segment. We thus propose that this NH_2-terminally anchored brush border membrane protein requires the same membrane translocation system, consisting of SRP and SRP-receptor protein, that has been shown to be involved in the translocation and/or integration of a variety of eukaryotic secretory and membrane proteins [13–15]. In contrast to the majority of the latter type of proteins, the signal sequence in transpeptidase remains uncleaved and embedded in the lipid bilayer to serve as the membrane-spanning domain. It is likely that other brush border enzymes which exhibit similar topogenic features [6,7,20] use the same translocation system. This mechanism seems to be applicable also to transmembrane proteins found in other types of cell membranes which are synthesized without a cleavable signal sequence and whose membrane-spanning domains are located near their NH_2-termini [19,21–23].

Although no strict consensus sequences have been identified at the signal peptidase cleavage sites in eukaryotic proteins, the sequence Ala-X-Ala, or other amino acids with small, uncharged side chains in place of alanine residues, seems to be the preferred sequence preceding the cleavage site [16]. The amino acid sequence of γ-glutamyl transpeptidase indicates that such a sequence (Thr-Thr-Ser-Gly) is indeed present at positions 28–31 [4]. This is preceded by the hydrophobic, anchor sequence (positions 5–26). Other structural features of γ-glutamyl transpeptidase must, therefore, render it resistant to the action of the signal peptidase.

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