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Synthesis and insertion of cytochrome *P*-450 into endoplasmic reticulum membranes

(membrane biogenesis/bound polysomes/*in vitro* protein synthesis/signal sequence/phenobarbital induction)

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ABSTRACT **Treatment of rats with phenobarbital leads to a substantial increase in levels of translatable liver cytochrome *P*-450 mRNA. This mRNA is primarily associated with ribosomes bound to endoplasmic reticulum membranes which in an *in vitro* system synthesized approximately 10 times more cytochrome *P*-450 than did free polysomes from the same animals. Cytochrome *P*-450 synthesized by rough microsomes *in vitro* appears to be directly inserted into the membranes because it was not released by a treatment with low detergent concentrations that released albumin and other microsomal content proteins. The amino-terminal amino acid sequence of cytochrome *P*-450 synthesized in an mRNA-dependent system resembles in hydrophobicity the signal segment of presecretory proteins and therefore may serve to insert the polypeptide into the membrane during synthesis. In contrast to the situation with secretory proteins and several other membrane proteins, however, the putative insertion signal of cytochrome *P*-450 is not removed by a membrane-associated peptidase and remains in the mature polypeptide.**

An understanding of the process of membrane biogenesis requires elucidation of the mechanisms by which newly synthesized integral membrane proteins are inserted into membranes and acquire the orientation necessary for their function. It has been proposed (1) that a general mechanism for the insertion of integral membrane proteins that acquire a transmembrane disposition or are located in the luminal side of membranes involves synthesis of the polypeptide on membrane-bound polysomes and its cotranslational incorporation into the rough endoplasmic reticulum (ER) membrane. After this insertion, proteins could be transferred to other membrane systems by lateral diffusion along continuous phospholipid bilayers or through vesicles that shuttle between compartments (1, 2). Cytochrome *P*-450 (cf. ref. 3) is a major integral membrane protein of both rough and smooth ER of hepatocytes and thus appears to provide an ideal model with which to examine aspects of the process by which proteins become incorporated into membranes.

Although the detailed orientation of cytochrome *P*-450 with respect to the phospholipid bilayer is not known, it is clear that a substantial portion of the molecule is exposed on the cytoplasmic face of the ER (4–7). Furthermore, labeling experiments suggest that the protein has a transmembrane disposition because its accessibility to macromolecular labeling probes appears to be enhanced when membranes are made permeable by detergents (8).

Treatment of Long–Evans rats with phenobarbital (PB) induces high levels of a molecular form of cytochrome *P*-450 that is present at much lower levels in untreated animals (9). It has been reported that this induction results, at least in part, from an increase in the rate of synthesis of the apoprotein (10, 11). We have found that mRNA preparations obtained from the livers of PB-treated animals have an enhanced template activity for directing the *in vitro* synthesis of this protein, and we have taken advantage of this fact to determine the site of synthesis of cytochrome *P*-450, to characterize the primary translation product of its mRNA, and to examine the mechanism by which the polypeptide is inserted into the ER membrane.

MATERIALS AND METHODS

Cytochrome *P*-450 from the livers of Long–Evans rats treated with PB was purified by chromatography on aminooctyl-Sepharose and hydroxylapatite (12, 13). A rabbit antiserum was raised as described (13).

Preparation of Total Polysomal mRNA. Polysomes were prepared from livers of unstarved rats (14) or starved animals (15) perfused with ice-cold 0.25 M sucrose/25 mM Tris·HCl, pH 7.5/25 mM NaCl/5 mM $MgCl_2$. Total RNA was extracted from polysomes (16) and polyadenylylated mRNA was prepared by oligo(dT)-cellulose chromatography (17).

Preparation of Free and Membrane-Bound Polysomes. Different procedures were used to prepare polysomes from starved (18) or unstarved animals (19). In the latter case, the entire procedure was carried out in medium containing 75 mM KCl; after incubation with α-amylase, 3 mM dithiothreitol was added. Supernates containing free polysomes were not treated with detergent.

***In Vitro* Protein Synthesis.** Polyadenylylated mRNA (0.4 A_{260} unit/ml) or polysomes (20.0 A_{260} units/ml) were used for translation at 37°C in a reticulocyte lysate system (20) supplemented with rat liver tRNA (55 μg/ml) and spermidine (0.2 mM).

RESULTS

Increased Levels of Translatable Cytochrome *P*-450 mRNA After PB Treatment. Analysis of total translation products of reticulocyte lysates programmed with polyadenylylated mRNA (Fig. 1, lanes a and b) showed that the synthesis of a polypeptide with the same electrophoretic mobility as mature cytochrome *P*-450 was enhanced substantially by PB treatment. This polypeptide was immunoprecipitated by specific antibodies against cytochrome *P*-450 (Fig. 1, lanes c and d) and, after induction, accounted for approximately 1–2% of the total radioactivity incorporated into cell-free products.

Synthesis of Cytochrome *P*-450 in Membrane-Bound Ribosomes. The sets of total translation products of free and

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Abbreviations: ER, endoplasmic reticulum; PB, sodium phenobarbital.

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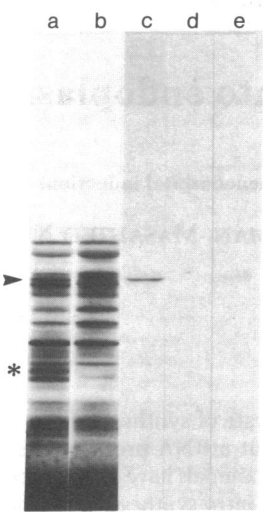

FIG. 1. Effect of PB treatment on levels of translatable cytochrome *P*-450 mRNA. Polysomal polyadenylylated mRNA was translated with [^{35}S]methionine as a label (500 μCi/ml; 1000 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels). Total translation products (5-μl aliquots) and immunoprecipitates (from 15 μl) were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis (13) followed by autoradiography. Samples for immunoprecipitation were brought to a volume of 50–100 μl containing 150 mM NaCl, 50 mM sodium phosphate buffer (pH 7.5), 5 mM EDTA, 1% (wt/vol) sodium deoxycholate, 1% (vol/vol) Triton X-100, and 100 units of Trasylol (Delbay Pharmaceutical) per ml; purified cytochrome *P*-450 (1–10 μg) and anti-cytochrome *P*-450 IgG (0.1–1.0 mg) were added. After incubation (1 hr at 25°C and 12 hr at 4°C), the immunoprecipitate was sedimented in a Microfuge (12,800 × *g*, 30 min), resuspended in 1.0 ml of phosphate-buffered saline, and washed by centrifugation through a step gradient (0.1 ml of 1.0 M sucrose and 0.05 ml of 0.5 M sucrose containing 1% sodium deoxycholate, 1% Triton X-100, and 20 unlabeled amino acids at 10 mM each). The recovered sample was dissolved and used directly for analysis. Lanes: a and b, total translation products with mRNA from PB-treated (a) and control (b) rats; c and d, immunoprecipitates obtained from a and b, respectively; e, cytochrome *P*-450 purified from liver microsomes of PB-treated rats, labeled with ^{125}I (21), and used as a marker. The position of *in vitro* synthesized cytochrome P-450 in the electropherogram of the translation mixture programmed with mRNA from PB-treated rats (lane a) is indicated by an arrowhead; that of an additional induced protein of M_r 25,000 is indicated by *.

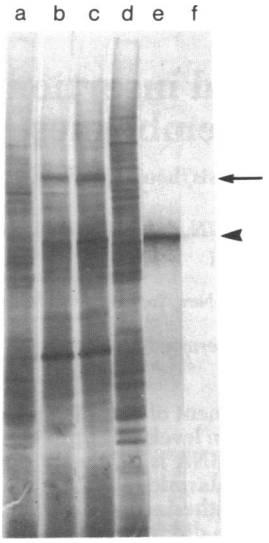

FIG. 2. Synthesis of cytochrome *P*-450 on membrane-bound ribosomes from PB-treated rats. Total translation products and immunoprecipitates were prepared and analyzed as described in the text and in the legend to Fig. 1. Lanes: a and d, total products of free polysomes from control (a) and PB-treated (d) rats; b and c, total products of bound polysomes from control (b) and PB-treated (c) animals; e and f, *in vitro* synthesized cytochrome *P*-450 immunoprecipitated from translation systems programmed with bound (e) or free (f) polysomes isolated from PB-treated rats. Arrowhead, position of cytochrome *P*-450; arrow, position of pre-proalbumin.

bound polysomes differed substantially and were affected specifically by the PB treatment (Fig. 2). The polypeptide representing the PB-induced form of cytochrome *P*-450 appeared to be synthesized at significant levels only in translation mixtures programmed with membrane-bound polysomes, as was the case with the secretory protein pre-proalbumin. Estimates from total immunoprecipitates showed that cytochrome *P*-450 and pre-proalbumin amounted to approximately 4 and 6.5%, respectively, of the total radioactivity incorporated in translation products of membrane-bound polysomes from PB-treated rats and less than 0.5 and 0.7% of the radioactivity in translation products of free polysomes. Similar results demonstrating the exclusive synthesis of cytochrome *P*-450 in membrane-bound polysomes of PB-treated rats were observed with both starved and unstarved animals and when media containing either 75 or 250 mM KCl were used during cell fractionation.

Insertion of Cytochrome *P*-450 into the ER Membrane. To determine if cytochrome *P*-450, like other integral membrane proteins (22–26), is inserted into the microsomal membrane cotranslationally, rat liver rough microsomes from PB-treated animals were used for *in vitro* protein synthesis, recovered, and subfractionated into luminal and membrane components by exposure to low detergent concentrations. Low concentrations of sodium deoxycholate (0.025–0.05%) release proteins from the vesicular lumen of microsomes whereas integral membrane proteins are solubilized only at much higher detergent levels (27). The data in Figs. 3 and 4 demonstrate that, whereas newly synthesized *in vitro* labeled albumin was re-

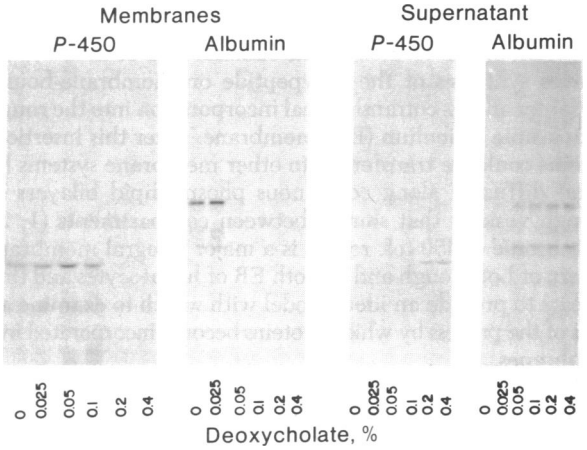

FIG. 3. Release of *in vitro* synthesized cytochrome *P*-450 and albumin from detergent-treated rough microsomes. Rough microsomes prepared from livers of PB-treated rats were used for protein synthesis in a system containing a rat liver G-100 high-speed supernatant (26) with 200 μCi [^{3}H]leucine (52 Ci/mmol) per ml as label. The labeled microsomes (10 ml) were diluted 1:10 with 0.5 M KCl/50 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$, recovered by centrifugation (100,000 × *g*, 60 min), and resuspended (3 mg of protein per ml) in 50 mM KCl/50 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$. Aliquots (0.5 ml) were incubated at 0°C for 30 min with different concentrations of sodium deoxycholate (27). Released (supernatant) and sedimentable (membranes) subfractions were separated by centrifugation (100,000 × *g*, 60 min). *In vitro* labeled cytochrome *P*-450 and albumin in the subfractions were immunoprecipitated with the corresponding IgG preparations and analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis and fluorography (28).

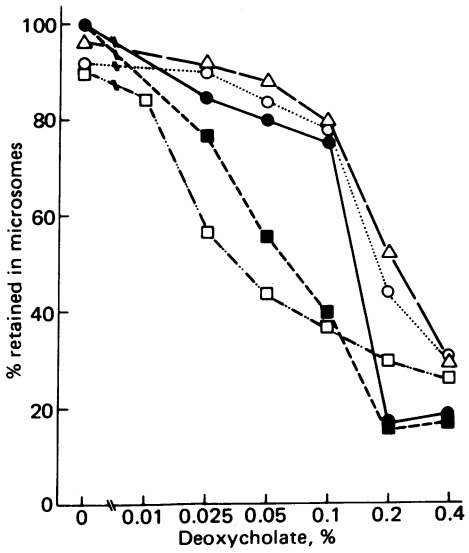

FIG. 4. Insertion of *in vitro* synthesized cytochrome *P*-450 into rough microsomal membranes. Microsomes with *in vivo* labeled content proteins were prepared from animals sacrificed 30 min after a single injection of [^{3}H]leucine (1 μCi/g; 58 Ci/mmol) (□); microsomes containing *in vivo* labeled membrane proteins were obtained from animals injected for 3 successive days with [^{14}C]leucine and sacrificed 24 hr after the last injection (△) (27). These preparations of labeled microsomes, as well as unlabeled microsomes and microsomes containing peptides labeled *in vitro* as described in Fig. 3, were subfractionated by the detergent treatment described in Fig. 3. The content of cytochrome *P*-450 in subfractions from the unlabeled microsome preparations was determined spectrophotometrically (○) (29). The distribution of total radioactivity in subfractions of the *in vivo* labeled samples was measured by liquid scintillation counting of trichloroacetic acid precipitates. The distribution of radioactivity in *in vitro* synthesized cytochrome *P*-450 (●) and albumin (■) was determined after immunoprecipitation and NaDodSO$_4$/polyacrylamide gel electrophoresis. Protein bands (see Fig. 3) were located by fluorography and their radioactivity was determined by assay of excised bands after solubilization of the protein in NCS (Amersham/Searle). At each detergent concentration the fraction of radioactivity or spectrophotometrically measured cytochrome *P*-450 retained in the sedimentable subfractions is presented as a percentage of the sums recovered in both fractions.

leased at low detergent concentrations, in parallel with proteins contained within the microsomal lumen (content proteins) which were labeled *in vivo* with a brief pulse of radioactive leucine, the *in vitro* synthesized cytochrome *P*-450 was released at much higher detergent concentrations, in parallel with the authentic mature cytochrome *P*-450 assayed spectrophotometrically and with the bulk of the membrane proteins which were labeled *in vivo* by an appropriate pulse–chase regimen.

Comparison of Amino-Terminal Sequences of *In Vitro* Synthesized and Mature Cytochrome *P*-450. Nascent polypeptides synthesized on membrane-bound ribosomes contain signals that determine the association of the ribosomes with the ER membrane (30, 31). The existence of amino-terminal signal peptides that are removed by a membrane-associated peptidase during translation has been demonstrated not only for numerous secretory proteins (32) but also for the membrane glycoprotein of vesicular stomatitis virus (33) and for several bacterial membrane proteins (34, 35). To determine if cytochrome *P*-450 contains a transient signal sequence, the amino-terminal amino acid sequence of the *in vitro* synthesized and mature proteins were compared. The partial sequence of PB-induced mature cytochrome *P*-450, presented in Fig. 5, shows substantial homology to that reported for PB-induced rabbit liver cytochrome P-450 (36). In particular, the amino-terminal portion of mature

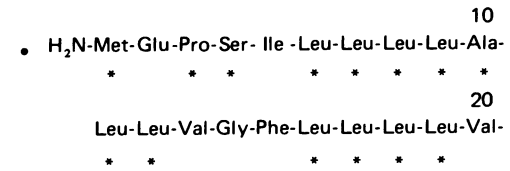

FIG. 5. Identity of amino-terminal amino acid sequences of mature cytochrome *P*-450 and the product of cell-free synthesis. An aliquot of cytochrome *P*-450 was dialyzed against 0.1 mM dithiothreitol and concentrated by using Aquacide IIA (Calbiochem). Approximately 4 mg was dissolved in 0.6 ml of 50% HCOOH, and 0.3 mg of Polybrene (Aldrich) was added as a carrier. Automated Edman degradation was carried out with a Beckman 890C sequencer and a 0.1M Quadrol program. Thiazolines recovered from each cycle were converted to phenylthiohydantoins by incubation with 0.1M HCl at 80°C for 10 min. These were identified by high-pressure liquid chromatography. Positions indicated by * correspond to residues identified in the *in vitro* synthesized radiolabeled cytochrome *P*-450, from the data in Fig. 6.

cytochrome *P*-450 begins with methionine and, as observed by Haugen *et al.* (36) for the rabbit cytochrome *P*-450, it is notable in that it resembles the transient amino-terminal sequence of presecretory proteins in its high proportion of hydrophobic amino acids. In addition, as is the case with most signal sequences (32), charged amino acids are located near the amino terminus. The partial sequence of the *in vitro* product, radiolabeled with five selected amino acids representing 14 of the first 20 residues of the mature protein, was also determined. The positions of the chosen amino acids in the *in vitro* synthesized cytochrome *P*-450 (Fig. 6) corresponded exactly to their positions in the mature protein (Fig. 5). These data suggest that the primary *in vivo* translation product of cytochrome *P*-450 mRNA is not proteolytically processed either co- or posttranslationally. A proteolytic modification therefore is not an obligatory requirement for the correct assembly of cytochrome *P*-450 into the ER.

DISCUSSION

The preceding observations demonstrate that membrane-bound polysomes from the livers of PB-treated rats are capable of synthesizing, in an *in vitro* system, approximately 10 times higher levels of cytochrome *P*-450 than do free polysomes. Because the ratio of bound to free polysomes is 2:1 in rat liver (38), our results suggest that, in the hepatocyte, cytochrome *P*-450 is synthesized exclusively (>95%) by polysomes associated with ER membranes. A similar conclusion has previously been reached from studies of the distribution of *in vivo* synthesized nascent cytochrome *P*-450 peptide chains in free and bound polysomes (39, 40).

We also found that cytochrome *P*-450 synthesized *in vitro* by ribosomes associated with microsomal membranes was incorporated into these membranes as an integral protein that could be solubilized only by detergent concentrations substantially higher than those required to release albumin and other content proteins from the microsomal lumen. It has recently been suggested (41–43) that, upon release from ribosomes, a fraction of the cytochrome *P*-450 molecules is first transiently transferred into the microsomal lumen from where it can be eventually incorporated into the ER membrane. Our observations indicate that such a mechanism could not apply to the bulk of newly synthesized cytochrome *P*-450. This mechanism is also rendered unlikely by the finding that, in contrast to nascent chains of secretory polypeptides (44, 45), incomplete cytochrome *P*-450 chains released from bound ribosomes by puromycin remain associated with the membranes and exposed on the outside of the vesicles (40).

As is the case with ovalbumin (46) and bovine retinal opsin

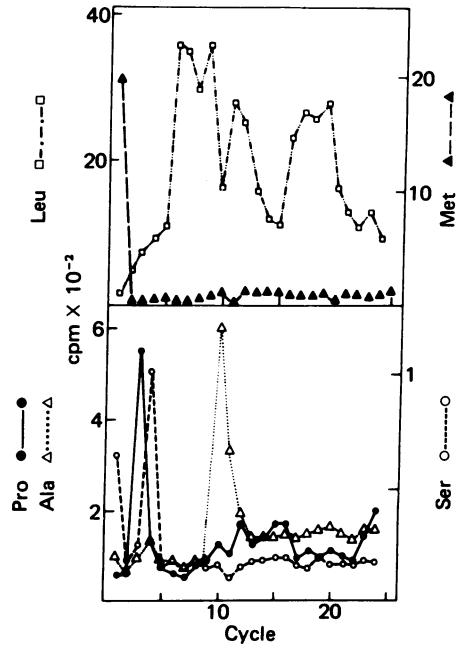

FIG. 6. Amino-terminal sequence analysis by automated Edman degradation of *in vitro* synthesized cytochrome *P*-450. Cytochrome *P*-450 labeled with different amino acids was synthesized in a rabbit reticulocyte cell-free system programmed with polyadenylylated mRNA obtained from livers of PB-treated rats. The cell-free systems contained, in 1 ml, citrate synthase (30 units) and oxaloacetate (1 mM) to prevent *N*-acetylation (37) and 500 μCi of [^{35}S]methionine (1000 Ci/mmol) or 1.5 mCi of [^{3}H]leucine (52 Ci/mmol) or a mixture of 440 μCi of [^{3}H]alanine (35 Ci/mmol), 750 μCi of [^{3}H]serine (19 Ci/mmol), and 150 μCi of [^{3}H]proline (117 Ci/mmol). A double immunoprecipitation was used to isolate the labeled *in vitro* product. In this case the first immunoprecipitate (see legend to Fig. 1) was dissolved in 0.1 ml of 50 mM sodium phosphate, pH 7.5/0.5% $NaDodSO_4$/Trasylol (100 units/ml) and the sample was incubated in a boiling water bath for 2 min. After dilution of the $NaDodSO_4$ to 0.1%, the immunoprecipitation procedure was repeated. The final immunoprecipitate, which showed a single band on electrophoresis, was dissolved in 0.1 ml of 0.5% $NaDodSO_4$/5% 2-mercaptoethanol, heated to 100°C for 2 min, and diluted 1:5 with 50% HCOOH. Sperm whale apomyoglobin (3 mg) was added and the samples were applied to the sequencer. When samples contained a single labeled amino acid, fractions from each cycle were evaporated to dryness and their radioactivity was assayed directly by liquid scintillation in 5.0 ml of ACS (Amersham/Searle) with 0.1 ml of water. Thiazolines generated by each cycle of Edman degradation from cytochrome *P*-450 samples containing several labeled amino acids were converted to their phenylthiohydantoin derivatives. These were subjected to high-pressure liquid chromatography, and effluents were collected for liquid scintillation counting. The phenylthiohydantoin amino acid derivatives in the main radioactive peaks were identified by comparison of their elution positions with those of known standards.

(47), the amino-terminal segment of the nascent chain of cytochrome *P*-450 is retained in the mature protein. In the case of cytochrome *P*-450, however, the amino-terminal segment resembles transient insertion signals of other proteins synthesized in bound polysomes. This hydrophobic region most likely establishes a relationship with the membrane early during synthesis of cytochrome *P*-450, retains the nascent peptide in the membrane throughout chain elongation, and may be one of the factors that prevents its discharge upon chain completion.

Because cytochrome *P*-450 is synthesized exclusively in bound polysomes and inserted directly into the rough membranes, its presence in the smooth portions of the ER, which proliferate after PB administration (48, 49), must result from lateral displacement of the polypeptide along the phospholipid bilayer of these two continuous membrane systems.

We have proposed that the orientation of a protein in a membrane is related to the process by which it is inserted into the membrane during synthesis (1). The mechanism of insertion of nascent cytochrome *P*-450 into the ER membrane may be regarded as akin to that by which secretory polypeptides are vectorially discharged. Some features of the cytochrome *P*-450 polypeptide, however, must ensure that its vectorial discharge is interrupted before translocation across the membrane is completed. Information to halt the transfer of nascent cytochrome *P*-450 before completion of synthesis could be contained anywhere in the polypeptide chain, including its amino-terminal portion which could act first as an insertion signal and subsequently as a signal that halts transfer. Alternatively, the amino-terminal portion of cytochrome *P*-450 could act only as a leader segment analogous to the one in secretory proteins which facilitates the passage of succeeding portions of the polypeptide across the membrane.

If this is the case, however, one should expect that, as has been suggested for the vesicular stomatitis virus glycoprotein (33), other signals that halt transfer are present in the interior of cytochrome *P*-450 to prevent complete passage of the polypeptide across the membrane. Such "halt transfer" signals would cause all subsequent regions to remain on the cytoplasmic side, unless a new interior (re)insertion signal appears in the distal region. Hydrophobic segments that strongly interact with the membrane phospholipid bilayer are likely to serve as halt transfer signals, although a similar effect could be exerted by a stretch of highly polar or charged residues which cannot enter the membrane.

In the model proposed here, the number and relative position of insertion and halt transfer signals within the cytochrome polypeptide would be responsible for the disposition of specific regions of the molecule with respect to the phospholipid bilayer and for the final orientation of the protein within the membrane.

Current evidence (1, 2, 22–26, 33, 47) suggests that membrane-bound ribosomes are involved in the synthesis of proteins with various subcellular destinations such as those that are transferred to the plasma membrane and others that are retained within the ER membrane itself. It is therefore reasonable to postulate that, in addition to insertion and halt transfer signals which function during polypeptide elongation, other structural features of the polypeptides exist which mediate posttranslational interactions with specific cellular components and in this way act as "sorting out" signals that determine the subcellular pathway and ultimate location of each polypeptide.

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