EARLY EVENTS IN THE CELLULAR FORMATION OF PROPARATHYROID HORMONE

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ABSTRACT

Early events in the cellular synthesis and subsequent transfer into membrane-limited compartments of pre-proparathyroid hormone (Pre-ProPTH) and proparathyroid hormone (ProPTH) were investigated by electrophoretic analyses of newly synthesized proteins in subcellular fractions of parathyroid gland slices pulse-labeled for 0.5–5 min with [^{35}S]methionine. During these short times of incubation, both Pre-ProPTH and ProPTH were confined to the microsomal fraction. Labeled Pre-ProPTH and ProPTH were detected in a 30-s interval between 0.5 and 1.0 min of incubation. The radioactivity in Pre-ProPTH became relatively constant between 3 and 5 min, whereas the radioactivity in ProPTH increased markedly over this period. When corrected for the known content of methionine in the prehormone and the prohormone, we found four times as much radiolabeled prohormone as prehormone between 0.5 and 1.0 min of synthesis. Sequestration of labeled prohormone into endoplasmic reticulum compartments was shown by treatment of the microsomal fraction with chymotrypsin and trypsin, which resulted in the degradation of the prehormone but not of the prohormone. Approximately 50% of pre-prohormone and 25% of prohormone were released from the microsomes by their extraction with 1.0 M KCl, whereas 80–90% of both was released by treatment with Triton X-100. These results in intact cells support the signal hypothesis proposed by Blobel and his co-workers in studies utilizing cell-free systems, inasmuch as the results indicate transfer of prohormone into the cisternal space of the rough endoplasmic reticulum concomitant with the growth of the nascent polypeptide chain. Appearance of membrane-sequestered ProPTH takes place without entry of Pre-ProPTH into the cisternal space, suggesting that proteolytic removal of the leader peptide occurs during transfer of the polypeptide through the lipid bilayer. Further evidence in support of this process is that Pre-ProPTH is only partly extracted from the microsomes by treatment with 1.0 M KCl, suggesting that a substantial fraction of the nascent Pre-ProPTH is integrally inserted into the membranes before it is cleaved to form ProPTH.

Parathyroid hormone (PTH), a polypeptide of 84 amino acids, is synthesized by way of two successive cleavages of NH_2-terminal sequences from a larger precursor, pre-proparathyroid hormone (Pre-ProPTH) of 115 amino acids (4, 6). The earliest cleavage occurs either co- or post-translation-

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ally, or both, in the rough endoplasmic reticulum (RER) and consists of the removal of an NH_2-terminal leader sequence of 25 amino acids, resulting in the formation of an intermediate precursor, proparathyroid hormone (ProPTH). The second cleavage occurs in the Golgi complex 12–15 min later by removal of an NH_2-terminal sequence of six amino acids, resulting in the formation of PTH (3, 8).

In accord with the "signal hypothesis" proposed by Blobel and Dobberstein (1), the leader sequence of Pre-ProPTH appears to serve as a signal that functions in the establishment of a polyribosome/membrane junction via the attachment of the nascent polypeptide to a transport element, as yet unidentified, located in the lipid bilayer of the RER. The growing polypeptide chain is then transferred in a unidirectional manner into the cisterna of the RER.

We reported previously on the synthesis of Pre-ProPTH and ProPTH in studies of hormone synthesis in vitro using slices of parathyroid glands (8). In this paper we report results of subsequent, more detailed studies of the early events in the cellular biosynthesis of Pre-ProPTH and ProPTH in parathyroid gland slices in which we investigated the processing and membrane-related translocational events involved in the biosynthesis of these precursors by analysis of microsomes prepared from the slices incubated for 0.5–5 min with [^{35}S]methionine. We have found that processing of nascent Pre-ProPTH to ProPTH is extensive during the interval between 0.5 and 1 min of synthesis. We conclude that the formation of ProPTH probably occurs to a large extent co-translationally during growth of the nascent polypeptide chains. Our observations made in studies of intact parathyroid cells support earlier findings obtained from studies in cell-free systems (1). The concept of co-translational processing of precursors of secretory proteins seems to be a valid model of biosynthetic mechanisms for these proteins in intact cells as well as in cell-free systems.

MATERIALS AND METHODS

Pulse-labeling and Homogenization of Parathyroid Gland Slices

Approximately 100 mg of parathyroid gland slices prepared from freshly collected bovine parathyroid glands was pre-incubated with gentle shaking for 40 min in 1.5 ml of Dulbecco's modified minimal essential medium obtained without methionine (Grand Island Biological Co., Grand Island, N. Y.). 0.1 ml of an aqueous solution of [^{35}S]methionine (450–650 Ci/mM, 7.5 mCi/ml, New England Nuclear, Boston, Mass.) was added to the shaking suspension of slices. 10 s before the termination of the pulse-labeling period, the slices were transferred to an ice-cold Teflon/glass homogenizer, the labeling medium was quickly removed, 1.5 ml of ice-cold STKM buffer (0.25 M sucrose, 50 mM Tris [pH 7.5], 25 mM KCl, 5 mM $MgCl_2$) was added, and the slices were immediately homogenized by 5–10 strokes with the pestle rotating at 1,200 rpm. It was possible to achieve almost complete disruption of the rather fibrous gland slices by this procedure. The homogenates were centrifuged at 1,000 *g* for 15 min to provide a postnuclear supernate.

Preparation and Treatment of Microsomal Fractions

Aliquots of the postnuclear supernates were centrifuged at 105,000 *g* for 60 min, providing a microsomal pellet and a cytosolic supernate. In certain experiments, aliquots of the postnuclear supernates were adjusted to 1.0 M KCl or 1% Triton X-100 before centrifugation. Microsomal pellets were resuspended by homogenization in a volume of STKM buffer equal to the original volume of postnuclear supernate that was centrifuged.

SDS Polyacrylamide Gel Electrophoresis

Cytosolic supernates and suspensions of microsomes were adjusted to 1% SDS, 5% 2-mercaptoethanol, and 0.05% bromphenol blue. Microsomes were dissolved by allowing them to incubate at 23°C for 2 h with frequent mixing. Total acid-precipitable radioactivity in the above-mentioned fractions was determined as described previously (5). Aliquots of the fractions were heated at 100°C for 2 min, and protein in the fractions was analyzed by electrophoresis on 10–20% gradient acrylamide gel slabs containing SDS (13). The gel slabs were stained with Coomassie Brilliant Blue, destained in 7% acetic acid and 25% methanol followed by a wash in 7% acetic acid, and finally were dried under vacuum or, alternatively, were treated with Enhance (New England Nuclear) and then dried. Autoradiograms or autoradiofluorograms were prepared at 25° or −70°C, respectively, on Kodak SB-5 film. Densitometric analyses were performed semi-quantitatively with a Zeiss PMQII scanner. Amounts of radioactivity in the gels were calculated both by measurements of peak heights and by determinations of areas under the curves described by labeled Pre-ProPTH and ProPTH.

RESULTS

Electrophoretic analyses of the labeled proteins in the microsomal and cytosolic fractions prepared from parathyroid gland slices incubated for 0.5–5 min with [^{35}S]methionine demonstrate the appearance of labeled Pre-ProPTH and ProPTH in the interval between 0.5 and 1.0 min of the labeling period (Fig. 1). No definite bands corresponding to labeled Pre-ProPTH or ProPTH were detected at 0.5 min by radiographic analysis of labeled proteins in the microsomal fraction using the more sensitive autoradiofluorography, although several faint bands appeared in the region of migration of the hormonal precursors; this region of the autoradiogram was obscured by a diffuse haze which probably represents nascent chains. The labeled

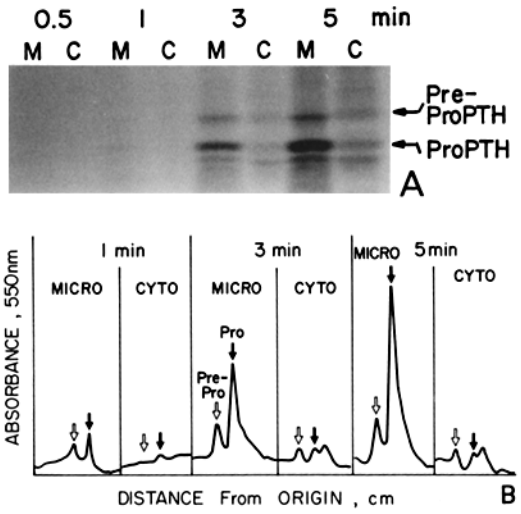

FIGURE 1 Labeled Pre-ProPTH and ProPTH in microsomal (*M*) and cytosolic (*C*) fractions prepared from parathyroid gland slices pulse-labeled for 0.5–5 min with [^{35}S]methionine. Patterns are from an autoradiofluorogram (*A*) prepared from 10–20% gradient polyacrylamide gels containing SDS. Only regions of the gel in the region of migration of the hormones are shown. Densitometric tracings of *A* are shown in *B*.

hormonal proteins detected from 1 to 5 min were found almost entirely in the microsomal fraction (Fig. 1) and were readily released from this fraction by treatment of the postnuclear supernate with Triton X-100 before the microsomes were sedimented (Fig. 2).

The data shown in Fig. 2, obtained from gland slices pulse-labeled for 0.5 min, further revealed the presence in the Triton X-100 cytosolic fraction of a faint band corresponding in mobility to ProPTH without any traces of Pre-ProPTH. The liberation by Triton X-100 of the labeled ProPTH from the radioactive background of ribosome-bound, labeled nascent chains has resulted in the appearance of a small amount of labeled ProPTH before the appearance of any labeled Pre-ProPTH (Fig. 2 *B* and *C*).

Fig. 3 provides a summary of the data shown in Figs. 1 and 2. Note that amounts of ProPTH, when corrected for the relative contents of methionine in the two proteins (Pre-ProPTH contains seven, and ProPTH two methionine residues [9]), always exceeded by three- to fourfold those of Pre-ProPTH even at 1 min of synthesis. Amounts of ProPTH increased rapidly during 5 min of incubation, whereas amounts of Pre-ProPTH tended to level off after 3 min. After longer incubations (not shown) for 10 and 25 min, the amounts of labeled Pre-ProPTH were not greater than those seen after 5 min of incubation.

With increasing times of incubation, a greater fraction of the total labeled proteins synthesized in the gland slices was released from the microsomal fraction into the cytosolic fraction (Fig. 3 *A*) by treatment with Triton X-100, a finding consistent with the growth and release of nascent chains from the polyribosomes and their transport into membrane-limited compartments, which are disrupted by the detergent. Pre-ProPTH and ProPTH were at all times of incubation readily released from the microsomal fraction by the detergent treatment (Fig. 3 *B* and *C*).

To determine the location of the newly synthesized proteins in the microsomal fraction, aliquots of the microsomes were subjected to limited proteolysis using a mixture of trypsin and chymotrypsin. When examined at each time of labeling, including 1 min, ProPTH, but not Pre-ProPTH, was found to be resistant to proteolysis (Fig. 4). When the microsomes were first disrupted by treatment with Triton X-100, however, both Pre-ProPTH and ProPTH were completely hydrolyzed by the enzymes (Fig. 4). These results are consistent with the location of the prohormone within the microsomal vesicles where it is protected against proteolysis (1, 15). Pre-ProPTH, however, remains available to enzymic attack and, by this criterion, is not sequestered, as is ProPTH, within vesicles.

The physical dispositions of Pre-ProPTH and ProPTH during their biosynthesis were analyzed further by a determination of the distribution of the polypeptides after treatment of the microsomes with high concentrations of salt (1.0 M KCl), a procedure that extracts most peripheral but not integral proteins from membranes (17). Approximately 50% of Pre-ProPTH was removed from the microsomal fraction by this procedure, whereas, in contrast to this finding, only ~25% of ProPTH was extracted by KCl (Fig. 5). Thus, a substantial fraction of the Pre-ProPTH associated with the microsomes appears to be inserted firmly in the membrane bilayer, and an approximately equal amount appears to be only superficially bound to the membrane. As expected, most of ProPTH (75%) enclosed predominantly within the microsomal vesicles is not liberated by exposure to high concentrations of KCl.

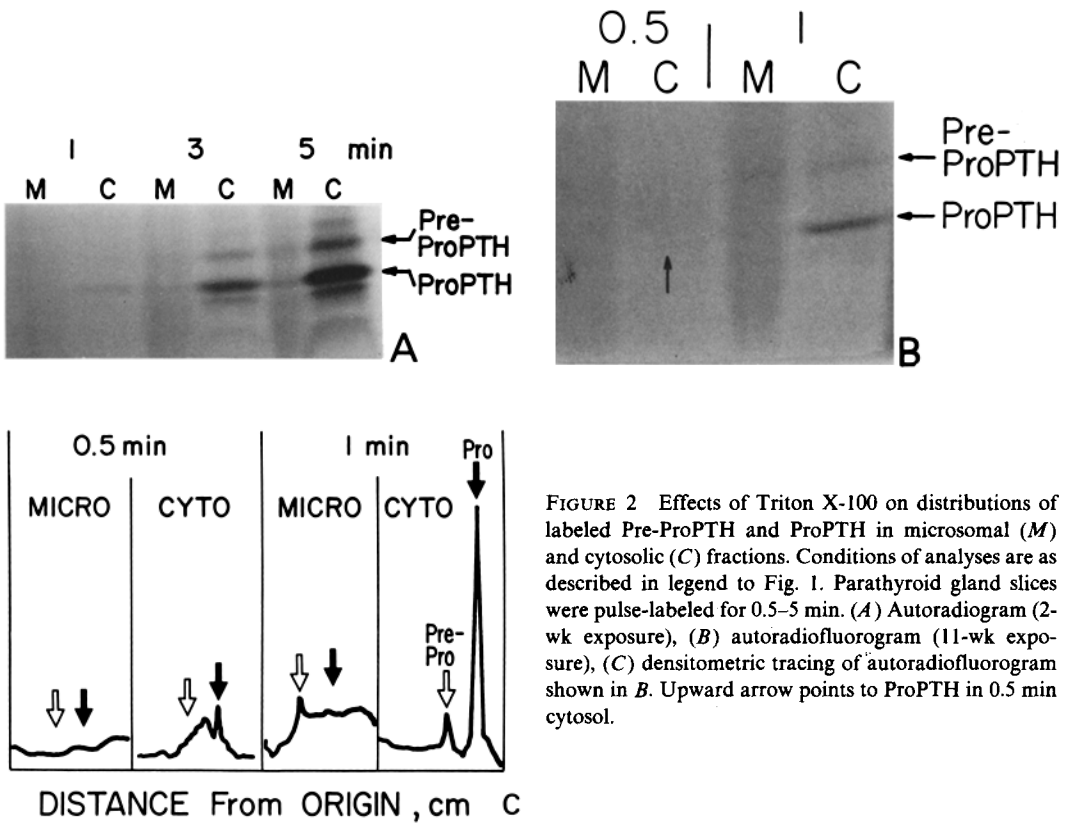

FIGURE 2 Effects of Triton X-100 on distributions of labeled Pre-ProPTH and ProPTH in microsomal (*M*) and cytosolic (*C*) fractions. Conditions of analyses are as described in legend to Fig. 1. Parathyroid gland slices were pulse-labeled for 0.5–5 min. (*A*) Autoradiogram (2-wk exposure), (*B*) autoradiofluorogram (11-wk exposure), (*C*) densitometric tracing of autoradiofluorogram shown in *B*. Upward arrow points to ProPTH in 0.5 min cytosol.

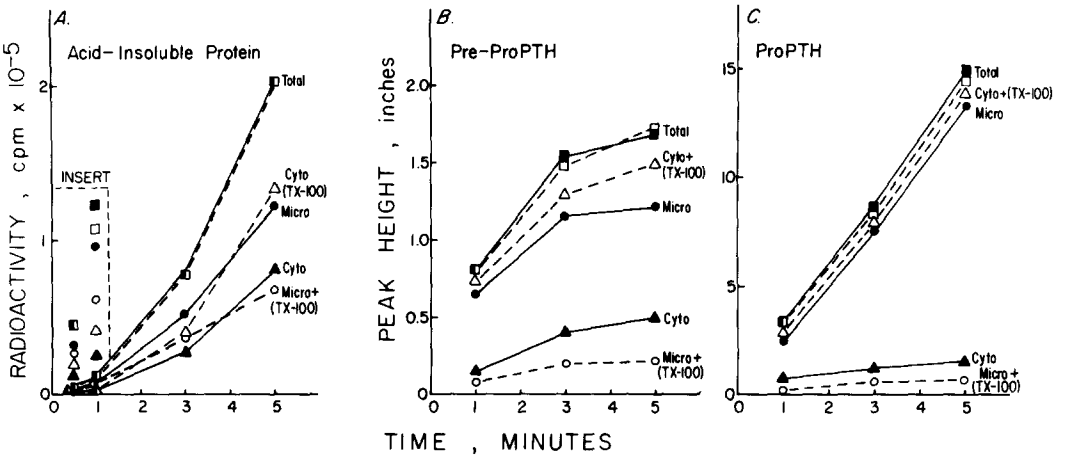

FIGURE 3 Kinetic analyses of the appearance of (*A*) labeled total of acid-insoluble proteins (*B*) of Pre-ProPTH, and (*C*) of ProPTH in microsomal and cytosolic fractions prepared from parathyroid gland slices pulse-labeled for 0.5–5 min with [^{35}S]methionine. Data were obtained by semi-quantitative densitometric scanning of SDS acrylamide gels, representatives of which are shown in Figs. 1 and 2. Insert on left panel *A* shows 0.5- and 1-min values on scales of ×10. Open symbols denote fractions after treatment of microsomes with Triton X-100 (*TX-100*). *Micro*, microsomal fraction; *Cyto*, cytosolic fraction.

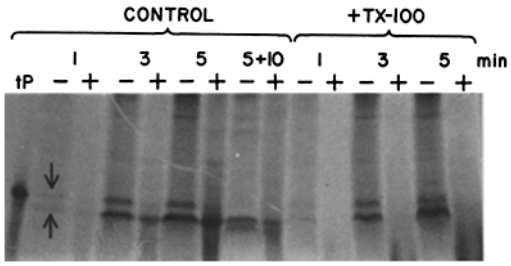

FIGURE 4 Evidence for rapid sequestration of newly synthesized ProPTH into cisterna of RER. Microsomes were prepared and parathyroid gland slices that were pulse-labeled for 1–5 min followed by a chase-incubation of 10 min (5 + 10). Microsomes were subjected to limited proteolysis with a mixture of trypsin and chymotrypsin before and after treatment of the microsomes with 0.5% Triton X-100 (*TX-100*). Labeled proteins were analyzed by electrophoresis on a 10–20% gradient acrylamide gel containing SDS. An autoradiogram is shown of the gel slab: (−) No enzymes added; (+) enzymes added. Downward arrow, Pre-ProPTH; upward arrow, ProPTH. *tP*, RNA translation products in wheat germ cell-free system.

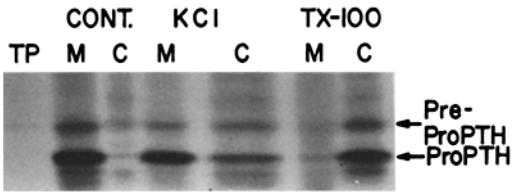

FIGURE 5 Effects of 1.0 M KCl and Triton X-100 on the distributions of Pre-ProPTH and ProPTH in microsomal (*M*) and cytosolic (*C*) fractions prepared as described in legends to Figs. 1 and 2 and in the text. Gland slices were incubated with [^{35}S]methionine for 5 min.

DISCUSSION

Results of studies from a number of different laboratories involved in investigations of the biosynthesis of secretory proteins have provided evidence in support of the signal hypothesis originally proposed by Blobel and Dobberstein (1). Inasmuch as this hypothesis arose as a result of studies carried out in reconstituted cell-free systems utilizing cellular components of diverse origins (1), it seemed necessary to us to attempt to test the hypothesis using an intact cell system.

In these pulse-labeling experiments in intact parathyroid gland slices, newly synthesized Pre-ProPTH and ProPTH were clearly demonstrated during the earliest 30-s period in which both these labeled proteins could be detected (between 30 and 60 s). The labeled ProPTH thus formed was found almost exclusively in association with the microsomal fraction of the parathyroid cell. Furthermore, rapid sequestration of the prohormone within the interior of microsomal vesicles at this early time of synthesis was evidenced when the prohormone was shown to be largely resistant to limited proteolysis or to extraction by 1.0 M KCl, yet was released readily after disruption of the microsomes by treatment with Triton X-100.

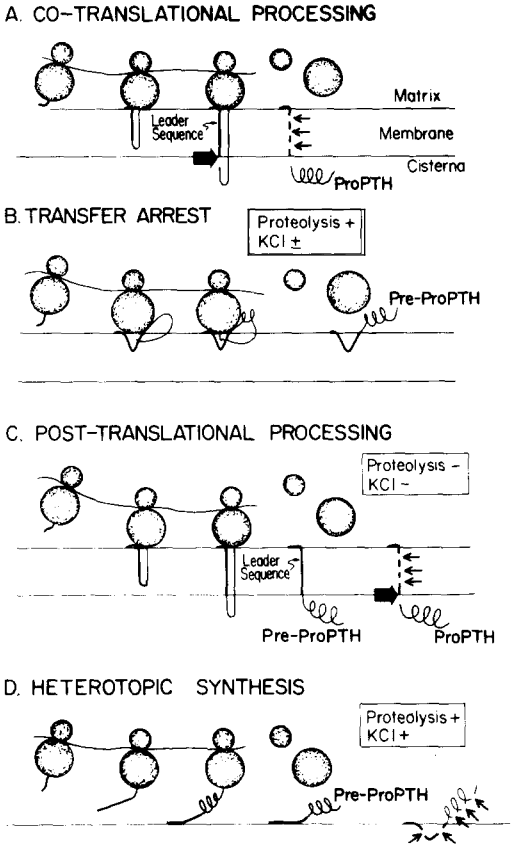

FIGURE 6 Schematic diagram of proposed events in the early formation of ProPTH. (*A*) Co-translational processing via enzymic removal of the NH_2-terminal leader sequence during growth of the nascent chains is the mechanism by which most of the prohormone is formed. (*B*) A small fraction of the chains undergo a transfer arrest and fail to translocate across the membrane bilayer. (*C*) Post-translational processing. (*D*) Heterotopic synthesis. The findings that the Pre-ProPTH is susceptible to limited proteolysis and partially extracted from the microsomes with 1.0 M KCl are most consistent with the mechanism shown in *B*. Small arrows denote hydrolysis of leader (or signal) sequence (10) and of unprocessed Pre-ProPTH.

During the 30-s interval (between 30 and 60 s) of protein labeling, the amounts of labeled ProPTH were at least four times greater than the amounts of labeled Pre-ProPTH when appropriate corrections for actual relative amounts of these two polypeptides were made, based on the known contents of methionine in the two proteins, seven and two residues in Pre-ProPTH and ProPTH, respectively (9).

Furthermore, a trace amount of labeled ProPTH but not Pre-ProPTH was detected at 30 s of labeling but only in the cytosolic fraction after treatment of the microsomes with Triton X-100. Thus, at this earliest time of labeling, ProPTH is formed before Pre-ProPTH appears. These observations strongly indicate that the formation of ProPTH via the cleavage of the NH_2-terminal leader sequence from the initial translation product, Pre-ProPTH, must occur, to a large extent, during growth of the nascent polypeptide chain (co-translational processing) (Fig. 6 *A*) rather than after completion of the polypeptide chain (post-translational processing) (Fig. 6 *C*). Several lines of evidence lend support to this contention. The polypeptide chain of Pre-ProPTH is 115 amino acids in length (9). From the NH_2-terminus, methionines occur at positions 1, 2, 7, 11, 14, 39, and 49 (9). Therefore, for a nascent chain to incorporate labeled methionine, it can be no longer than 48 amino acids, and, to appear as an intact labeled chain of ProPTH identifiable by electrophoretic analyses, it must undergo polymerization for 67 additional amino acids. For this to occur in a period of 30 s, a rate of chain elongation of at least 135 amino acids per minute is required, which is consistent with rates reported in eukaryotes (11, 12). Inasmuch as all chains are synthesized initially with the NH_2-terminal leader sequence of Pre-ProPTH, the extraordinarily rapid formation of such large amounts of the product, ProPTH, relative to the precursor, Pre-ProPTH, makes it likely that to a large extent the processing events take place during rather than after the unidirectional transfer of the nascent chain across the lipid bilayer of the RER.

These observations and conclusions derived from our studies of PTH biosynthesis are in agreement with those proposed by other workers who have reported evidence for co-translational processing of presecretory proteins in systems other than the parathyroid gland. Of particular note in this regard are the studies of Dobberstein and Blobel (1) who, in a series of "read-out" experiments involving translations of immunoglobulin mRNA under cell-free conditions utilizing microsomal membranes, demonstrated removal of the leader sequence before completion of the growth of the polypeptide chain. Similarly, Spielman and Bancroft (16) and Boime et al. (2) observed co-translational processing in cell-free systems supplemented with membranes and primed with mRNAs coding for growth hormone and placental lactogen. In addition to our studies using intact cells, Patzelt et al. (14) studied proinsulin synthesis in intact pancreatic islets, with results qualitatively similar to ours. They observed a rapid accumulation of ^{3}H-labeled rat proinsulin, greater than the amounts of preproinsulin accumulated during pulse-labeling periods of from 1 to 20 min.

The evidence favoring co-translational processing of Pre-ProPTH leaves unanswered the origin of the small quantities of Pre-ProPTH detected in these and our earlier (3, 7, 8) pulse-labeling studies. An apparently reasonable explanation for this phenomenon is that the Pre-ProPTH detected represents a small fraction of the polypeptide chains that escape processing during their growth. Such an occurrence could result from a small but finite degree of inefficiency of the responsible transport and processing enzymic systems believed to exist within the membrane (17, 18), resulting in an arrest of the transfer of the newly synthesized chains across the membrane (Fig. 6 *B*). It is difficult to envision a model whereby actual transfer of the completed Pre-ProPTH chain takes place across the membrane into the cisternal space where enzymic processing then takes place post-translationally or even fails to occur (Fig. 6 *C*) because, unlike our findings for ProPTH, we found no evidence that the pre-prohormone was resistant to limited proteolysis. Therefore, by this criterion Pre-ProPTH could not be sequestered within microsomes.

Another possible, but in our view less likely, explanation for the apparent survival of unprocessed Pre-ProPTH chains is that these particular chains are synthesized on polyribosomes that fail to attach to the membrane and, as a consequence, are released into the cell matrix (heterotopic synthesis) (Fig. 6 *D*) (14). This explanation, however, must take into account the observation that the Pre-ProPTH was found in the subcellular fractions in association with the microsomes and not in the cell sol. It must be considered, however, that, if released into the cell matrix, the Pre-ProPTH might adsorb nonspecifically to the microsomes.

We showed previously that Pre-ProPTH, and to a lesser extent ProPTH and PTH, will adsorb nonspecifically to subcellular particulate fractions containing membranes (7). Such nonspecific adsorption leaves these proteins susceptible to proteolysis when the particulate is treated with proteolytic enzymes (7).

The results of our experiments using 1.0 M KCl, a concentration of KCl sufficient to extract many peripheral proteins from membranes, i.e., proteins adsorbed to the surface of membranes (17), led to the finding that 50% or greater of the Pre-ProPTH chains remained with the microsomes. These results suggest that at least 50% of the Pre-ProPTH chains are inserted integrally into the lipid bilayer of the membrane and favor the first explanation given above that at least a considerable portion of the Pre-ProPTH observed in our studies represents chains that have arrested in transit across the membrane because of a failure of the appropriate translocation process (Fig. 6 *B*) and/or enzymic cleavage (Fig. 6 *C*). The remaining 50% of the Pre-ProPTH chains as well as the 25% fraction of ProPTH, solubilized by treatment of the microsomes with 1.0 M KCl, can be envisioned to represent chains that are attached only peripherally to the microsomal membrane because of either incomplete insertion of the leader sequence of the Pre-ProPTH into the membrane or nonspecific adsorption (7) of chains synthesized heterotopically in the cell matrix to the membrane during the disruption of the tissue (Fig. 6 *D*) or, perhaps, both circumstances. It is possible, of course, that all of the Pre-ProPTH is adsorbed peripherally onto the membranes and that the KCl treatment failed to release all of the chains. The fact that all of the Pre-ProPTH, independent of extractability from microsomes with KCl, was susceptible to proteolytic digestion suggests that any "transfer-arrested" chains, as well as any chains adsorbed peripherally from the cytosol onto microsomes, must have considerable portions of the chain exposed to the aqueous environment and available for proteolysis. Clearly, much additional study will be required to provide a more accurate and detailed model of the processes involved in the nature of the recognition and transport elements in the membrane, the forces involved in the discharge of polypeptide chains across the membrane, and the nature of the enzymic recognition and cleavage of the leader peptide from Pre-ProPTH.

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