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Early events in the biosynthesis and subcellular consignment of secretory and membrane proteins

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy at The Rockefeller University

by

Vishwanath R. Lingappa

March 1, 1979

The Rockefeller University
New York, New York

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Abstract

The biosynthetic mechanisms responsible for the segregation of secreted proteins and for the insertion of integral membrane proteins have been examined in light of the signal hypothesis. Model systems for the study of the biosynthesis of these different classes of proteins, as well as for the study of specific events common to both, have been developed. Their common as well as distinctive features have been probed by a variety of different experimental approaches involving cell free protein synthesizing systems supplemented with heterologous microsomal membranes either during or after translation:

a) proteolytic enzymes, SDS-PAGE and autoradiography have been used to characterize various biosynthetic forms of bovine pituitary growth hormone and prolactin, vesicular stomatitis virus glycoprotein, rat mammary α-lactalbumin and chicken oviduct ovalbumin.

b) amino terminal sequence analysis has been performed on various forms of these proteins synthesized in cell free systems. The sequence data presented demonstrate that nascent precursors to both secretory and membrane proteins contain "signal peptides" at the NH_2 terminus; which display common structural features.

In most--but not all--cases of both classes of proteins the signal sequence is proteolytically cleaved (correctly) during the process of insertion into microsomal membranes prior to chain completion. The transmembrane orientation of the inserted cleaved and glycosylated membrane protein is identical to that of the authentic glycoprotein from virions, while the secreted proteins are completely segregated within the vesicular spaces of the heterologous microsomal membranes.

c) Nascent chain competition experiments were performed in order to functionally characterize and contrast these various mechanisms. The data suggest that an initially common pathway is involved in the biogenesis of secretory proteins (both with and without cleaved signals) and at least one class of integral membrane proteins.

This thesis is dedicated to the two scientists who have had the greatest influence on my intellectual and personal development. They have been my greatest source of strength in the past and are my greatest source of inspiration for the future: my parents.

Acknowledgements

The studies described in Chapter II were carried out in collaboration with Anne Devillers-Thiery; in Chapter III with Flora N. Katz and James E. Rothman from the laboratory of Harvey F. Lodish; in Chapter IV and V with Jaisri R. Lingappa. I am indebted to Drs. Kurt E. Ebner and Rajani Prasad of the University of Kansas Medical School for the rat α-lactalbumin and α-lactalbumin antiserum used in Chapter IV; to Dennis Shields of Albert Einstein Medical College and Savio L. C. Woo of Baylor School of Medicine for the globin and purified ovalbumin mRNAs, respectively, used in Chapter V. I would like to thank Helen DesRuisseaux for help with the Beckman Sequencer and Marion Boudreau for providing the latter two thirds of the approximately 750 SDS gels used in the course of my thesis projects.

Finally, I would like to thank Günter Blobel, my thesis advisor, in whose laboratory and under whose guidance the studies described here in were carried out.

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I Historical Introduction

RER and the Secretory Pathway

Morphologically the rough endoplasmic reticulum (RER) is seen to be a cavitary intracellular membrane system in continuity with both the outer nuclear membrane as well as the smooth endoplasmic reticulum (SER) and is distinguished from the latter by the presence of bound ribosomes and polysomes. These "small ribonucleoprotein particulate components of the cytoplasm" (1) later called ribosomes, (2) were first discovered and demonstrated to have an association with the endoplasmic reticulum in 1955 by Palade (1). Such early breakthroughs, part of the massive wave of advances in our understanding of cells made possible by the application of electron microscopy to cell biology, propelled the study of protein biosynthesis into its modern phase.

Coupled to those advances were biochemical studies on cell fractions which supplemented the morphological findings. Through such studies, in particular those by Siekevitz and Palade (3) and later by Jamieson and Palade (4), was born the currently accepted view of protein secretion. As excellent reviews on the subject are available (5, 6) these will be only briefly summarized here.

The presence of ribosomes bound to membranes was correlated with the synthesis of proteins for secretion, in particular in tissues such as pancreas and liver (5). Free polysomes, however, were associated primarily with the synthesis of proteins destined to remain in the cytoplasm (7). Secretory proteins, synthesized

on bound ribosomes, were demonstrated to appear first in the lumen of the endoplasmic reticulum. They were found to be transported by a process of pinching off and refusion of surrounding membrane, through a series of compartments, namely from RER, via transitional elements to the cis face of the Golgi apparatus; from the trans face of the Golgi apparatus via condensing vacuoles (later to become secretion granules) to the plasma membrane. Fusion of secretion granules to the plasma membrane resulted in deposition of the secreted product outside of the cell--the topological equivalent of the ER cisternal space (6).

This process proceeded against a concentration gradient (e.g. secreted products did not equilibrate with the cytoplasm, but instead, maintained their location in the lumenal space or its topological equivalent). Secretion could also be dissociated from protein synthesis. Subsequent to arrest of protein synthesis products already synthesized and deposited in the RER were seen to "chase out" into the later stages of the secretory pathway (8). The process was seen to be provided with metabolic "locks," that is, steps which required the expenditure of energy and which would not proceed if energy production were blocked (9). These energy dependent steps were: a) protein synthesis per se; b) transfer from RER to Golgi; and c) fusion of granules to the plasma membrane.

Viewed simplistically the biosynthesis of secretory proteins poses a paradox. On the one hand, segregation of proteins into the ER cisternae requires transfer of large hydrophilic domains across

the hydrophobic lipid bilayer. On the other hand, this same membrane serves as a barrier to the reequilibration of secretory proteins into the cytoplasm--and indeed the secretory pathway as a directional process could not otherwise be maintained.

One solution would be to propose a specialized mechanism, perhaps obligatorily coupled to biosynthesis, by which these physical chemical "barrier" features of the lipid bilayer are overcome. Before considering the nature of this solution in depth, let us contrast this problem with a related one, seen in the assembly of biological membranes.

The asymmetry of biological membranes

One characteristic feature of living cells is the segregation of various vital activities within distinct subcellular compartments. This is true for procaryotes as well as eukaryotes but has attained such a remarkable degree of diversification in the latter so as to constitute the defining feature of eukaryotic organization.

The structural basis for this subcellular "rationalization of labor" are the biological membranes and the asymmetric distribution of enzymes, soluble and membrane bound, both with respect to the plane of lipid bilayer, as well as from one membrane system to another. How cells establish and maintain this asymmetry has been one of the most fascinating riddles of modern cell biology (10). At the present time, one can distinguish at least 10 distinct membrane systems in animal cells, namely the inner and outer membranes

of the nuclear envelope, RER, SER, Golgi, lysosomal and peroxisomal membrane, secretory granule membrane, mitochondrial inner and outer membranes and plasma membrane.

In addition, plant cells contain 3 distinct membranes of the chloroplast (outer membrane, inner membrane and thylacoid membranes). Each of these membrane systems contains or delimits enzymatic activities not only characteristic of the various organelles but which in fact define the organelles' role in the physiology of the cell. An important task of cell biological research has been, and continues to be, to understand the structure and biogenesis of these various membranes--how are membrane proteins inserted, how are they sorted, and how are they anchored?

Structurally, biological membranes consist of a lipid bilayer with associated protein and carbohydrate components. Some of these proteins, termed integral membrane proteins, interact with hydrophobic regions of the bilayer either by being embedded on one side or actually spanning the bilayer (11). Operationally they are distinguished by the requirement of detergents to remove them from the bilayer. Other membrane proteins are associated with the membrane indirectly, e.g. via association with integral membrane proteins (12) or via non-covalent interaction with the polar heads of the lipid component of the bilayer. These are the so called peripheral membrane proteins (11). Typically they can be removed by agents that affect electrostatic, H-bonding or other interactions without disrupting the lipid bilayer (12).

While the proteins of biological membranes exhibit a greater or lesser mobility in the plane of the bilayer, they are permanently and asymmetrically oriented structures when considered perpendicular to the plane of the bilayer. This asymmetry is a consequence of the fact that all copies of any given species of membrane protein have the identical topological orientation with respect to the bilayer. This asymmetric organization is maintained by the fact that the hydrophobic regions of the lipid bilayer are a total barrier to the diffusion of proteins across the membrane. Many biosynthetic processes besides secretion are vectorial with respect to a membrane (e.g. energy production) and this is a consequence of membrane protein orientation.

Without such an organization the distinction between "inside" and "outside," between organism and environment, would be lost. Thus, membrane asymmetry is not only required for organelle function but also is responsible for the problem of the "external world", with which philosophers have grappled for ages.

The phenomenon of membrane asymmetry seems to pose a variant of the paradox stated earlier for secretory proteins: If the physico-chemical features of the lipid bilayer are responsible for this asymmetry, how are these same forces overcome in the process of biosynthesis, during which polypeptide chain growth originates in the cytoplasm. In both processes, secretion and membrane protein insertion, hydrophilic polypeptide domains must traverse a hydrophobic lipid bilayer.

Early studies on the mechanism of nascent chain translocation

A reasonable proposition in attempting to account for secretory and membrane protein biosynthesis was that specialized mechanisms operated to overcome the normal physico-chemical features of a lipid bilayer. For example, the formation of a specific pore or gate might provide such means.

The demonstration by Redman et al (13), and Redman and Sabatini (14) that nascent chains were vectorially discharged into the RER lumen provided the first insight into the specific solution achieved in the course of evolution: cotranslational transfer via a ribosome membrane junction. The demonstration by Sabatini et al (15) that the ribosome membrane junction involved a specific interaction with the large subunit but not the small subunit provided specific information on the orientation of this junction. Similarly, the work of Blobel and Sabatini demonstrated a proteolysis resistant space within the ribosome able to protect 40 amino acids. (16)

In addition to the problem of how a polypeptide chain could traverse a membrane was the mystery of how only certain polypeptide species were selected to be substrates for this process-- Why was albumin always secreted while, for example, ferritin always retained in the cytosol of the same hepatocyte? Selectivity could reside in interaction with membranes of: a) mRNA directly; b) polypeptide coded by specific sequences in the mRNA; c) classes of ribosomes which recognized sequences of mRNA.

The direct association of mRNA for secretory proteins with the

membrane was first proposed by Redman (7) and later based on experimental findings, by Sabatini (17). More recent work, however, has called the significance of this association into question (18). The proposal that the nascent chain guided nascent secretory proteins to the membrane was first proposed by Blobel and Sabatini (19). The subsequent discovery by Leder et al (20) and Milstein et al (21) that one secretory protein, namely light chain of MOPC myeloma was biosynthesized as a larger precursor suggested that this hypothesis might be correct. Milstein et al further observed that translation of light chain mRNA in an ascites S-30 cell free system resulted in synthesis of the authentic light chain rather than the percursor observed in the reticulocyte lysate. They hypothesized that membranous material present in the ascites S-30 but not the reticulocyte lysate was involved in proteolytic cleavage of the precursor and that this precursor might be involved in the committment to secretion. However, Milstein et al failed to provide evidence for the suggested role of membranes.

The signal hypothesis for secretory proteins

In 1975, Blobel and Dobberstein proposed a detailed hypothesis, termed the signal hypothesis, which was in part based on the earlier proposal by Blobel and Sabatini (19) and which attempted to provide a general and comprehensive molecular explanation of how specific proteins were transferred across the membrane of the endoplasmic reticulum (22).

They proposed (Fig. 1) that the 5' end of all mRNAs coding for secretory proteins contained a set of codons, after the initiation site, which coded for a so called signal sequence. As the signal sequence was translated and subsequently emerged from the large ribosomal subunit of a free ribosome, it recognized a specific receptor in the membranes, catalysing formation of a ribosome membrane junction and tunnel for translocation of the nascent polypeptide chain across the bilayer, into the cisternal space. They proposed that, in most cases, the signal peptide was cleaved from the nascent chain and that upon chain completion and detachment of ribosomal subunits to join the recirculating free pool, the pore or tunnel in the membrane was dissolved by disaggregation of component proteins in the plane of the bilayer. This hypothesis accounted not only for the phenomenon of specific and vectorial translocation of secretory proteins across the ER (during biosynthesis), but also accounted for the observation of precursors (20, 21, 23) to secreted protein in cell-free systems but not in vivo.

The signal hypothesis for membrane proteins

In 1977, Blobel presented an expanded discussion of a notion that was suggested in the signal hypothesis (24). The proposal was made that the hydrophilic domains of integral transmembrane proteins achieve their correct orientation across the bilayer by a modification of the signal hypothesis. He proposed (Fig. 2) that, like secretory proteins, integral membrane proteins are biosynthesized with amino terminal signal sequences that direct the formation of

functional ribosome-membrane junctions for chain translocation. However, in contrast to the case of secretory proteins, chain transfer does not proceed to completion. Rather, translocation of integral membrane proteins is arrested at a point in their polypeptide sequence characteristic for each protein species. To account for this difference he proposed the existence of another "signal" -- termed a "stop transfer" sequence -- which would abrogate transfer, perhaps by dissolving the tunnel, resulting in transmembrane deposition of the polypeptide chain upon completion of protein synthesis.

The studies to be presented in this thesis represent my attempts to study several fundamental aspects of the signal hypothesis. The chapters represent self contained sets of experimental findings which constitute concrete progress towards the larger goals of our laboratory in general and my thesis project in particular. The participation of co-workers and collaborators is indicated in the acknowledgement section.

Fig. 1 Schematic illustration of the signal hypothesis for the translocation of secretory proteins across the RER membrane.

Various stages of the translation of a mRNA for a secretory protein on a membrane-bound polysome are indicated. The polysome contains six ribosomes. The first two ribosomes near the 5' end of mRNA are not yet bound to the membrane. The nascent chain is indicated to grow in a tunnel in the large ribosomal subunit. The signal peptide portion is indicated as a zig-zagged line at the amino terminus of the nascent chain (see second and third ribosome) or cleaved from the nascent chain and located in the membrane (see membrane between ribosome 3 and 4). Ribosome receptor and signal receptor activity are represented arbitrarily by integral membrane proteins.

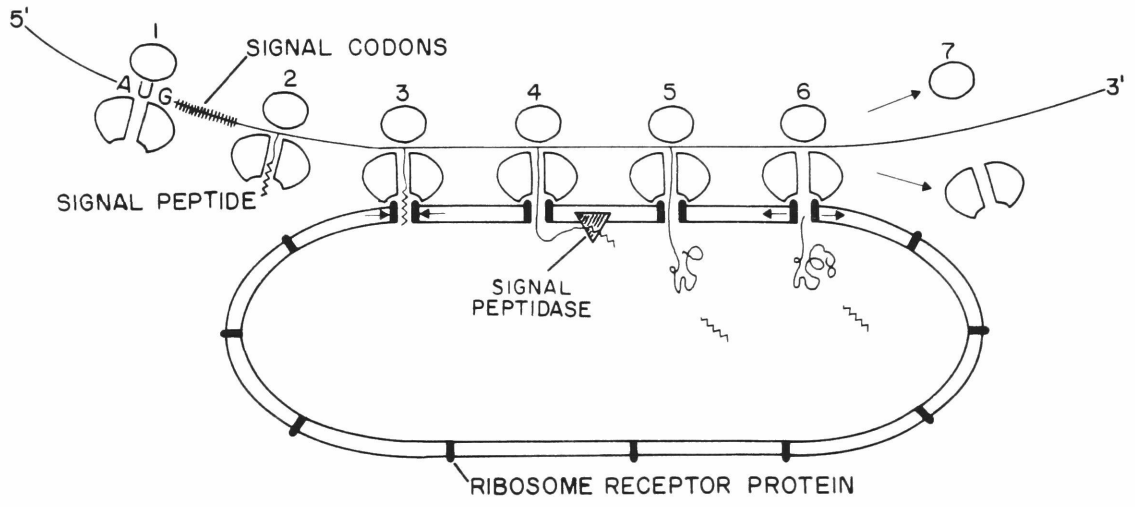
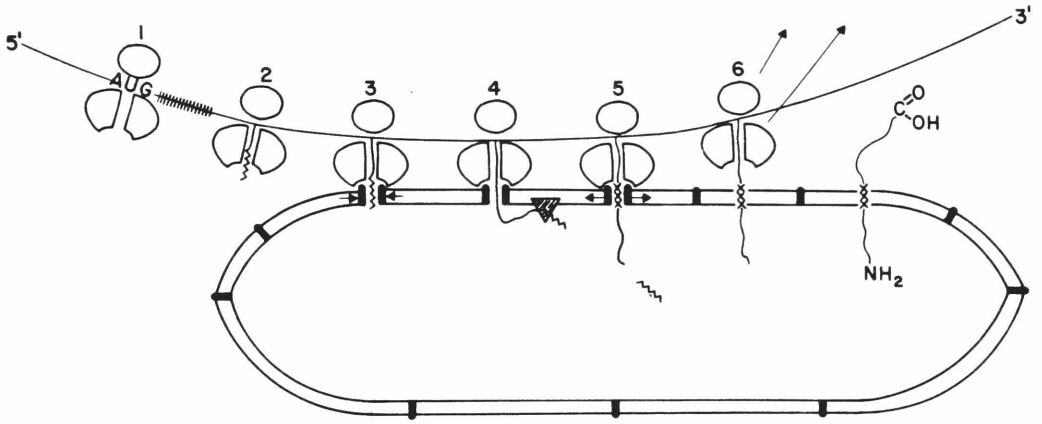


Fig. 2 Schematic illustration for the cotranslational insertion of a bitopic integral membrane protein.

This scheme is taken from ref. 24. The signal and ribosome receptor activities are represented here by a single integral membrane protein. The "stop-transfer" sequence of the nascent chain is indicated by xxx.



II Secretory Proteins:

Nascent prehormones are intermediates in the biosynthesis of authentic bovine pituitary growth hormone and prolactin.

Introduction

At the time that work to be presented in this chapter was initiated, evidence for the signal hypothesis rested on studies from only 2 experimental systems, the MOPC myeloma light chain (21, 22, 25) and the dog exocrine pancreas (26). In order for this hypothesis to be accepted as a model for secretion in general, it would be necessary to investigate a variety of systems and evaluate their similarities and differences. In particular, polypeptide hormones seemed good candidates since polypeptide hormone "precursors" resulting from in vitro translation had been obtained as early as 1974 (23). Yet, the physiological relationship between these precursor and the authentic forms remained a mystery. On the hypothesis that some of these precursors might be involved in early biosynthetic events as described in the signal hypothesis, we attempted to set up cell-free systems to study such endocrine secretory proteins.

Bovine pituitaries were chosen for study for several reasons. Weighing approximately 1 gram each, they were readily available in large quantity and freshly prepared, from local abattoirs. Moreover, having been taken from a physiological setting, i.e., from the animal, comparison to the results from cultured or tumor cell lines which were being studied by other laboratories would be of interest.

Results

RNA extracted from the anterior lobe of bovine pituitary resulted in a 15-20 fold stimulation of amino acid incorporation when added to a wheat germ cell-free system (Fig. 3). Analysis of the translation products by polyacrylamide gel electrophoresis in $NaDodSO_4$, and subsequent autoradiography of the dried gel yielded two major bands with apparent molecular weights of 25,000 and 24,000 respectively (Fig. 4, lane 3). A corresponding preponderance of two polypeptides with apparent molecular weights of 22,000 and 20,000 was observed when the entire homogenate of anterior pituitary was analyzed (Fig. 4, lane 2). The latter two bands were tentatively identified as prolactin (22,000 dalton band) and growth hormone (20,000 dalton band) on the basis of co-migration with commercially obtained hormones (data not shown). On the assumption that the abundance of proteins in the homogenate might reflect the abundance of translatable mRNAs, we tentatively identified the two major translation products of bovine anterior pituitary mRNA (Fig. 4, lane 3) as larger forms of prolactin (25,000 dalton band) and of growth hormone (24,000 dalton band). This assignment was supported by immunoprecipitation of the translation products with an antibody against ovine prolactin: only the 25,000 dalton polypeptide was precipitated (data not shown). These tentative identifications were shown to be correct by the sequence data described below.

From the predictions of the signal hypothesis (22) it could be anticipated that the two larger forms of bovine prolactin and growth hormone contain the sequence of the respective authentic hormones plus

a characteristic amino terminal sequence extension. This assumption was verified (Figs. 5 and 6) by subjecting the radioactively labeled molecules to automated sequential Edman degradations. In the case of the 24,000 dalton band which was synthesized in the presence of (^{35}S) Met and several tritiated amino acids (Leu, Pro, Phe), liquid chromatography was used ([27]) after each Edman degradation cycle in order to identify the PTH derivative of the labeled amino acids. The discrete peaks of radioactivity (Fig. 5) enabled us to identify leucine as the amino acid at residues 10, 11, 12, 16, 17, 19 and 33; Pro at residues 6, 20, and 29; Phe at residues 14 and 28; and Met at residues 1, 2 and 31. The identification of Phe_{28}, Pro_{29}, Met_{31}, and Leu_{33} permitted alignment (Fig.[12]) with the amino terminal sequence of authentic bovine growth hormone and thus provided strong evidence that the 24,000 dalton band is pregrowth hormone in which the amino terminus of authentic growth hormone is preceded by 26 or 27 (see below) amino acid residues.

In the case of the 25,000 dalton band which was labeled with (^{35}S) Met and only one tritiated amino acid (Pro), double labeling counting procedures after each Edman degradation cycle were sufficient to identify Met and Pro. From the peaks of radioactivity (Fig. 6) proline was identified as the amino acid at residues 32, 35, and 38 and methionine at residues 1 and 54. The identification of Pro_{32}, Pro_{35}, Pro_{38}, and Met_{54} permitted alignment with the authentic amino terminal sequence of bovine prolactin (Fig.12). Together with the immunological identification these data provide strong evidence

that the 25,000 dalton band is preprolactin. In this case, the amino terminus of authentic prolactin is preceded by 30 amino acid residues.

It has previously been shown that *in vitro* translation of mRNAs for secretory proteins in the presence of microsomal membranes resulted in reconstitution of functional rough microsomes from heterologous components. Reconstitution was inferred from the fact that most of the translation products were "processed", i.e. were shortened to the size of authentic proteins and "segregated," i.e. were resistant to post-translational proteolysis (25). It can be seen (Fig. 7) that translation of bovine anterior pituitary RNA in the wheat germ cell-free system but in the presence of microsomal membranes from either dog pancreas (lane 3) or bovine pituitary (lane 6) resulted in the appearance of two new, faster moving bands (indicated by vertical arrows) which had an apparent mol. wt. of 22,000 and 20,000 and co-migrated with authentic prolactin and growth hormone. The co-translational presence of increasing amounts of microsomal membranes resulted in an increased synthesis of authentic growth hormone and prolactin, and in a correspondingly decreased synthesis of the prehormone bands (Fig. 7).

These results suggested that translation in the presence of microsomal membranes, heterologous or homologous with respect to the mRNA, resulted in "processing", which was more (lane 3) or less (lane 6) complete. Moreover, the processed chains (lanes 4 and 7) were found to be resistant to subsequent proteolysis by added trypsin and chymotrypsin presumably because they were segregated in

the lumen of the membrane vesicles (lanes 4 and 7). In contrast, the remaining unprocessed chains (lanes 3 and 6, horizontal arrows) were degraded (lanes 4 and 7) because they were presumably not segregated. However, after solubilization of the protecting microsomal membrane by detergent, even the processed chains were sensitive to proteolysis (lane 5). Furthermore, polypeptides present in a high speed supernatant (1 hr. 100,000 x g) of a bovine anterior pituitary homogenate (lane 8) were digested (lane 9) during proteolysis in the absence of detergent. Thus, sensitivity to proteolysis was not due to detergents *per se* nor was protection from proteolysis an intrinsic property of the authentic hormone. Most likely, therefore, it was a result of localization within membrane vesicles. Processing and segregation only occur when the microsomal membranes are present *during* translation; post-translational incubation with microsomal membranes did not result in processing (lane 1), i.e., there were no bands in the position of the authentic hormones; nor does it result in segergation i.e., there was no protection from proteolytic enzymes (lane 2).

In order to investigate the fidelity of processing, we determined the amino terminal sequence (Figs. 9, 10, 11) of the processed chains which resulted from translation of mRNA in the presence of either heterologous or homologous microsomal membranes. The sequence data which were obtained showed that the two processed chains resulted from a *bona fide* cleavage since their partially determined amino terminal sequence was identical to that of the authentic prolactin and growth hormone, respectively (Fig. 12).

Thus, it was found (Fig. 9) that the 22,000 band (Fig. 7, lanes 3 and6), synthesized in the presence of (^{35}S) Met and (^{3}H) Pro,

contained proline at residues 2, 5 and 8 regardless of whether homologous (Fig. 9, section B) or heterologous (Fig. 9, section C) membranes were used for processing. Likewise, when synthesized in the presence of (^{35}S) Met and three tritiated amino acids (Pro, Thr, Asn) but in addition subjected to post-translational proteolysis by trypsin and chymotrypsin (Fig. 7, lane 4), the 22,000 dalton band contained (Fig. 9, section D): proline at residues 2, 5 and 8; threonine at residue 1; asparagine at residue 6; and methionine at residue 24.

In the case of the 20,000 dalton band (Fig. 7, lane 3) synthesized in the presence of (^{35}S) Met and three tritiated amino acids (Phe, Pro, Ala), the distribution of radioactivity (Fig. 10) was not confined to single cycle peaks (as for instance in the case of the 22,000 dalton band, see Fig. 9); instead, each peak was followed by a shoulder in the subsequent cyle. When peak and shoulder radioactivities were expressed as "relative" radioactivities (i.e. cpm divided by specific activity of respective amino acid) and plotted separately (Fig. 11) it became evident that the 20,000 dalton band consisted of two discrete populations of chains, which differed from each other by the presence or absence of an initial Ala. This heterogeneity is similar to that found in authentic bovine growth hormone preparations (28). Thus identification of the processed chains as authentic bovine growth hormone was made on the basis of alignment of Ala_1, $Phe_{1/2}$, $Pro_{2/3}$, $Ala_{3/4}$, $Met_{4/5}$.

Another experimental design previously used (8) to probe the sequence of events suggested in the signal hypothesis is to investigate the nature of the nascent chains associated with ribosome containing cell fractions. To this intent the nascent chains contained in these fractions were completed in vitro in a "readout" system (8). The completed chains could then be detected (using SDS-PAGE and AR) by virtue of the radioactive amino acid residues they acquired during their in vitro completion. It can be seen (Fig. 13) that the readout products of rough microsomes (slot RM) contain two prominent bands co-migrating with authentic growth hormone and prolactin but do not contain bands co-migrating with the unprocessed prehormones. In contrast, detached ribosomes (slot DR) yield both the processed as well as the unprocessed forms of these chains. Our interpretation of these results is that chain completion in rough microsomes simulated topological translation while chains in detached ribosomes (prepared from rough microsomes by detergent solubilization of the microsomal membrane and of signal peptidase) were completed in a non-topological manner. Therefore, in the case of rough microsomes, the signal peptide, still part of those nascent chains which are carried by polysomal ribosomes not yet attached to the membrane and located near the 5' end of mRNA (22), will induce in vitro ribosome attachment to the membrane, its own removal by the membrane associated signal peptidase, and co-translational intravesicular segregation of the remainder of the chain. These processes cannot occur when in the case of detached ribosomes the microsomal membrane was solubilized and

therefore resulted in these chains being completed without co-translational removal of the signal peptide. Since signal peptidase acts co-translationally some nascent chains will have already had their signal peptide removed prior to solubilization of the membrane. Thus, both precursor and processed chains were seen in the detached ribosome translation products. Completion of the chains contained in free ribosomes did not reveal detectable distinct bands in the position of the unprocessed or processed forms of growth hormone and prolactin (Fig. 13, slot FR). This indicated that little if any mRNA for these two hormones was engaged with free ribosomes and that *in vivo* their translation occurs on rough ER.

Finally, the observed resistance to proteolysis of the two processed chains synthesized by rough microsomes (Fig. 14, slot RM+) is due to their being segregated rather than being an intrinsic property of the processed chains. This was shown by detergent solubilization of the protective membranes of rough microsomes, followed by incubation with proteolytic enzymes which caused complete degradation of the processed chains (Fig. 14, slot RM DOC+).

One feature of the pitutary system deserves mention from a purely technical point of view. Depending on the physiological states and source of the animals it is possible to manipulate the relative preponderance of mRNA coding for pregrowth hormone as preprolactin. In Fig. 15, lane 1 is displayed total translation products of pituitary gland in RNA from (castrated) steers. Preprolactin is the single dominant product while pregrowth hormone is

barely identifiable as a discrete band. Lane 3 presents translation products from young non-castrated animals where growth hormone is the predominant species with a greatly reduced preprolactin band visible. Lane 2 represents a commercially obtained "mixed butcher run" containing approximately equal amounts of each product (pre-growth hormone and preprolactin) presumably due to the heterogeneous source of animals and/or physiological state. The experiments presented in this chapter were largely carried out with mRNA whose products resemble lane 2. However, it is clear that selection of castrated steers should provide mRNA that is operationally homogeneous and of particular value as a general assay for early biosynthetic events.

Conclusion

Consistent with earlier studies on rat prolactin (29, 30, 31, 32) and growth hormone (33), the data presented here demonstrate that the translation products of mRNA coding for bovine growth hormone and prolactin are larger molecules containing amino terminal extensions of 26/27 and 30 amino acid residues, respectively (see Fig. 12). More significantly, however, we have been able to define the conditions under which the authentic rather than the larger form of these two hormones can be synthesized and segregated in a cell-free system. This, in fact, amounts to an *in vitro* reconstruction of the initial steps of the secretory pathway (5), compatible with the sequence of events outlined in the signal hypothesis (22).

Particularly remarkable was the fidelity of cleavage, which converted *nascent* preprolactin into *nascent* prolactin. Even more intriguing was the cleavage of *nascent* bovine pregrowth hormone. The authentic hormone is known to occur in two forms which differ by the presence or absence of an initial alanine (28). Thus, the faithful *in vitro* reproduction of this heterogeneity by a heterologous signal peptidase strongly suggests that the cleavage site in nascent pregrowth hormone may be sufficiently ambiguous so as to result in random cleavage either before or after alanine. Fidelity of cleavage has also been observed with nascent fish preproinsulin (34) and with nascent human placental prelactogen (35).

It should be noted that cleavage as well as segregation only operates on *nascent* not on *completed* chains, i.e. it occurs only

when membranes are present during but not after translation (see Fig. 7). This, together with other previously presented evidence (22, 25) implies that it is not the larger completed molecules (synthesized in vitro in the absence of membranes, and often referred to as "precursors"), but rather their uncompleted nascent forms which are the physiological intermediates in the synthesis of authentic secretory proteins.

Fig. 3 Time course of polypeptide synthesis in a wheat germ system in the absence (-Pit) or presence (+Pit) of bovine anterior pituitary mRNA.

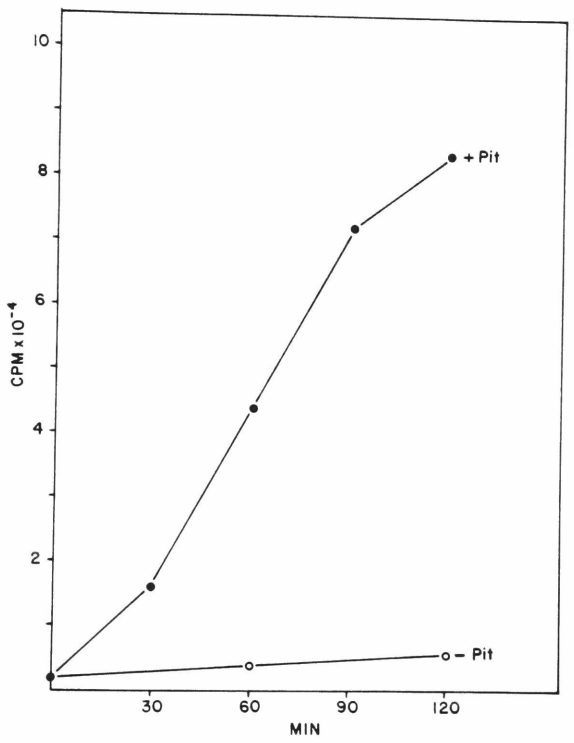
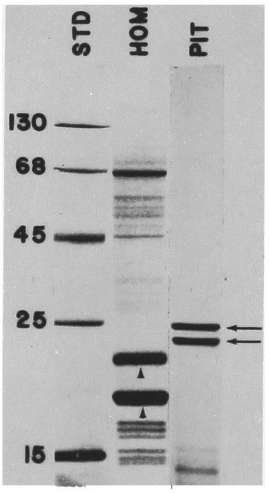


Fig. 4 Characterization of polypeptides synthesized by translation of anterior pituitary RNA in the wheat germ cell-free system.

Analysis was by $NaDodSO_4$/polyacrylamide gel electrophoresis. Bands in lanes 1 and 2 are due to Coomassie brilliant blue stain while those in lanes 3 and 4 are from an autoradiograph. Lane 1: mol. wt. standards: *E. coli* β-galactosidase (130,000), bovine serum albumin (68,000), ovalbumin (45,000), porcine chymotrypsinogen (25,000) and rabbit globin (15,000); lane 2: anterior pituitary homogenate; land 3: translation products of anterior pituitary mRNA;

Arrowheads (lane 3) point to bands with a mobility identical to prolactin (upper) and growth hormone (lower). Horizontal arrows point to preprolactin (upper) and pregrowth hormone (lower).



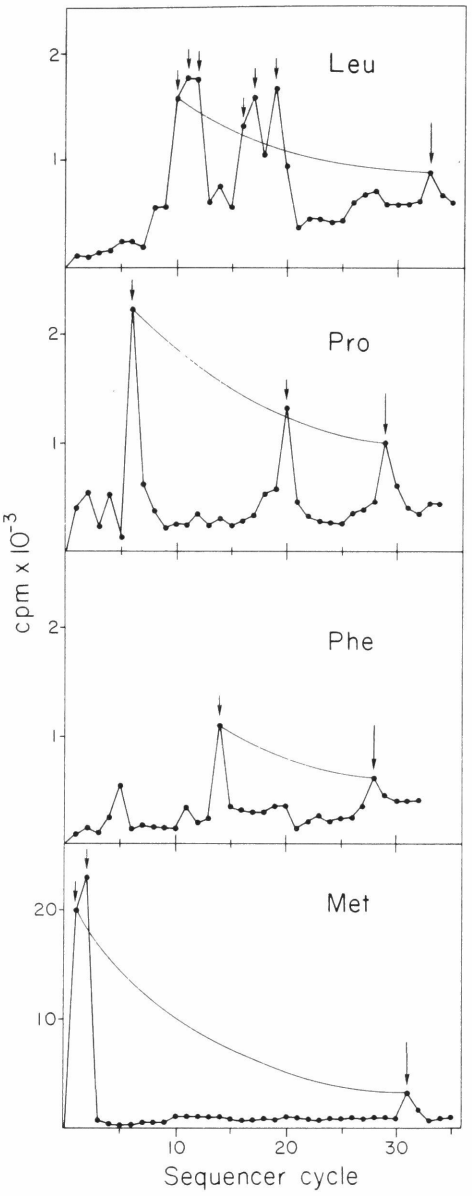

Leu
Pro
Phe
Met
cpm x 10^-3
Sequencer cycle
10
20
30

Fig. 5 Partial amino terminal sequence analysis of pregrowth hormone.

Bovine anterior pituitary RNA was translated in the wheat germ cell-free system containing 100 μc of [^{3}H] Leu, 100 μc of [^{3}H] Pro, 60 μc of [^{3}H] Phe and 400 μc of [^{35}S] Met in 2 ml final volume. The pregrowth hormone band (lower of the horizontal arrows in lane 3 of Fig. 4) was taken through 35 cycles of Edman degradation. The curved line indicates the theoretical yield of radioactivity based on the calculated repetitive yield (20) normalized to the first radioactive peak. Sequence positions in the signal peptide region are indicated by short arrows, those in the authentic region by long arrows.

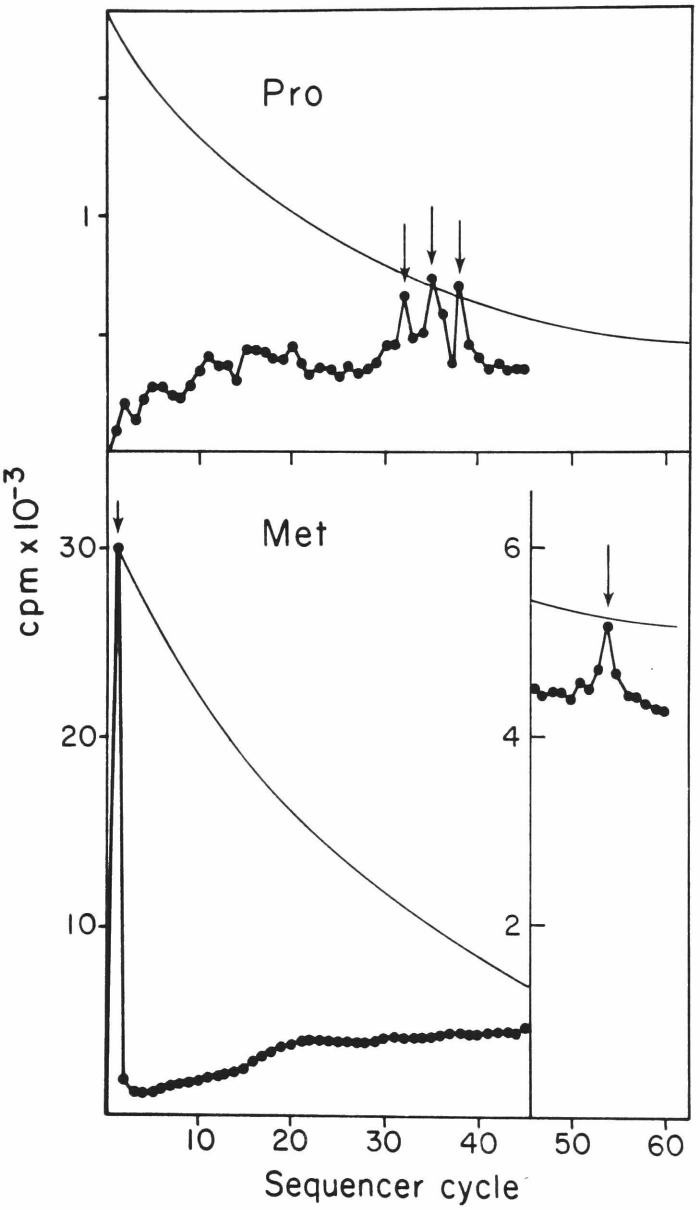

Pro
1
Met
30
20
10
6
4
2
cpm x 10^-3
10
20
30
40
50
60
Sequencer cycle

Fig. 6 Partial amino terminal sequence analysis of preprolactin.

Bovine anterior pituitary RNA was translated in the wheat germ cell-free system containing 100 μc of [^{3}H] Pro and 400 μc of [^{35}S]Met in 2 ml final volume. The preprolactin band (upper of the horizontal arrows in lane 3 of Fig. 4) was taken through 60 cycles of Edman degradation.

Fig. 7 In vitro cleavage and segregation of nascent bovine preprolactin and pregrowth hormone by dog pancreas microsomal membranes. Analysis was by $NaDodSO_4$/polyacrylamide gel electrophoresis and staining only (lanes 8 and 9) or subsequent autoradiography of dried slab gels (lanes 1-7).

Bovine anterior pituitary RNA was translated in the wheat germ cell-free system. Lane 1: post-translational incubation for 90 min at 27° with dog pancreas microsomal membranes (2.5 A_{260} units per ml); lane 2: as in lane 1, but followed by incubation with proteolytic enzymes; lane 3: dog pancreas microsomal membranes (2.5 A_{260} units per ml) were present during translation; lane 4: as in lane 3, but followed by incubation with proteolytic enzymes; lane 5: as in lane 4, except that proteolysis was in the presence of 0.5% sodium deoxycholate; lane 6: bovine pituitary microsomal membranes (5.0 A_{260} units per ml) were present during translation; lane 7: as in lane 6, but followed by incubation with proteolytic enzymes; lane 8: high speed supernate (1 hr, 100,000 x g_{av}) of pituitary homogenate; lane 9: as in lane 8, but incubated with proteolytic enzymes. Incubation with proteolytic enzymes (200 µg each of trypsin and chymotrypsin per ml) was for 60 min at 27°. Horizontal arrows point to their "processed" forms: prolactin (upper) and growth hormone (lower) with a mobility identical to their presumptive counterparts in a pituitary homogenate (lane 8).

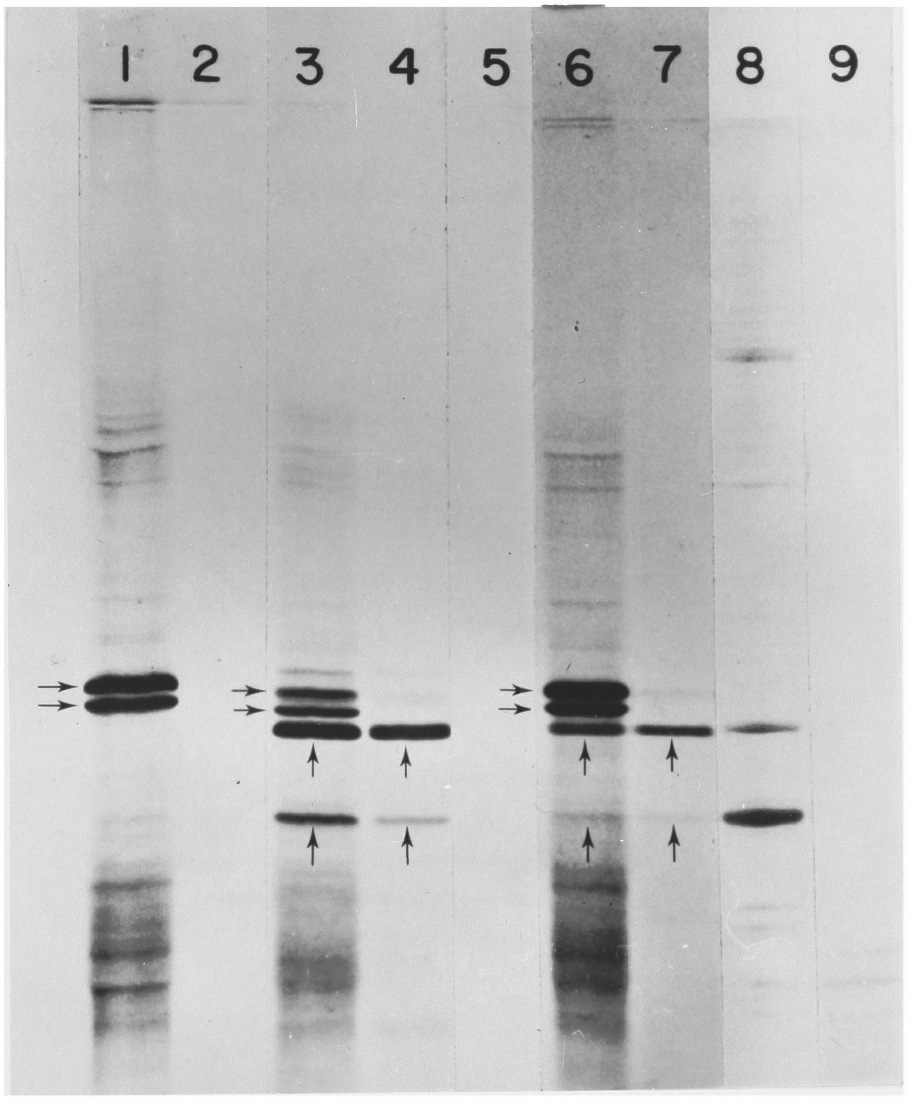
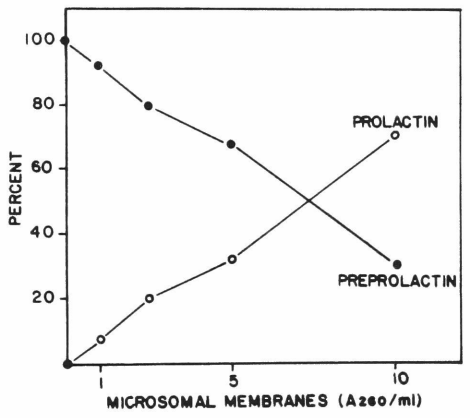

1
2
3
4
5
6
7
8
9

Fig. 8 Effect of membrane concentration on the extent of co-translational processing.

Shown in the distribution of radioactivity between the putative preprolactin (o-o) and prolactin (o-o) band as a function of membrane concentration per ml of cell free system. Data were obtained by densitometric analysis. (For details, see text.) The sum of the radioactivity in preprolactin and prolactin in each of the lanes was defined as 100%.



		1	2	3	4	5	6	7	8	9 ... 23	24	25
A. authentic bovine prolactin		Thr	Pro	Val	Cys	Pro	Asn	Gly	Pro	Gly...Val	Met	Val
B. processed chain: pituitary membranes	[^{3}H] Pro	112	750	169	220	450	187	173	435	151		
C. processed chain: pancreatic membranes	[^{3}H] Pro	80	1,850	360	240	2,450	390	265	1,940	n.d.		
D. processed chain: pancreatic membranes; protected from proteolysis	[^{35}S]Met	63	21	14	16	17	n.d.	16	20	22...33	273	85
	[^{3}H] Pro	24	297	43	48	274	72	37	175	52		
	[^{3}H] Thr	28	15	n.d.	10	14	12	10	14	14		
	[^{3}H] Asn	34	35	n.d.	36	24	96	32	14	17		

Fig. 9 Partial amino terminal sequence analysis of *in vitro* synthesized prolactin and alignment with the amino terminal sequence of authentic bovine prolactin (A).

Bovine anterior pituitary RNA was translated in the wheat germ cell-free system containing in 2 ml final volume: B. 100 μc of [^{3}H] Pro, 400 μc of [^{35}S] Met and 10.0 A_{260} units of microsomal membranes from bovine anterior pituitary; C. as B. except that the microsomal membranes (5.0 A_{260} units) were from dog pancreas; D. 100 μc of [^{3}H] Pro, 50 μc of [^{3}H] Thr, 80 μc of [^{3}H] Asn, 400 μc of [^{35}S] Met and 5.0 A_{260} units of dog pancreas microsomal membranes. In this case translation was followed by incubation with proteolytic enzymes (see Fig. 7). The prolactin band (upper of the vertical arrows in Fig. 7) was taken through 9-25 cycles of Edman degradation and the data shown are cpm, with assigned positions underlined.

		1	2	3	4	5	
A. authentic bovine growth hormone		Phe / Ala	Pro / Phe	Ala / Pro	Met / Ala	Ser / Met	/ Ser
B. processed chain: pancreatic membranes	[^{3}H] Ala:	23	8	32	22	8	10
	[^{3}H] Phe:	110	70	17	0	5	20
	[^{3}H] Pro:	27	173	110	40	36	n.d.
	[^{35}S]Met:	205	60	55	1,440	1,060	160

Fig. 10 Partial amino terminal sequence analysis of *in vitro* synthesized growth hormone and alignment with the amino terminal sequence of the two forms of authentic bovine growth hormone (A), differing in the absence or presence of an initial Ala (17).

Bovine anterior pituitary RNA was translated in a wheat germ cell-free system containing in 2 ml final volume 50 μc of [^{3}H] Ala, 60 μc of [^{3}H] Phe, 100 μc of [^{3}H] Pro and 400 μc of [^{35}S] Met and 5.0 A_{260} units of dog pancreas microsomal membranes. The growth hormone band (lower of the vertical arrows in Fig. 4) was taken through 5 cycles of Edman degradation and the data shown are cpm with assigned positions underlined (see text).

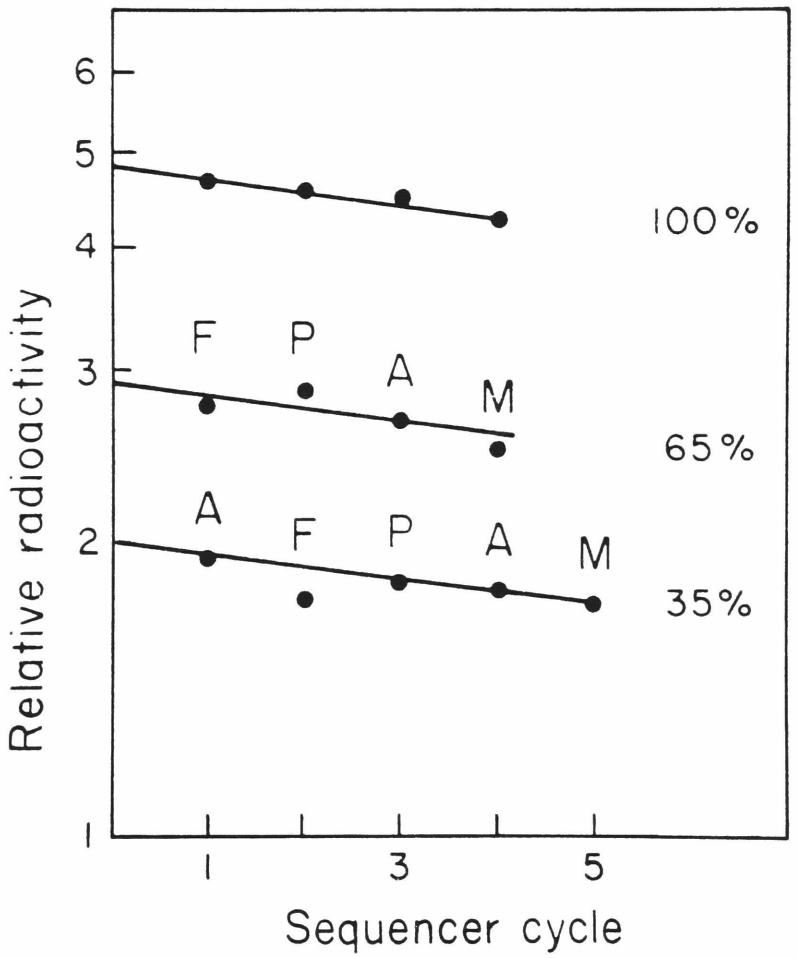

6
5
4
3
2
1
Relative radioactivity
100%
F P A M
65%
A F P A M
35%
1
3
5
Sequencer cycle

Fig. 11 Heterogeneity of *in vitro* synthesized growth hormone with respect to amino terminal residue.

Data shown in Fig. 6 were converted into arbitrary units of relative readoactivity (cpm divided by specific activity of radioactive amino acid). Note logarithmic ordinate. The uppermost line (100%) represents the calculated sum of the relative radioactivity of two amino acid residues released in each cycle of the sequence. The lower lines represent the relative radioactivities of individual residues in each cycle (see text).

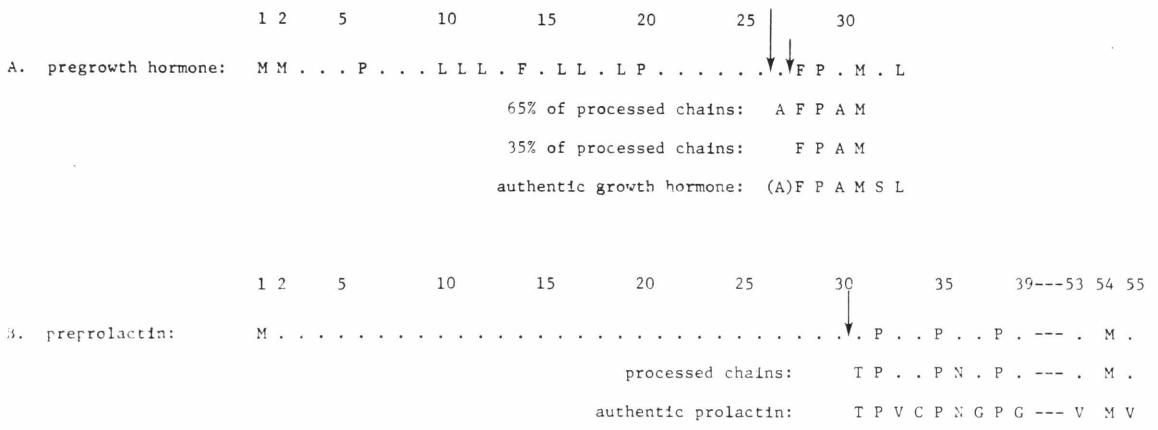

1 2 5 10 15 20 25 30
A. pregrowth hormone: M M . . . P . . . L L L . F . L L . L P . . . . . F P . M . L
65% of processed chains: A F P A M
35% of processed chains: F P A M
authentic growth hormone: (A)F P A M S L
1 2 5 10 15 20 25 30 35 39---53 54 55
B. preprolactin: M . . . . . P . . P . . P . --- . M .
processed chains: T P . . P N . P . --- . M .
authentic prolactin: T P V C P N G P G --- V M V

Fig. 12 Summary of radiosequence data of *in vitro* synthesized (A) pregrowth hormone and growth hormone as well as (B) preprolactin and prolactin (see Figs. 2, 3, 5, 6, 7). Arrows indicate cleavage site for signal peptidase. A=Ala, C=Cys, F=Phe, G=Gly, L=Leu, M=Met, N=Asn, P=Pro, S=Ser, T=Thr, V=Val.

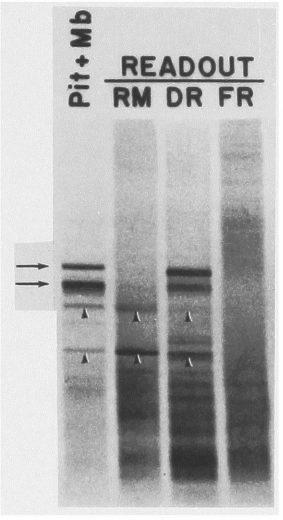

Pit + Mb
READOUT
RM DR FR

Fig. 13 In vitro completion of nascent polypeptide chains present in isolated rough microsomes (lane RM), detached ribosomes (lane DR) and free ribosomes (lane FR) of bovine anterior pituitary. Included for comparison are the translation products of isolated pituitary mRNA in the wheat germ cell free system containing 2.5 A_{260} units of microsomal membranes from canine pancreas (see also Fig. 7, lane 3). Analysis was by SDS-PAGE and AR. Designation by arrows and arrow heads was as in Fig. 7.

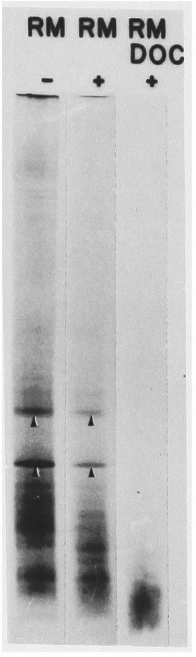

RM
RM
RM
DOC
-
+
+

Fig. 14 Resistance to post-translational proteolysis is dependent on the integrity of the microsomal membrane. Analysis was by SDS-PAGE and AR following post-translational incubation of readout products of rough microsomes in the absence of proteolytic enzymes (lane RM-), in the presence of proteolytic enzymes (lane RM+) or in the presence of sodium deoxycholate and proteolytic enzymes (lane RM DOC+). Designation by arrowheads as in Fig. 7.

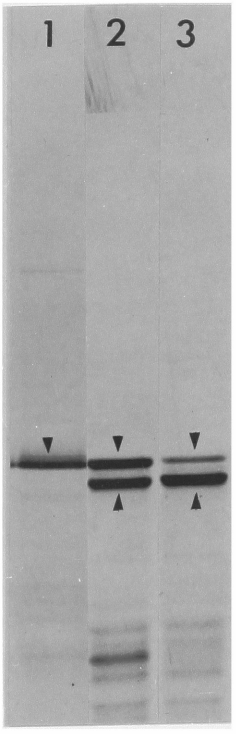

1
2
3

Fig. 15 Relative synthesis of preprolactin and pregrowth hormone varies with physiological state of animals used for mRNA preparation. Analysis by SDS-PAGE and AR. Lane 1, total translation products of mRNA from castrated steers; lane 2, from mixed butcher run; lane 3 from young immature animals.

III Integral membrane proteins:

A signal sequence for the insertion of a transmembrane glycoprotein

Introduction

Studies on the biosynthesis of membrane proteins have lagged behind work on the secreted proteins. The existance of organs devoted to massive secretion of specific polypeptides (e.g. pancreas or liver) has facilitated studies on the latter class of proteins. There is however, one set of cases in which cells become "factories" devoted to the massive synthesis of, among other polypeptides, specific integral membrane proteins.

Enveloped viruses are enclosed by a lipid bilayer derived from the host but including proteins coded by viral genes. When cells are infected with such agents their synthetic capacities are commandeered to make enormous quantities of these viral proteins -- including envelope polypeptides. These are used to assemble virus at the plasma membrane where, upon maturation viral particles bud off enclosed within modified plasma membrane. It seems to be a good assumption that the general mechanism by which such viral coded membrane proteins are synthesized, inserted and transported are common to those processes used in the biogenesis of host plasma membrane proteins. Some of these viral genomes (e.g. VSV) encode a small number of polypeptides all of which are found in the mature virion. It stands to reason therefore that general cellular machinery must be used in the assembly and maturation of these viruses. Evidence suggests (36, 37, 38) that these membrane proteins follow a pathway similar to that of secretory proteins--biosynthesis

on membrane bound ribosomes, initial appearance in RER, migration in vesicles to smooth membranes before appearance on the cell surface. Never are these integral membrane proteins found soluble in the cytoplasm.

In view of forementioned considerations and previous studies (36) which have demonstrated its synthesis on membrane bound ribosomes, the glycoprotein G of vesicular stomatitis virus seemed a good model system for testing the hypothesis (10, 25, 39) that integral membrane proteins are integrated into the lipid bilayer by a modification of the signal hypothesis (Fig. 2). Our approach, in collaboration with Flora N. Katz, James E. Rothman and Harvey F. Lodish, was to construct a cell free protein synthesizing system capable of membrane assembly.

In this chapter, amino terminal sequence analysis of various forms of in vitro synthesized G protein as well as nascent chain competition experiments are presented which allow us to extend the comparison of membrane assembly and protein secretion. We demonstrate that this integral transmembrane glcoprotein like most secretory proteins, is biosynthesized with a transient "signal sequence;" that this signal sequence is proteolytically removed during insertion into microsomal membranes; and that the resulting new amino terminal resides exclusively within the lumen of the membrane vesicles. We also present evidence that nascent G and nascent boving pituitary prolactin compete for membrane sites involved in chain segregation. These results are interpreted to provide further support for a conception of membrane protein biogenesis based on the signal hypothesis (24).

Results

Previously, translation of VSV mRNA in the wheat germ cell-free protein synthesizing system in the absence of a microsomal membrane supplement has yielded as major products the virus coded proteins n, NS, M, and G. Except for the G protein, all of the proteins synthesized *in vitro* co-migrated with their authentic viral counterparts (36, 40). The identical pattern of four major polypeptides was observed upon translation of VSV mRNA in the reticulocyte lysate cell-free system (Fig. 16 lane 3). Moreover, in either cell-free system, translation in the presence or absence of a microsomal membrane fraction *during* translation resulted in the synthesis of two distinct intermediates in the biogenesis of G. Pre G_0 (Fig. 16 lane 3 upward pointing arrow) synthesized in the absence of microsomal membranes is a 60,000 dalton, non-glycosylated, putative precursor of the 67,000 dalton authentic glycoprotein (G_2) isolated from virions. G_1 (Fig. 16, lane 5, downward pointing arrow) synthesized in the presence of membranes has been previously characterized (41) to represent a partially glycosylated form of G which is inserted into the microsomal membrane with an asymmetric, transmembrane orientation identical to that of the pulse labelled intermediate of G observed in rough microsomes of infected cells. When membranes were added after translation, the conversion of preG_0 to G_1 did not take place, i.e. the completed pre G_0 was not a substrate for insertion nor hence for glycosylation, an exclusively lumenal event (10).

In the case of secretory proteins, the information for translation coupled segregation (see chapters 2 and 4, ref. 42) usually resides in an amino terminal "signal sequence" which is proteolytically removed before chain completion (22). In the case of the G protein, initial studies demonstrated a requirement for the amino terminal region of the nascent chain in the process of insertion (43, 44), but were unable to resolve the question of whether proteolytic cleavage of such information also took place. Indeed, it was suggested (45, 46) that the signal sequence of integral membrane proteins might not be removed.

To define the nature and fate of the amino terminal information required for insertion of this membrane protein, we undertook partial amino terminal sequence determination of the various forms of the G protein.

Pre G_o labelled with one tritiated amino acid and either ^{35}(S) Met or ^{35}(S) Cys was identified and purified from total translation products by immunoprecipitation (Fig. 16,lane 2) with a monospecific antiserum prepared against G_2, and by subsequent polyacrylamide gel electrophoresis in sodium dodecyl sulfate and autoradiography. Samples were prepared for sequencing as described in methods and subjected to from 17 to 35 cycles of Edman degradation. The amino terminal sequence was established (Fig.17) to be: Met-Lys-Cys-Leu-Leu-Tyr-Leu-Ala-Phe-Leu-Phe-Ile-His-Val-Asn-Cys-Lys-Phe-X-Ile-Val-Phe-Pro-X-X-X-Lys-X-X-X-Lys-X-. Due to the low specific activity of radioactive amino acids available and hence, low recovery of radioactivity in pre G_o, the assignments of His-Val-Asn in positions 13, 14, and 15 must be considered tentative.

Similarly, G_1 synthesized in the presence of EDTA-stripped and nuclease-digested dog pancreas microsomal membranes and radiolabelled with either (^{3}H) Lys, Phe or Pro and ^{35}(S) Met or Cys, was purified from total translation products by immunoprecipitation (Fig. 16, lane 6) and polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Double label counting of thiazolinones at each cycle of Edman degradation yielded a sequence (Fig. 17) of : Lys-Phe-X-X-X-Phe-Pro-X-X-X-Lys-X-X-X-Lys-X-. Alignment with the sequence of Pre G_0 (Fig. 18) indicated that conversion of nascent pre G_0 and G_1 was accompanied by proteolytic removal of a 16 amino acid long "signal sequence" from the amino terminus of pre G_0, as well as insertion and glycosylation of the growing chain.

In order to determine whether the cleavage of nascent pre G_0 by dog pancreas microsomes was "correct", i.e. whether the authentic amino terminus of G_2 had been generated, partial sequence analysis was performed on G_2 isolated from virions by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The identification of Lys in position 1 and Phe in position 2 (data not shown) consistent with earlier peptide mapping studies (Lodish, H.F., unpublished observations) suggested not only fidelity of cleavage but also, more importantly, that further processing of the amino terminus does not take place during maturation of G and its transport from the rough ER to the plasma membrane.

Correct asymmetric orientation of G_1 synthesized <u>in vitro</u> and inserted during translation into microsomal membranes has been demonstrated by experiments in which microsomal vesicles were

digested with proteases (41). These indicated that the same carboxy terminal tryptic peptides were removed from G_1 synthesized and inserted *in vitro* as from isolated rough microsomes of infected cells. Removal of these tryptic peptides from the exposed region of the carboxy terminus resulted in a partially digested form of G_1, designated G_1', (Fig. 16,lane 4, arrowhead), which is identified here by immunoprecipitation with rabbit anti G_2 serum (Fig. 16,lane 7, arrowhead). When detergent (0.5% Triton X-100) was added to solubilize the membranes, total digestion of G_1 resulted (Fig. 16,lane 8). When pre G_0 was treated with proteolytic enzymes in a similar fashion (Fig. 16,lane 1), no G- related polypeptides (by immunoprecipitation) were found resistant to proteolysis. While indicating fidelity of insertion *in vitro* the previous studies on G_1' (1) provided no information on the location of the amino terminus. In order to resolve this question, and to further characterize the orientation of G across the bilayer, automated Edman degradation of G_1' was performed (Fig. 18). The amino terminal sequence was found to be identical to that of G_1 (Fig. 18, Fig. 19). This strongly suggests that the amino terminal sequence of G lies within the lumen of the microsomal vesicles, and moreover that G spans the bilayer only once, since a multiple transmembrane orientation would most likely have resulted in sequence heterogeneity of the amino terminus after proteolytic digestion.

From the data presented here and previously (43,44) it seems clear that the information which initiates the process of translation-

coupled insertion of the G protein resides in the amino terminal signal sequence, and moreover, that proteolytic removal of this information takes place before chain completion. It seemed possible however, that separate sets of membranous receptors were involved in segregation and insertion, the latter set allowing only partial transfer of proteins across membranes and hence resulting in a transmembrane deposition. To investigate this possibility, competition experiments were designed involving nascent chains of G, or bovine pituitary prolactin (a non glycosylated secretory protein), or rabbit globin (a cytosolic protein lacking a signal sequence). In these experiments the behavior of nascent prolactin in the presence of varying amounts of nascent G protein is evaluated. Except where indicated the amount of membranes was kept constant and subsaturating with respect to segregation activity. Competition was monitored by assaying for processing of prolactin which is an accurate and readily quantifiable indicator of the extent of segregation.

It can be seen that the presence of low (Fig. 20, lane 2) or high (Fig. 20, lane 3) concentrations of VSV mRNA resulted in decreased levels of processed prolactin relative to preprolactin, when compared to translation of prolactin mRNA in the absence of added VSV mRNA (Fig. 20, lane 1). Similar experiments with increasing amounts of globin mRNA had no such effect at either low (Fig. 20, lane 4) or high (Fig. 20, lane 5) globin mRNA concentration. That competition between nascent pre G_0 and nascent preprolactin was most likely for membrane sites and not for soluble factors was

supported by the demonstration (Fig. 20, lane 6) that increasing membrane concentrations abolished the competition (Fig. 20, lane 3). In order to quantitate the extent of inhibition of segregation of nascent prolactin by nascent pre G_0, the radioactivity in preprolactin and in processed prolactin was determined separately and segregation was expressed as a percentage of total radioactivity in completed prolactin chains (processed prolactin/pre-prolactin + processed prolactin). It can be seen in Fig. 21, that increasing the amount of VSV mRNA changed the ratio of processed prolactin to preprolactin from approx 3:1 to 1:1 (i.e. reduced the percentage of processed prolactin chains from 77% to 52%). In contrast, globin mRNA had no effect on the percentage prolactin segregation (Fig. 21). In either case, increasing the concentration of the competing mRNA resulted in an increase in both total protein synthesis (data not shown) and in specific products coded for by the competing species (arrowheads, Fig. 20). For example, at 16 ug/ml of globin mRNA approximately 182,000 cpms of gobin were synthesized while at the same mRNA concentration 122,000 cpms of VSV products were made of which G comprised less than 5 percent. Thus, even a vast excess of nascent globin did not affect the percentage processing of prolactin.

Conclusion

The demonstration (43, 47) that cell-free systems are capable of membrane assembly and that asymmetric integration into the bilayer proceeds only during translation has permitted detailed studies into this fundamental and ubiquitous intracellular process. Kinetic studies (44) have indicated a requirement for the amino terminal region in the insertion process. The structural studies presented in this communication demonstrate conclusively that nascent G protein of VSV contains a hydrophobic sequence of 16 amino acids at the amino terminus which is proteolytically removed during insertion prior to chain completion.

A comparison of the VSV signal with sequence data on signals of secretory proteins reveals several common features including a typical region of leucine-rich hydrophobic amino acids in positions 4 through 12 and the presence at either end of the signal, e.g., at residues 2, 3, 13, 15, 16, of hydrophobic or polar amino acids. Similarly, the presence of a tyrosine in position 6 is consistent with the presence of at least one free hydroxyl containing side group in most signal sequences to date. Apart from these general features, few specific homologies with other signal sequences can be discerned, which is not surprising in view of the fact that secretory signals which have been demonstrated to recognize similar receptors (chapter 5) often have little apparent homology with each other. For this reason, certain distinctive features of the VSV pre G_0 signal may not be a significant indication of functional distinction. For example, the presence of Cys in positions 3 and 16 while unusual,

is not unique, in that preconalbumin has been demonstrated to contain Cys in positions 6 and 17 of its 19 residue-long signal peptide (48) and pre MOPC 41 a and b light chains both have a cysteine, as does pre G_0 at the penultimate site before cleavage (49).

In general, then, the primary structural studies suggest similarities between the structural prerequisites for the early events in protein secretion of those of membrane assembly, two processes which pose conceptually related biosynthetic problems. In both processes, correct topological biogenesis involves exclusively the nascent form of the polypeptide and in both processes the information for transfer usually resides in a cleavable hydrophobic amino terminal sequence. However, that a signal sequence need be neither cleavable nor highly hydrophobic in order to function in the transfer of proteins across membranes in cell-free systems has been demonstrated. It seems possible that hydrophobicity plays a role in the recognition by signal peptidase, a membrane bound enzyme, and that recognition by receptors for transfer across the membrane is mediated by as yet poorly understood conformational features common to all signal sequences.

From our data it appears that nascent G and nascent prolactin recognize at least one common membranous component. The component seems unlikely to be involved in processes of protein synthesis other than transfer across (into) membranes, since nascent globin, translated by the same pool of (initially) free ribosomes as G and prolactin, did not interfere with segregation of prolactin. Rather,

it seems likely that competition was for a receptor on the cytoplasmic face of the membrane which recognizes at least in part, nascent, signal-containing chains and participates in their transfer across (into) the membrane. Although we cannot rule out competition for other components, e.g., signal peptidase on the lumenal aspect of the membranes, in view of previous studies which show that nascent ovalbumin (glycosylated but not processed by signal peptidase) and nascent prolactin (processed by signal peptidase but not glycosylated) compete for common receptors, these explanations seem unlikely. Moreover, no new intermediates of G were seen (e.g. unprocessed but segregated and glycosylated precursors).

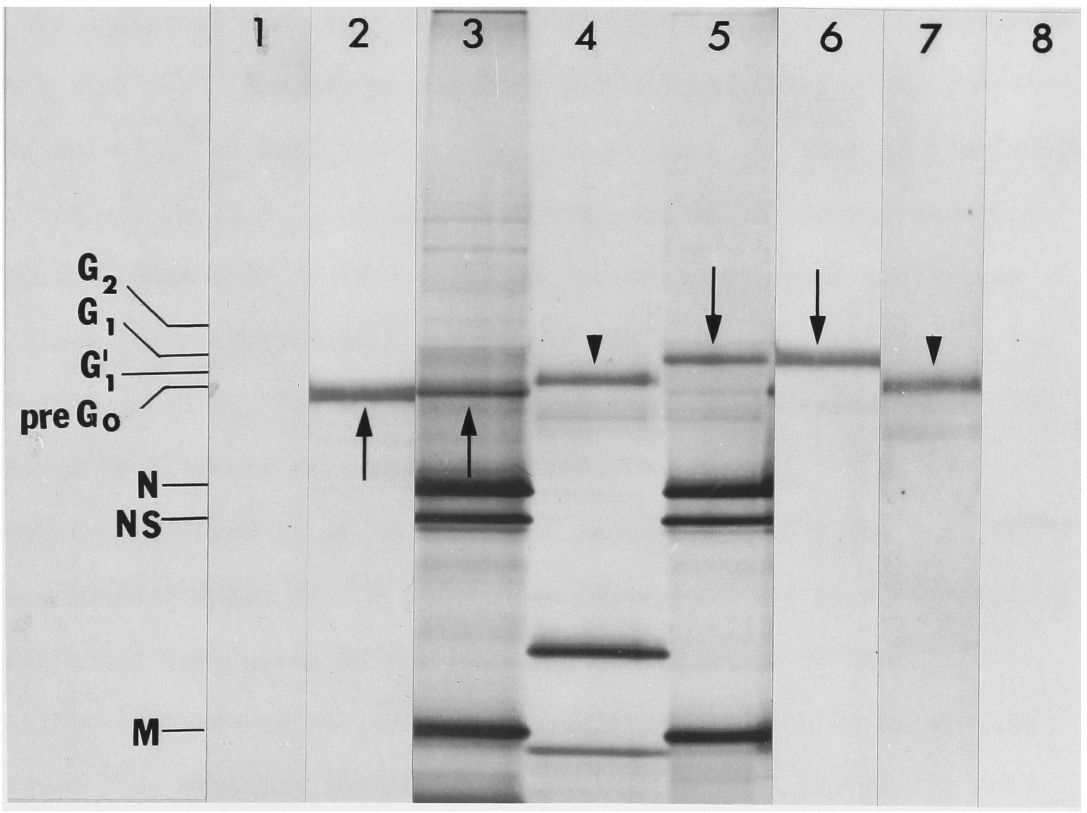

1
2
3
4
5
6
7
8
G2
G1
G'1
pre Go
N
NS
M

Fig. 16 Synthesis, insertion and core-glycosylation of VSV G. VSV mRNA was translated in the staphylococcal nuclease treated rabbit reticulocyte lysate in the absence or presence of EDTA-stripped nuclease-digested dog pancreas microsomal membranes. This was followed by various post-translational assays as specified for each lane, and by preparation of aliquots for polyacrylamide slab gel electrophoresis in sodium dodecyl sulfate Lanes were derived from two different slab gels and aligned according to protein standards on each slab gel. Incubation mixtures for translation (50 µl) containing 12 µCi of [^{35}S] Met, energy generating system and ions as previously specified 0.05 A_{260} units of VSV mRNA and either no microsomal membranes (lanes 1-3) or EDTA-stripped, nuclease-digested microsomes at a final concentration of 4 A_{260}/ml (lanes 4-8). Incubation was for 90 minutes at 25°C. The following post-translational assays were performed on aliquots of translation products. Lane 1: a 25 µl aliquot was adjusted to 10 mM $CaCl_2$ and incubated at 0°C for 1 hr with a final concentration of 250 µg/ml each of trypsin and of chymotrypsin. Digestion was terminated by the addition of 200 units of trasylol. The entire mixture was subjected to immunoprecipitation as previously described with the following modifications. Trichloroactic acid precipitation and resolubilization in 1% sodium dodecyl sulfate was omitted. Instead, the sample was adjusted to the ionic conditions of buffer A, namely, 1% Triton X-100, 10 mM EDTA, 50 mM Tris HCl, pH 8.3, 150 mM NaCl; rabbit anti-serum was raised against authentic G which was purified from virions by polyacrylamide gel electrophoresis in sodium dodecyl sulfate; instead of formalin-fixed Staphylococcus aureus,

Fig. 16 (continued)

protein-A sepharose was used as an affinity adsorbent for isolation of antigen-antibody complexes. Immunoprecipitates were dissociated from sepharose as previously (15). Lane 2: as lane 1, except that the post-translational digestion with trypsin and chymotrypsin was omitted. Lane 3: 10 µl aliquot was treated as in lane 2 except that immunoprecipitation was omitted. Lane 4: 10 µl aliquot was treated as in lane 1, except that immunoprecipitation was omitted. Lane 5: 10 µl aliquot was treated as in lane 3. Lane 6: aliquot was treated as in lane 2. Lane 7: aliquot was treated as in lane 1. Lane 8: as in lane 1, except that Triton X-100 was added to a final concentration of 0.5% before digestion with trypsin and chymotrypsin and subsequent immunoprecipitation. Upward pointing arrows indicate pre G_0; Downward pointing arrows indicate G_1; Downward pointing arrowheads indicate G_1'.

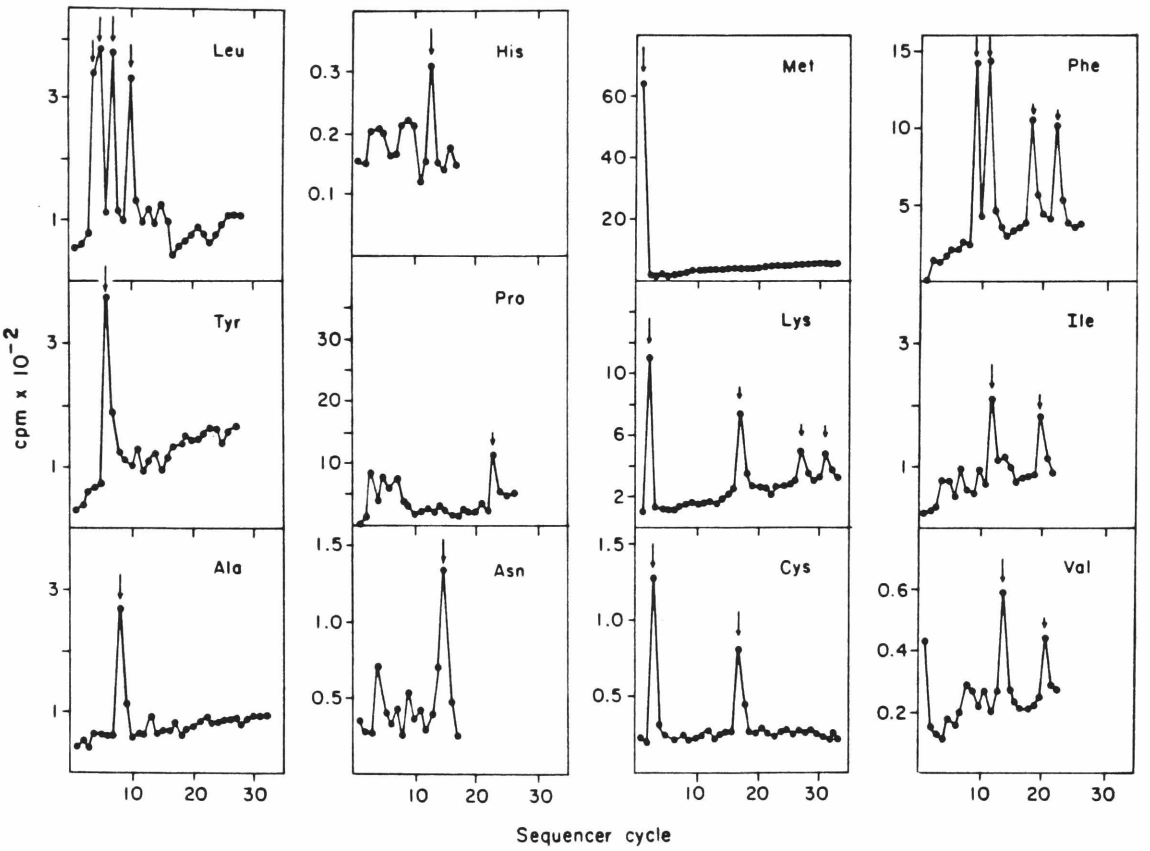

Leu
His
Met
Phe
Tyr
Pro
Lys
Ile
Ala
Asn
Cys
Val
cpm x 10⁻²
Sequencer cycle

Fig. 17 Amino terminal sequence analysis of VSV pre G_0. 1 ml translation reactions containing 250 μCi of a single radioactive amino acid, 250 μCi of [^{35}S] Met or Cys and 1.0 A_{260} μ/ml of VSV mRNA were carried out as previously described. Sample preparation was as described in methods. The pre G_0 was taken through 20-35 cycles of Edman degradation and residues analysed as described in methods. Sequence assignments in the signal region of pre G_0 are indicated by long arrows, those in the authentic region of G_1 by short arrows. Input radioactivity was: [^{35}S] Met, 120,000 cpm; [^{3}H] Lys, [^{3}H] Pro, and [^{3}H] Phe, 40,000 cpm; [^{3}H] Leu, [^{3}H] Ile, [^{3}H] Ala, [^{3}H] Tyr, 10-15,000 cpm; [^{3}H] Val, [^{3}H] Asn, [^{35}S] Cys, and [^{3}H] His, 5,000 cpm. Repetitive yields of 96% were routinely obtained.

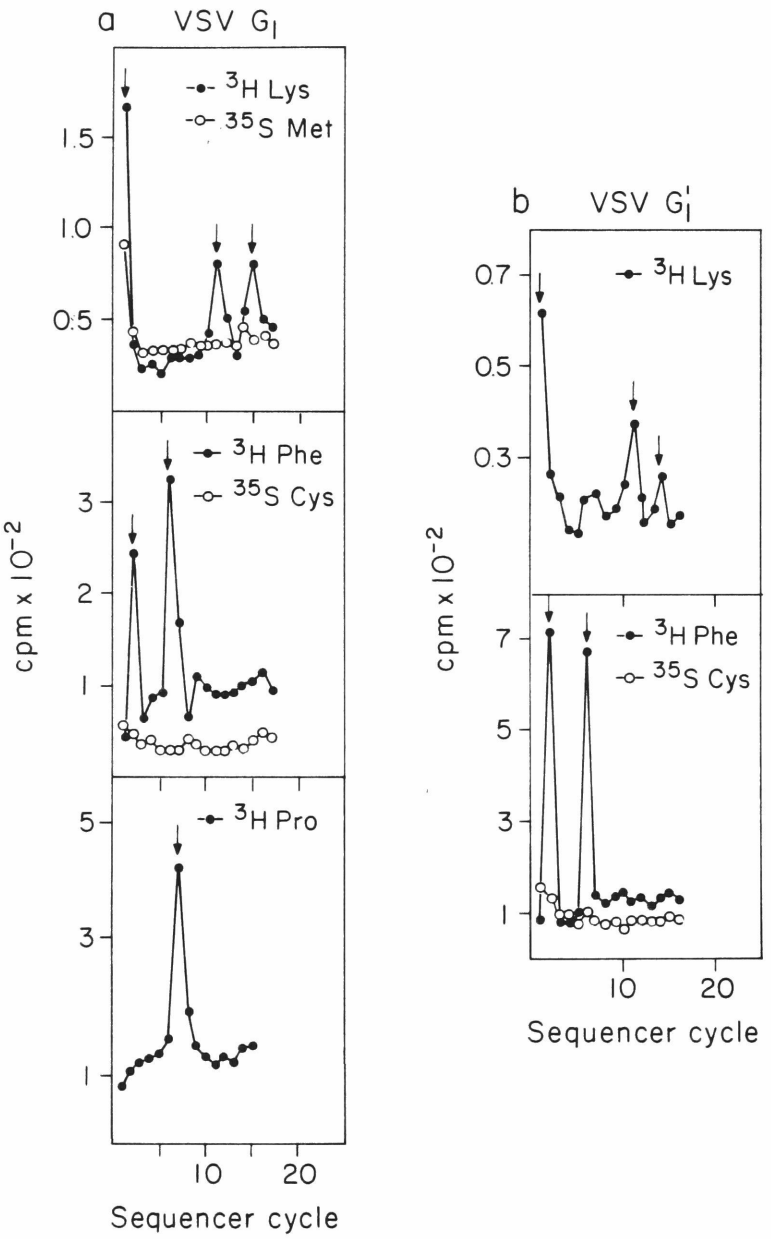

a VSV G_1
^{3}H Lys
^{35}S Met
^{3}H Phe
^{35}S Cys
^{3}H Pro
cpm x 10^{-2}
Sequencer cycle
b VSV G_1'
^{3}H Lys
^{3}H Phe
^{35}S Cys
cpm x 10^{-2}
Sequencer cycle

Fig. 18 Partial amino terminal sequence analysis of: a) VSV G_1 and b) VSV G_1'. 0.5 ml translation of reactions containing 100 μCi of [^{3}H] Pro, [^{3}H] Phe, or [^{3}H] Lys and 200 μCi of either [^{35}S] Met or [^{35}S] Cys, and containing 2 A_{260} μ/ml of EDTA-stripped nuclease-digested dog pancreas microsomal membranes were performed as in Fig. 17. Following translation aliquots were subjected to post-translational incubations and immunoprecipitations as described in Fig. 16, lane 6 (for G_1) and land 7 (for G_1'). Sample preparation for sequencing was described in methods. 17 cycles of automated Edman degradation were performed. Input radioactivity for G_1 was [^{3}H] Lys, 13,000 cpm, [^{3}H] Pro, 12,000 cpm, [^{3}H] Phe, 10,000 cpm, [^{35}S] Met, 60,000 cpm, [^{35}S] Cys, 15,000 cpm. Input radioactivity for G_1' was [^{3}H] Phe, 15,000 cpm and [^{3}H] Lys, 4,000 cpm. Arrows indicate assigned positions.

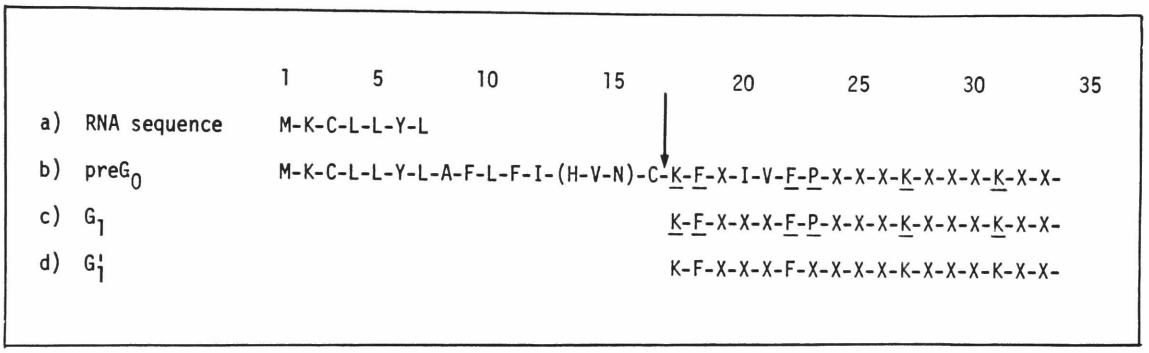
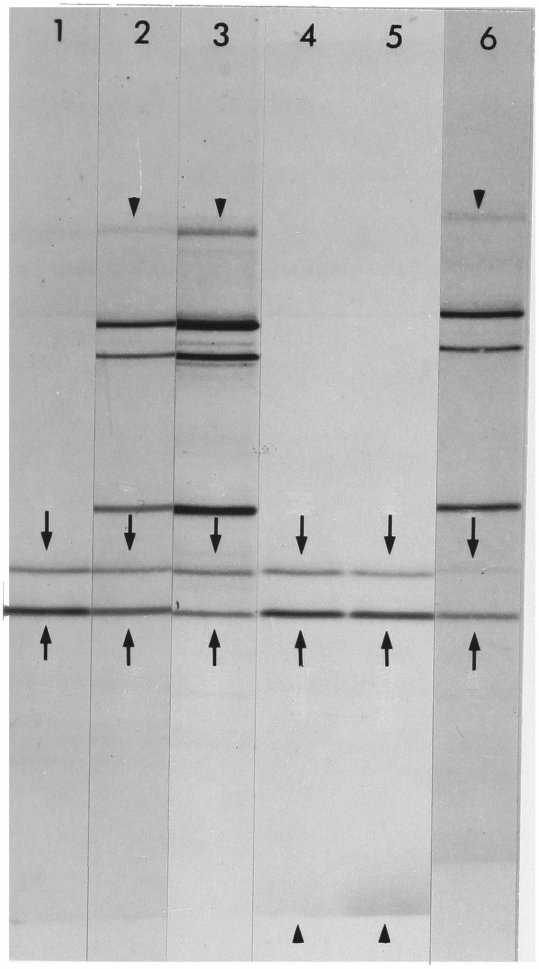

1 5 10 15 20 25 30 35
a) RNA sequence M-K-C-L-L-Y-L
b) preG0 M-K-C-L-L-Y-L-A-F-L-F-I-(H-V-N)-C-K-F-X-I-V-F-P-X-X-K-X-X-X-K-X-X-
c) G1 K-F-X-X-X-F-P-X-X-X-K-X-X-X-K-X-X-
d) G'1 K-F-X-X-X-F-X-X-X-X-K-X-X-X-K-X-X-

Fig. 19 Summary of sequence data on VSV G. a) from partial RNA sequence (ref. 21); b) VSV pre G_0 (see Fig. 2); c) VSV G_1 (see Fig. 3a); d) VSV G_1' (see Fig. 3b). Arrow indicates cleavage site for signal peptidase. Underlined residues indicate basis for alignment of pre G_0 with G_1. M=Met, K=Lys, C=Cys, L=Leu, Y=Tyr, A=Ala, F=Phe, I=Ile, H=His, V=Val, N=Asn, P=Pro, X=unknown.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6

Fig. 20 Competition for membranous receptors by nascent VSV G and nascent prolactin. 0.5 A_{260} μ/ml of prolactin mRNA was translated in the reticulocyte lysate system (6) containing 5 A_{260} μ/ml (lanes 1-5) or 10 A_{260} μ/ml (lane 6) of EDTA-stripped nuclease-digested dog pancreas microsomal membranes in the presence of 0.1 (lane 2) or 0.4 (lane 3 and 6) A_{260} μ/ml of VSV mRNA; or 0.1 (lane 4) or 0.4 (lane 5) A_{260} μ/ml globin mRNA. Downward pointing arrows refer to preprolactin and upward pointing arrows refer to processed prolactin. Downward pointing arrowheads refer to G_1, upward pointing arrowheads refer to globin. Lanes 1-5 were derived from one slab gel while lane 6 was derived from a different slab gel and realigned according to the migration of processed prolactin.

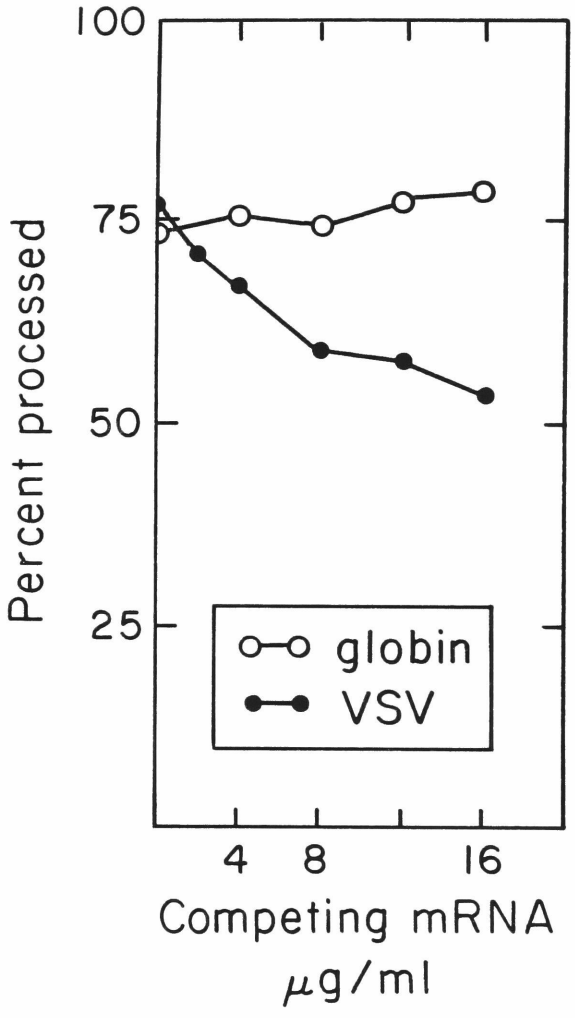
100
75
50
25
Percent processed
globin
VSV
4
8
16
Competing mRNA
μg/ml

Fig. 21 Quantitation of competition for membranous receptors by nascent G of VSV and nascent prolactin. A constant amount of prolactin mRNA (0.5 A_{260} µ/ml) was translated in the presence of 5 A_{260} µ/ml of microsomal membranes in the absence or presence, of increasing amounts of an additional mRNA (closed circles, VSV mRNA; open circles, globin mRNA). Translation products were analysed (see for example Fig.) and the radioactivity of gel slices containing preprolactin and prolactin was determined as previously (6). The extent of processing is expressed on the ordinate as a percentage of processed prolactin over that of total prolactin (which is preprolactin +processed prolactin) and is plotted as a function of competing mRNA concentration. As an example, 28,750 cpm were found in total prolactin and 21,800 cpm in processed prolactin when no competing mRNA was added.

Chapter IV:

Glycosylation: A cotranslational event coupled to the transfer of proteins across the microsomal membrane

Introduction

A useful approach for the investigation of the temporal and topological relationships between protein biosynthesis and core glycosylation has been described, (chapter III, ref. 43). It was demonstrated that translation of mRNA for a membrane protein--the glycoprotein G of vesicular stomatitis virus (VSV)--in a cell-free protein synthesis system containing (45) or supplemented with (43) microsomal membranes resulted in the synthesis of a core glycosylated form of this protein. Furthermore, it was demonstrated (43) that core glycosylation in this system was dependent on the presence of microsomal membranes during translation: translation in the absence of membranes followed by post-translational incubation with microsomal membranes did not result in core glycosylation of newly synthesized G. A subsequent analysis of the sequence of events, employing several modifications of that system, showed that core glycosylation occurs co-translationally on nascent chains (44). The results obtained employing the reconstituted in vitro system were consistent with data (51) which demonstrated that nascent ovalbumin chains contained carbohydrate, suggesting that in vivo glycosylation takes place similarly on nascent chains.

The aim of our present study was to investigate whether core glycosylation that was observed to occur in the reconstituted system for a membrane glycoprotein could be demonstrated also for a secretory glycoprotein. As a representative for this group of

glycoproteins, we chose rat α-lactalbumin, a secretory glycoprotein of milk. Our results demonstrate that core glycosylation of newly synthesized rat α-lactalbumin does occur *in vitro* in the microsomal membrane supplemented system.

Results

Cell-free synthesis of intermediates of rat α-lactalbumin

Rat α-lactalbumin (hereafter referred to as LA) is a milk glycoprotein which contains 123 amino acid residues and whose predominant carbohydrate moiety is linked to a single asparagine at position 45 (52, 53). mRNA isolated from mammary glands of rats and incubated in a cell-free wheat germ system was found to result in a 30-fold stimulation of (^{35}S) Met incorporation into hot trichloroacetic acid insoluble product (data not shown). Analysis of the translation products by polyacrylamide gel electrophoresis in sodium dodecyl sulfate followed by autoradiography of the dried slab gels showed that numerous polypeptides had been synthesized (Fig. 22, lane 1) but that only a single of these was immunoprecipitable by a rabbit antiserum raised against rat LA (Fig. 22, lane 2). This band, however, migrated considerably faster than secreted LA (Fig. 22, lane 6). When membranes prepared from isolated rough microsomes of dog pancreas were present in the cell-free system during translation (Fig. 22, lane 3) the immunoreactive translation products contained an additional, slower moving polypeptide (Fig. 22, lane 4). By analogy to previous findings (43) and in view of the signal hypothesis our interpretation of these results was that in the absence of microsomal membranes we obtained an unglycosylated form of LA (pre LA_0) which contained an amino terminal extension ("signal peptide"), while in the

presence of microsomal membranes we had synthesized an additional product which was processed (i.e., lacked the signal peptide), segregated within the intravesicular space and core-glycosylated. In order to distinguish this additional product from the secreted and fully glycosylated LA, which in addition (52) contains peripheral sugars (such as galactose, fucose and sialic acid) we designated this presumably core-glycosylated intermediate as LA_1.

Characterization of intermediates

The following experiments were designed to substantiate our interpretation. Partial amino terminal sequence analysis was performed on the putative $preLA_0$ and LA_1. Our results (Fig. 23) showed that LA_1 contained Lys in position 5 and Met in position 15. The position of these two residues in LA_1 is identical with that in secreted and fully glycosylated LA (Prasad, R., Butkowski, R., Hudson, B.G., and Ebner, K.E., unpublished) and was aligned with Lys_{24}, and Met_{34}, in $preLA_0$ (see Fig. 23). These data strongly suggested that $preLA_0$ contained an amino terminal extension of 19 amino acid residues that has a partial amino acid sequence of MetMetxxxxLeuxLeuxxxxLeuxxxxx and that is removed from nascent $preLA_0$ at the correct site when heterologous microsomal membranes from dog pancreas were present during translation.

In order to substantiate the assumption that LA_1 is core-glycosylated we subjected both $preLA_0$ and LA_1 to affinity chromatography on Con-A sepharose. From the data shown in Fig. 24 it can be seen that of the total immunoreactive product which was applied to the column (lane 2) only LA_1 was specifically retained by Con-A sepharose while $preLA_0$ was eluted (lane 1). Furthermore, Con-A bound LA_1 was specifically eluted by α -methylmannoside (lane 3). That the binding of LA_1 was sugar-specific was demonstrated by the finding that presaturation of Con-A sepharose with α -methylmannoside did not result in the binding of LA_1 (lane 4). In order to further characterize the carbohydrate moiety of LA_1, affinity chromatography was performed on columns of Ricinus communis

lectin (which binds specifically to galactose) linked to agarose. It can be seen (Fig. 24 lane 5) that neither LA_1 nor $preLA_0$ were bound by this lectin. Taken together, these data strongly suggested that LA_1 was core-glycosylated, i.e., it did contain mannose but not galactose, the former being a typical core sugar and the latter exclusively peripheral. In view of the evidence (55) that core glycosylation takes place in the rough endoplasmic reticulum while peripheral sugars are added in the Golgi membranes, this differential pattern of lectin binding was not surprising. Nevertheless the precise sequence of the attached sugar residues as well as the site of attachment on the polypeptide remain to be investigated in order to establish the fidelity of core-glycosylation which we presumably obtained in our system.

From the results of previous studies (Chapter II) it was expected that the extent of topological translation which results in processing, segregation, and in the present case also in glycosylation would depend on the concentration of microsomal membranes present in the cell-free system. When increasing amounts of microsomal membranes were present during translation, there was an increased synthesis of LA_1 relative to $preLA_0$ (Fig. 25).

That only LA_1 but not LA_0 was segregated and therefore resistant to post-translational proteolysis by trypsin is shown in Fig. 22, lane 5. As expected, protection was abolished (Fig. 22,lane 9) when post-translational proteolysis was done in the presence of the

detergent sodium deoxycholate to solubilize the microsomal membranes. Furthermore, processing, segregation and glycosylation were co-translational and not post-translational events: when microsomal membranes were added to the cell-free system after completion of protein synthesis there was no conversion of nascent $preLA_0$ to LA_1 (Fig. 22, lane 7) nor was there any protection of $preLA_0$ from proteolysis (Fig. 22, lane 8).

Conclusion

Our results demonstrating glycosylation of newly synthesized rat LA in a cell-free and membrane-supplemented protein synthesizing system should be compared with data that have previously been obtained, also from *in vitro* studies, although by an entirely different approach. The most notable difference is that glycosylation was investigated in those studies not by an assay that was coupled to the cell-free synthesis of the substrate, as reported here, but by an assay that was uncoupled from protein biosynthesis. For the sake of brevity, the latter will therefore be referred to as the "uncoupled" assay in order to distinguish it from the "coupled" assay. One of the earlier variants (56) of the uncoupled consisted essentially of isolated oligosaccharide lipid that was radiolabeled in its oligosaccharide moiety, unlabeled microsomal membranes, low concentrations of the detergent sodium deoxycholate (0.8%), and high concentrations of $MnCl_2$ (15 mM). Using microsomes isolated from hen oviduct, it was observed (56) that only a few but distinct molecular weight species of endogenous *membrane* glycoproteins were labeled. However, ovalbumin, a secretory protein, that also was present in the isolated microsomes remained essentially unlabeled (56). It was therefore suggested that the added oligosaccharide lipid may serve as a precursor only for membrane glycoproteins but not for secretory glycoproteins (57). Subsequently, however, it was demonstrated (58) that a variety of secretory glycoproteins could serve as carbohydrate acceptors as well, provided that they were added to

the uncoupled system in their unglycosylated and chemically denatured forms. These results supported an earlier proposal (59) that core glycosylation of membrane--as well as of secretory glycoproteins--proceeds via common lipid-linked oligosaccharide cores. The requirement for unfolded proteins in an uncoupled system supports the conclusion drawn from in vitro studies using the coupled system (44), that glydosylation occurs on nascent chains which presumably have not yet acquired their native configuration. It furthermore explains the previously ovserved inability (57) of native ovalbumin present in the isolated hen oviduct microsomes to serve as carbohydrate acceptors in the uncoupled assay. Although the data obtained from all the studies taken together strongly suggest that core glycosylation is coupled to translation and therefore occurs on nascent chains, it remains to be seen whether this is a rule for all glycoproteins. It is conceivable that potential glycosylation sites remain sufficiently exposed for glycosylation to occur in vivo as well as in vitro also on completed chains, due e.g. to a particular post-translational configuration of the completed chain which could be intermediate to that of the native protein. In this regard it is conceivable that the few distinct molecular weight species of membrane glycoproteins which were found by Pless and Lennarz (56) to serve as "endogenous" carbohydrate acceptors in the uncoupled assay could belong to the category of post-translationally glycosylated glycoproteins. An

alternative and very interesting possibility, however, which has to our knowlege not been proposed and which cannot yet be ruled out, is that these few distinct species are not the conventional end products of glycosylation but rather represent distinct carriers which function in the transfer of sugars to proteins and therefore serve as physiological intermediates in the glycosylation pathway.

Apart from the different protein substrates--newly synthesized versus unglycosylated and chemically denatured protein--that are used in the coupled and uncoupled core glycosylation assay, respectively, other differences should be noted. In the coupled system, core glycosylation of the newly synthesized protein occurred under conditions which were optimal for cell-free protein biosynthesis and by this criterion presumably therefore proceeded under "physiological" conditions. This is clearly not the case for core glycosylation in the uncoupled system which requires low concentrations of detergent and high concentration of $MmCl_2$. It is known that detergents in low concentrations make membranes "leaky" to macromolecules (60). Therefore, the requirement for detergent could be rationalized in that leaky vesicles are necessary to provide access for the unfolded protein substrate and most likely also for the oligosaccaride lipid to the lumenal aspects of the membrane with which the glycosyl transferase is presumably associated. In the coupled assay, on the other hand, access of the newly synthesized nascent chain to glycosyl transferase proceeds physiologically via co-translational segregation of the

nascent chain and therefore the need for detergent is bypassed. The requirement, however, for high concentrations of $MnCl_2$ in the uncoupled assay cannot be readily explained. In the coupled assay these high $MnCl_2$ concentrations result in a complete inhibition of translation (data not shown) and by this criterion therefore have to be considered unphysiological.

Our observation here that processing (removal of signal peptide), glycosylation (presumably of the core type), and segregation occur during translation suggests that these reactions proceed in an interlocking cascade wherein the product of one co-translational event is the substrate for another. In view of the signal hypothesis (22) the crucial initial event in this cascade is the establishment of a functional ribosome-membrane junction by the emerging signal peptide region of the nascent presecretory polypeptide, in the present case nascent $preLA_0$. If for lack of sufficient quantities of added microsomal vesicles in vitro this event is abolished, translation will proceed in a non-topological fashion, i.e. on free ribosomes, and all the subsequent events will be aborted, resulting in the synthesis of a complete and unglycosylated $preLA_0$ molecule. However, once the initial commitment is made and a functional ribosome-membrane junction is established, subsequent processing, glycosylation and segregation occur in vitro to a remarkably complete extent. That essentially all of these reactions did indeed go to completion can be inferred from the fact that no partial reaction products could be detected such as segregated (i.e. proteolysis resistant) and unglycosylated

$preLA_0$ molecules, or glycosylated and segregated but unprocessed $preLA_1$ molecules (which would migrate slower than $preLA_0$). In particular, the absence of an unglycosylated but processed and segregated intermediate (LA_0) indicates that insufficient quantities of the endogenous carbohydrate donor were present in the added microsomal membranes to permit glycosylation of all processed and segregated nascent chains in the wheat germ cell-free system. It should be noted, however, that the amount of newly synthesized LA is in the range of only a few nanomoles. Cell-free translation systems that yield a more efficient translation may result in a rapid depletion of the pool of the endogenous donor present in the added microsomal membranes and therefore may also yield processed and segregated but unglycosylated forms of secretory proteins.

Glycosylation of a newly synthesized secretory protein--a kappa-type light chain of mouse IgG--has been reported previously (61). However, glycosylation was achieved in a post-translational and uncoupled sytem and was observed in only one out of 40 newly synthesized chains (61).

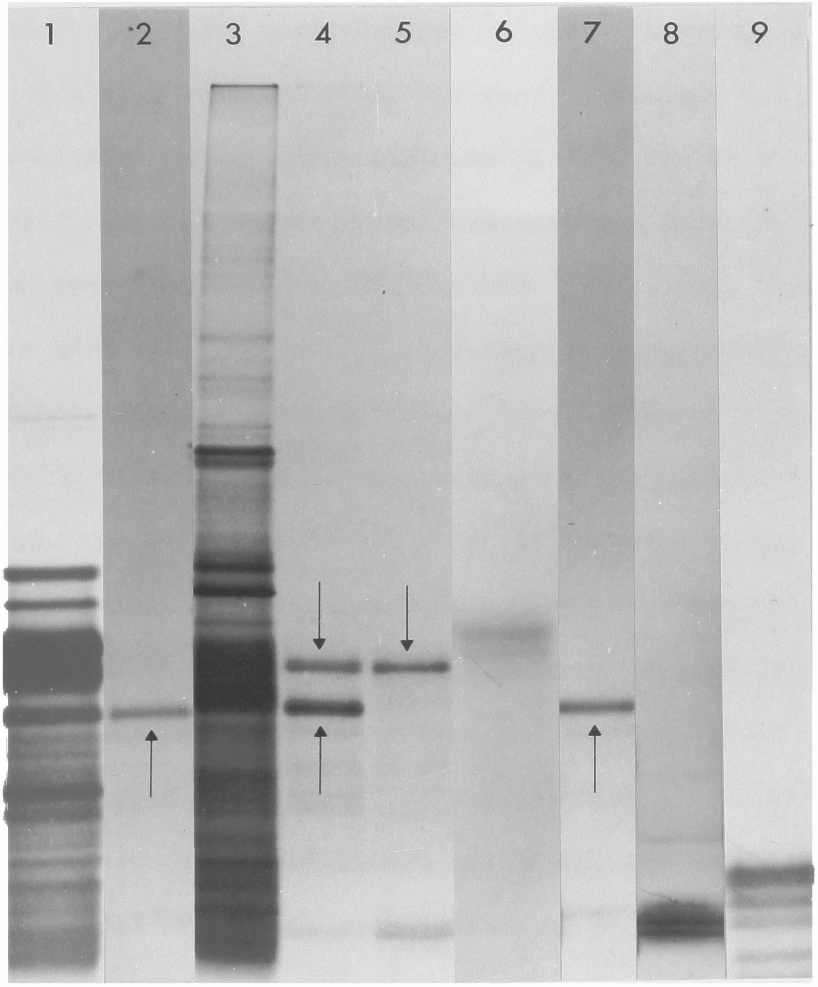
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Fig. 22 Characterization of polypeptides synthesized by translation of rat mammary mRNA in the wheat germ cell-free system. Analysis was by polyacrylamide gel electrophoresis in sodium dodecyl sulfate ($NaDodSO_4$) and autoradiography of the dried slab gels, except for lane 6, which represents a Coomassie brilliant blue staining pattern. mRNA isolated from mammary glands was translated in a wheat germ cell-free system as described previously (8). 100 µl incubation volumes contained 20 µCi of [^{35}S]methionine, 0.1 A_{260} units of mRNA, and when specified, 0.5 A_{260} units of microsomal membranes. Incubation was at 25°C for 60 min. after which aliquots were prepared for electrophoresis either directly or following various post-translational incubations (see below). Lane 1: total rat mammary mRNA translation products; lane 2: immunoreactive translation products; for immunoprecipitation 50 µl aliquots of the cell-free incubation mixture were precipitated with ice cold 5% TCA; the TCA precipitate was dissolved in 100 µl of 1% ($NaDodSO_4$); the solution was adjusted to ∿pH 8 with 1.0 M Tris base and incubated in a boiling water bath for 2 min; after cooling to room temperature, 0.9 ml of solution A (150 mM NaCl, 10 mM Tris·HCl, pH 8.3, 10 mM EDTA, 1% Triton X-100, 100 units of Trasylol) as well as 3 µl of a rabbit antiserum raised against rat α-lactalbumin was added; following incubation for 12 hr at 25°C and 12 hr at 4°C, a suspension of Staphylococcus aureus, prepared as described by Kessler (12) was added and incubation was added and incubation was continued for 3 hr at 25°C, after which the staphylococci with the attached antigen-antibody complexes were sedimented by centrifugation at 8,000 x g for 2 min. The sediment was washed by three cycles of resuspension in solution A and centrifugation. The antigen-antibody complexes were dissociated from the staphylococci (see legend

Fig. 22 (continued)

to Fig. 3) and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. Lane 3: <u>total</u> rat mammary translation products when microsomal membranes were present <u>during</u> translation; lane 4: <u>immunoreactive</u> polypeptides contained in the total translation products of those shown in lane 3; lane 5: as lane 4, except that immunoprecipitation was preceded by a post-translational incubation with trypsin (100 μg/μl for 1 hr at 25°C) after which Trasylol (500 units/ml) was added prior to TCA precipitation. Lane 6: rat α-lactalbumin purified from milk; lane 7: as lane 2, except that immunoprecipitation was preceded by a post-translational incubation with microsomal membranes (5.0 A_{260} units/ml) for 60 min at 25°C. Lane 8: as lane 7, except that post-translational incubation with microsomal membranes was followed by an incubation with trypsin (see lane 5). Lane 9: as lane 4, except that the post-translational incubation with trypsin was in the presence of 0.5% sodium deoxycholate. Downward pointing arrows indicate LA_1 and upward pointing arrows indicate $preLA_0$. Lanes were derived from several slab gels with composite alignment based on comigration of identical immunoprecipitates and protein standards on each slab gel.

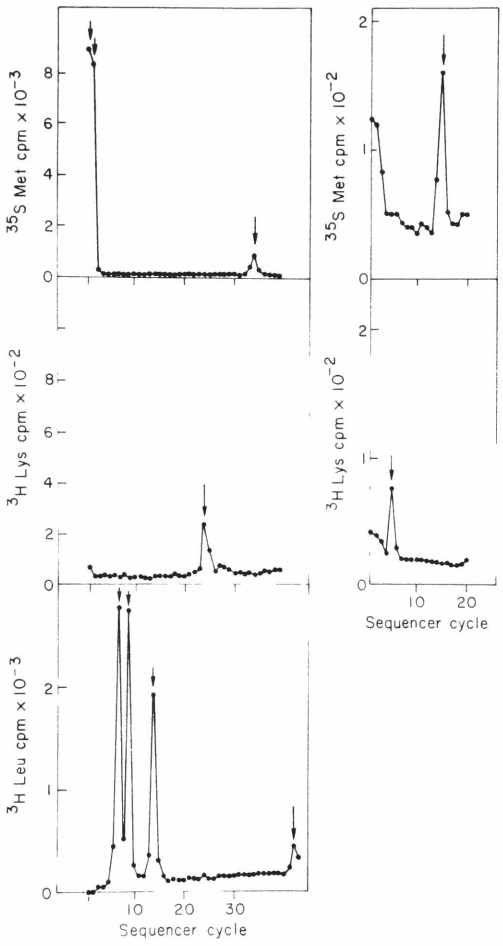
^{35}S Met cpm x 10^{-3}
^{3}H Lys cpm x 10^{-2}
^{3}H Leu cpm x 10^{-3}
Sequencer cycle
^{35}S Met cpm x 10^{-2}
^{3}H Lys cpm x 10^{-2}
Sequencer cycle

Fig. 23 Partial amino terminal sequence of $preLA_0$ and LA_1. Rat mammary mRNA was translated in the wheat germ cell-free system. One ml incubation volumes contained 50 µCi of [^{3}H]lysine and 200 µCi of [^{35}S]methionine or 50 µCi of [^{3}H]leucine and 200 µCi of [^{35}S]methionine. One of the two incubation mixtures with [^{3}H]lys and [^{35}S]Met contained 5.0 A_{260} units of microsomal membranes. $NaDodSO_4$/polyacrylamide gel electrophoresis of the immunoreactive polypeptides gave patterns as shown in Fig. 1, lane 2 (when microsomal membranes were absent) and lane 4 (when microsomal membranes were present. The $preLA_0$ band (see Fig. 1, lane 2) and the LA_1 band (see Fig. 1, lane 4) were excised from the dried slab gels, eluted (9) and subjected to consecutive Edman degradation for 20 cycles (in the case of LA_1, see right hand panel) or 39/42 cycles (in the case of $preLA_0$ (see left hand panel. Radioactive amino acids were determined by double label counting of the samples from each cycle. Arrows indicate positions of radioactive amino acid residues.

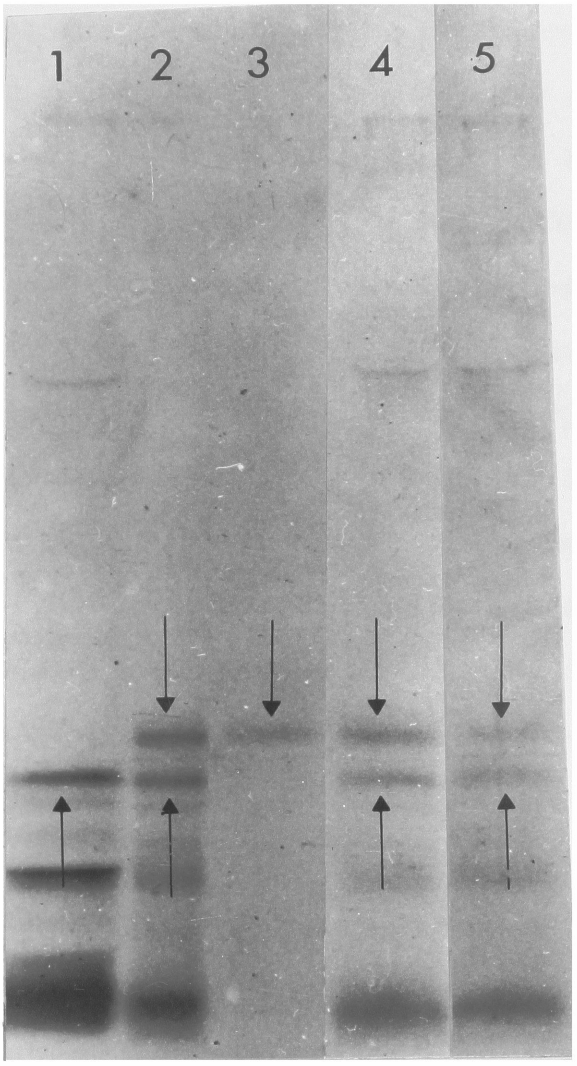
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Fig. 24 Characterization of the immunoreactive translation products by affinity chromatography with lectin-bound sepharose. Rat mammary mRNA was translated in the wheat germ cell-free system in the presence of microsomal membranes (see Fig. 22) and the immunoreactive polypeptides were separated from the total translation products as described in the legend to Fig. 1, except that the three washing cycles were omitted. The antigen-antibody complexes were dissociated from the staphylococci as follows: the sedimented staphylococci containing the Ag-Ab complexes were resuspended in solution B (2% $NaDodSO_4$, 10 mM Tris·HCl, pH 8.3, 5 mM DTT) and the suspension was incubated in a boiling water bath for 2 min. After removing the staphylococci by centrifugation at 8,000 x g for 2 min, the supernate was diluted with 4 ml of solution C (150 mM NaCl, 10 mM Tris·HCl, pH 7.5, 0.5 mM $MgCl_2$, 1 mM DTT and 50 µg of bovine serum albumin). This solution was then applied to a column containing ∿0.1 ml of packed Con-A sepharose which had been equilibrated with solution D (solution C containing 0.05% $NaDodSO_4$). The Con-A sepharose was washed with 15 ml of solution D and the eluant represented the unbound translation product. The Con-A sepharose was then washed with 5 ml of solution D, which contained 0.2 M α-methylmannoside. The eluant was collected and represented the sugar eluted translation products. Analysis was by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography. Lane 1, unbound immunoreactive translation products; lane 2, total, immunoreactive translation products, as applied to the Con-A sepharose column; lane 3, bound and α-methylmannoside-eluted immunoreactive translation products. Lane 4, as lane 1, except that Con-A sepharose was equilibrated with solution D containing 0.2 M α-methylmannoside; lane 5: as lane 1, except that affinity chromatography

Fig. 24 (continued)

was done on RCA (Ricinus communis)-agarose. Designation by arrows as in Fig. 22) Note that omission of the three washing cycles in the immunoprecipitation step (see Fig. 1) resulted in additional contaminating bands (compare lane 2 of Fig. 24 with lane 4 of Fig. 22).

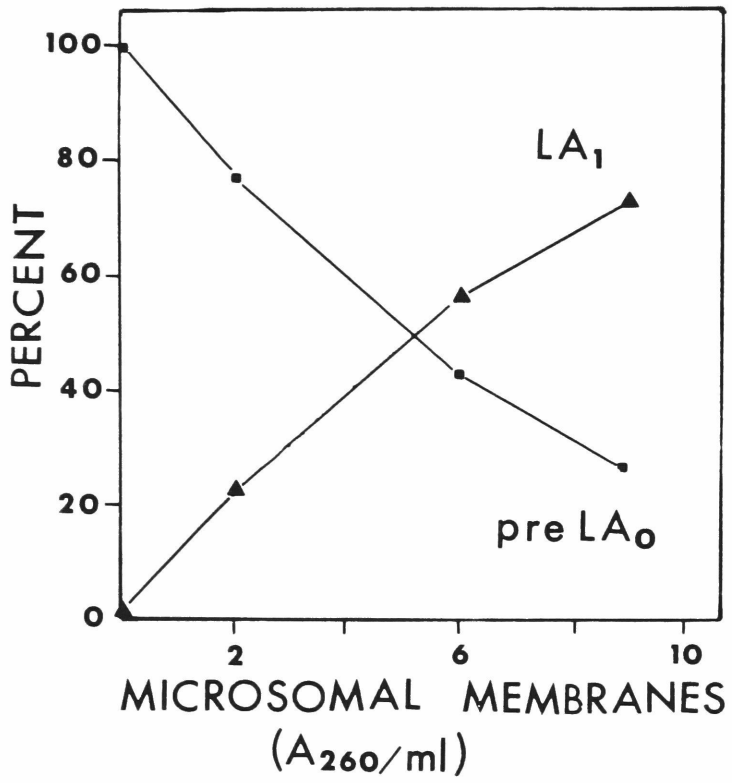

100
80
60
40
20
0
PERCENT
LA1
pre LA0
2
6
10
MICROSOMAL MEMBRANES
(A260/ml)

Fig. 25 Effect of membrane concentration on the extent of co-translational processing, glycosylation and segregation of nascent $preLA_0$. Rat mammary mRNA was translated in the absence or in the presence of increasing amounts of microsomal membranes. The immunoreactive translation products (see Fig. 22) were analyzed by $NaDodSO_4$ / polyacrylamide gel electrophoresis and autoradiography. Gel slices containing $preLA_0$ and LA_1 were excised, rehydrated, incubated for 1 hour at 70°C in 0.1 ml of a quaternary ammonium base ("tissue solubilizer"), neutralized with glacial acetic acid and counted in 10 ml of Liquifluor scintillation cocktail. From sequence data presented in Fig. 2 and from the amino acid composition of secreted rat LA(10), it is evident that two of the four [^{35}S] methionine residues present in $preLA_0$ occur in the "signal peptide" (residues 1 and 2). Thus "processing" of nascent $preLA_0$ molecules (see text) will result in a 50% reduction of radioactivity which was corrected for by multiplying the radioactivity obtained in LA_1 by a factor of two. The radioactivity in $preLA_0$ and in LA_1 (corrected) was plotted as the percentage of the sum of the radioactivity in both forms.

Chapter V:

Ovalbumin: A secretory protein with an uncleaved signal sequence

In contrast to studies reported in this thesis (Chapters II, and III) and elsewhere on the biosynthesis of secretory and membrane proteins, it was reported recently (62) that chicken ovalbumin is not synthesized with a cleavable amino terminal signal sequence. However, other hen oviduct secretory proteins such as lysozyme, conalbumin and ovomucoid, that are produced by the same cells as ovalbumin, were shown to be synthesized with cleavable signal sequences (63). The suggestion was therefore made that in chick oviduct there are at least two distinct mechanisms of protein secretion (62). However, an alternative interpretation of these results was that chick ovalbumin represented a protein whose signal sequence was not cleaved and might be retained in the mature molecule. It was, therefore, of interest to investigate whether ovalbumin could be segregated in the membrane-supplemented cell-free system.

Our results demonstrate that ovalbumin is indeed segregated in this system, and furthermore, that nascent ovalbumin competes for segregation with another nascent secretory protein (bovine prolactin) which contains a cleavable signal sequence. These results will be discussed in terms of the signal hypothesis.

Results

Translation-coupled core-glycosylation and segregation of ovalbumin

Translation of highly purified ovalbumin mRNA in a staphylococcal nuclease-treated reticulocyte lysate system yielded as expected, a major polypeptide (Fig. 26, lane 2) of approximately 40,000 daltons that represents an unglycosylated form (see below) of ovalbumin, designated here as Ov_0. When dog pancreas microsomal membranes were present from the start of translation an additional and slower-moving polypeptide of about 45,000 daltons was synthesized (Fig. 26, lane 4, downward pointing arrow). By analogy to our previous data on the cell-free synthesis of glycoproteins (Chapters III and IV) it was likely that this slower-moving band--refered to as Ov_1-represented a core-glycosylated form of ovalbumin that was segregated within the dog pancreas microsomal vesicles.

That segregation had indeed occurred was indicated by the following results: (1) Ov_1 but not Ov_0 could be quantitatively sedimented with the microsomal vesicles (Fig. 26, lane 7). (2) Post-translational incubation with proteolytic enzymes yielded protection of Ov_1 (Fig. 26, lane 5) but not of Ov_0 (Fig. 26, lanes 1 and 5). (3) Protection was abolished when post-translational incubation with proteolytic enzymes was performed in the presence of detergents that solubilized the microsomal vesicles (Fig. 26, lane 6), indicating that resistance of Ov_1 to proteolysis was not an intrinsic property of Ov_1 but is due to the impermeability of the membrane vesicle to the proteolytic probe.

That Ov_0 presented an unglycosylated form, and Ov_1 a glycosylated form of ovalbumin was suggested by the fact that Ov_0 did not bind to Con A-Sepharose whereas Ov_1 did (Fig. 26, lane 8) and that Ov_1 could be specifically eluted by α-methyl mannoside (Fig. 26, lane 9).

As previously observed for other glycoproteins (Chapters III, IV) segregation and glycosylation of ovalbumin is strictly coupled to translation. When membranes were added to the cell-free system only after completion of translation, followed by an additional incubation period, there was no synthesis of Ov_1 (Fig. 26, lane 3). Furthermore, the Ov_0 form that was synthesized in the absence of membranes was not segregated by a post-translational incubation with membranes and, therefore, was found to be sensitive to added proteolytic enzymes (Fig. 26, lane 1).

The extent of segregation and glycosylation was, as previously observed, dependent on the amount of microsomal membranes that were added to the cell-free system. Increasing amounts of microsomal membranes resulted in increased synthesis of Ov_1 and in decreased synthesis of Ov_0 (Fig. 27).

Competition for membraneous receptors

We have demonstrated previously that nascent secretory proteins from a variety of sources can be segregated by dog pancreas microsomal membranes (Chapters II, IV). Remarkable, however, in the present case is that a secretory protein was segregated, i.e., transferred across the microsomal membrane, that is not synthesized

with a cleavable signal sequence. It is conceivable that similar secretory proteins are synthesized also in dog pancreas. Microsomal membranes from dog pancreas could therefore, be endowed with a separate set of receptors which function only in the transfer of secretory proteins that are not synthesized with cleavable signal sequences.

In order to investigate this possibility, experiments were designed to determine whether nascent ovalbumin would compete for membraneous receptors with a nascent secretory protein (bovine prolactin) that contains a cleavable signal sequence and with a cytosolic protein (rabbit globin) that contains no signal sequence at all. In all experiments the amount of membranes was kept constant and subsaturating with respect to segregation activity. However, since different batches of membranes were used, slightly different segregation activities were observed (e.g., see open and closed circles on Fig. 28a and 28b). Competition was monitored by assaying for either glycosylation of ovalbumin or processing of prolactin. Both events, glycosylation and processing, have been shown previously to occur exclusively on the lumenal aspect of microsomal membranes and to be tightly coupled to segregation (Chapter IV). Thus, the extent of glycosylation and processing are accurate and readily quantifiable indicators of the extent of segregation.

In the first experiments, the concentration of ovalbumin mRNA was kept constant and the amount of a second species of mRNA, either for globin or prolactin, was varied. The radioactivity in the Ov_0 and Ov_1 bands was determined separately and segregation was expressed as a percentage of radioactivity in Ov_1 (Ov_1/Ov_0+Ov_1). It can be seen from Fig. 28 that increasing amounts of prolactin mRNA reduced the percentage of Ov_1 from approximately 60% to 30% (i.e. by one half) whereas increasing amounts of globin mRNA had no significant effect on percent ovalbumin segregated. In subsequent experiments the concentration of prolactin mRNA was kept constant and increasing amounts of ovalbumin mRNA or of globin mRNA were used as a second species of mRNA. The radioactivity in the preprolactin and processed prolactin bands was determined and segregation was expressed as percentage of processed prolactin. It can be seen from Fig. 28 that increasing amounts of ovalbumin mRNA reduced the percentage of processed prolactin from 76% to 45%, whereas increasing amounts of globin mRNA had no effect. It should be pointed out that the addition of increasing amounts of either second mRNA yielded approximately proportional translation of these mRNAs (data not shown). Moreover, competition was observed only under conditions of limiting membrane concentration and could be abolished by raising the membrane concentration to a saturating level (data not shown).

Conclusion

Our results demonstrate that nascent chicken ovalbumin chains are recognized by receptors in dog pancreas microsomes involved in the transfer of proteins across the microsomal membrane. Furthermore, as in the case of other secretory proteins with cleavable signal sequences, transfer cannot be accomplished after completion of ovalbumin chains. This suggests that the information for segregation is expressed only in the nascent obalbumin chain but not in the completed and presumably folded molecule.

The most plausible interpretation of these findings is that ovalbumin contains the functional equivalent of a signal peptide, that is not removed, and therefore is retained in the mature ovalbumin molecule. It should be noted here that such a possibility had been clearly anticipated in the signal hypothesis (22), where it was proposed that point mutations could conceivably affect only the signal peptidase site so that the membrane recognition site of the signal peptide could remain functional for transfer across the microsomal membrane. Implicit in this prediction is that removal of the signal peptide is not a prerequisite for transfer of the nascent chain across the membrane. Thus, ovalbumin could be the first representative of a number of secretory proteins where--in the course of evolution--the amino terminal signal sequence has remained functional for membrane transfer but has lost its site for signal peptidase. This conjecture is supported by a recent report (64) on an E. coli mutant with structural alterations in the outer membrane lipoprotein. It was observed (64) that replacement of a glycl-by an aspartyl

residue at position 14 of the signal sequence that comprises 20 amino acid residues, resulted in a loss of cleavage but not of transfer across the inner membrane.

Although Palmiter and his colleagues have considered the possibility that ovalbumin contains an uncleaved signal sequence they have argued against it because of a lack of similarity of the amino terminal sequence of ovalbumin to the signal sequences of various presecretory proteins (63). However, in view of the considerable differences in the primary structure of signal sequences thus far elucidated, it is not yet possible to discern in them those unique structural features which confer on them their common biological function.

The competition for segregation observed in our in vitro assay indicates that at least one membranous component is common in the catalytic assembly engaged in segregation of prolactin and of ovalbumin. It is likely that this common component is a receptor involved in the initial recognition of the signal region of nascent chains. Since the two competing polypeptides undergo different and non-overlapping co-translational modifications (ovalbumin is glycosylated but not processed by signal peptidase, while prolactin is proteolytically processed but not glycosylated), the competition observed is unlikely to be for access to modifying enzymes on the luminal face of the membranes.

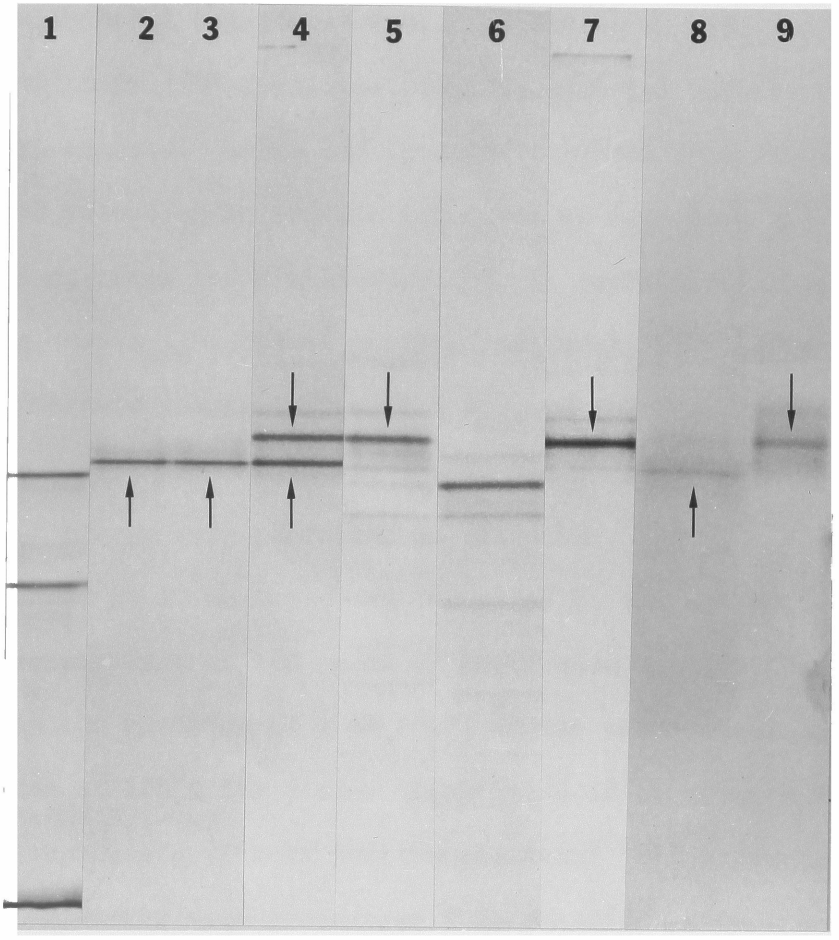

1
2
3
4
5
6
7
8
9

Fig. 26 Coupled cell-free synthesis, core-glycosylation and segregation of ovalbumin.

Ovalbumin mRNA was translated in the staphylococcal nuclease-treated rabbit reticulocyte lysate (73), in the absence or presence of nuclease-treated dog pancreas microsomal membranes (72). This was followed by various posttranslational assays, as specified for each lane, and by preparation of aliquots (72) for polyacrylamide slab gel electrophoresis in sodium dodecyl sulfate. Lanes 1-9 were derived from several slab gels; they were aligned according to protein standards on each slab gel.

Incubation mixtures for translation (50 µl) contained 12 µCi of [^{35}S] methionine, 0.5 A_{260} units of purified ovalbumin mRNA and either no microsomal membranes (lanes 1-3) or 0.5 A_{260} units of microsomal membranes (lanes 4-9). Incubation was for 90 min at 22°C. The following posttranslational assays were performed on aliquots. Lane 1: a 10 µl aliquot was adjusted to 10 mM $CaCl_2$ and incubated at 0°C and for 4 hrs with a final concentration of 100 µg/ml of proteinase K; digestion was terminated by the addition of 2 mM PMSF, sodium dodecyl sulfate to 2%, and incubation at 100°C for 5 min. Lane 2: a 10 µl aliquot was processed for electrophoresis without posttranslational incubation. Lane 5: a 10 µl aliquot, digested with proteinase K as detailed above (Lane 1). Lane 6: as Lane 5, except that digestion was in the presence of 0.5% Triton X-100. Lane 7: a 50 µl aliquot, adjusted to 0.01 M $CaCl_2$ and layered over 500 µl of a solution of 0.6M sucrose, 0.5 M NaCl, 0.01 M Tris·HCl, pH 7.5, 0.005 M $CaCl_2$ and 0.002 M $MgCl_2$ was centrifuged for 1 hr at 105,000 x g in a Spinco No. 40 rotor. The resulting pellet was rinsed with H_2O, and prepared for electrophoresis. Lane 8: a 50 µl

Fig. 26 (continued)

aliquot was adjusted to 2% sodium dodecyl sulfate, incubated at 60°C for 2 min, diluted with 4 ml of Solution A (0.15 M NaCl, 0.02 M Tris·HCl, pH 7.5, 0.001 M $MgCl_2$ and 0.001 M dithiothreitol) and loaded onto a column of Con-A Sepharose 4B (50 µl bed volume) that was equilibrated with Solution B (Solution A containing 0.05% sodium dodecyl sulfate). The unbound fraction was collected; a 0.8 ml aliquot was incubated at 0° for 1 hr with 2 volumes of ethanol/ether (50:50). The precipitate was collected and prepared for electrophoresis. Lane 9: following collection of the unbound fraction (see Lane 8) the column was washed with 5 ml of Solution B. The bound fraction was then eluted with 5 ml of Solution B containing 0.4 M α-methylmannoside, and a one-ml aliquot of this eluate was precipitated as in Lane 8, but with 25 µg of tRNA as a carrier. Upward pointing arrows indicate unglycosylated ovalbumin (Ov_o) and downward pointing arrows indicate core-glycosylated ovalbumin (Ov_1).

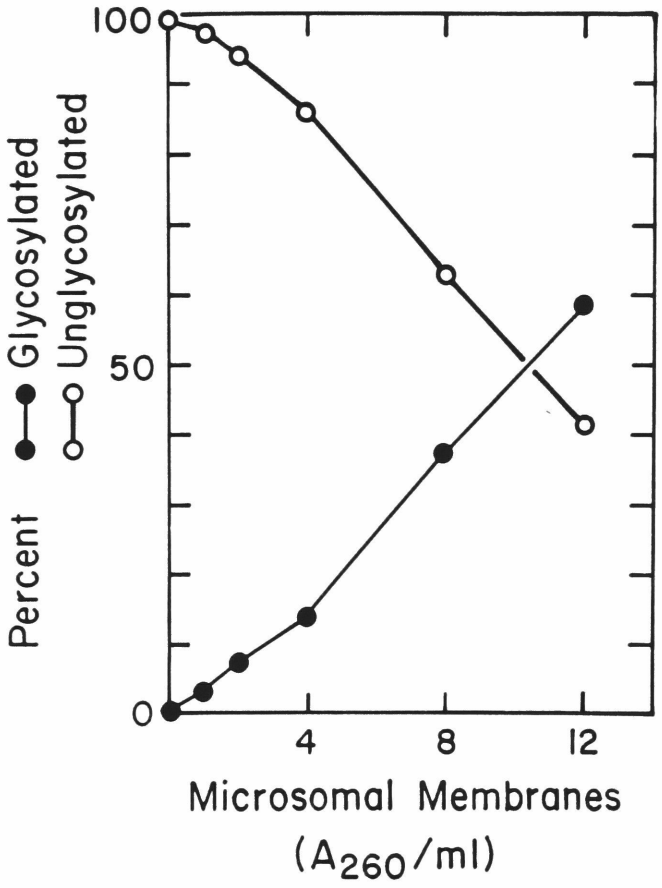
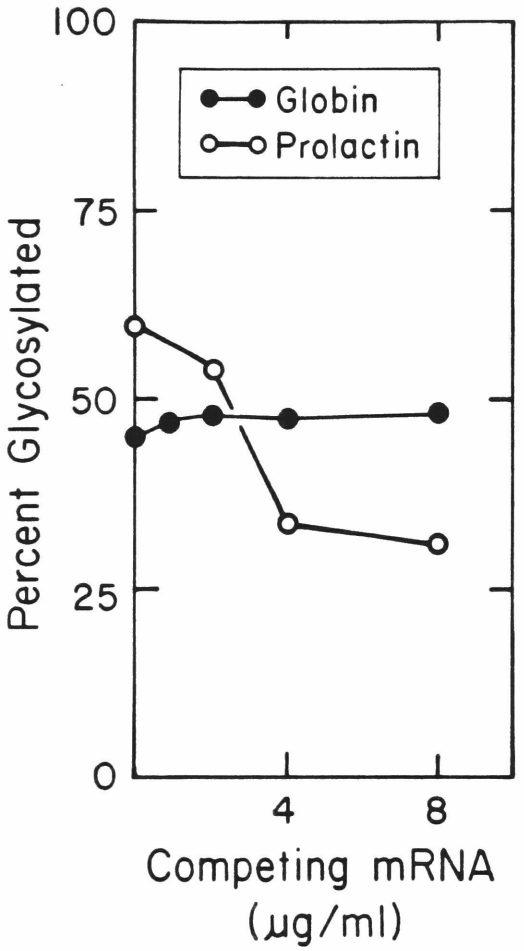

100
50
0
Percent
Glycosylated
Unglycosylated
4
8
12
Microsomal Membranes
(A260/ml)

Fig. 27 Effect of membrane concentration on the extent of cotranslational glycosylation and segregation of nascent ovalbumin. Ovalbumin mRNA was translated in the absence or in the presence of increasing amounts of microsomal membranes as indicated on the abscissa. Translation products were analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and autoradiography. Gel slices containing Ov_o (unglycosylated) and Ov_1 (glycosylated) were excised, and radioactivity determined as previously described (11). Extent of ovalbumin glycosylated (closed circles) or unglycosylated (open circles) is expressed on the ordinate as a percentage of total ovalbumin synthesized. Percent glycosylated ovalbumin $(Ov_1) = Ov_1/Ov_o + Ov_1$; percent unglycosylated ovalbumin $(Ov_o) = Ov_o/Ov_o + Ov_1$.


100
75
50
25
0
Globin
Prolactin
Percent Glycosylated
4
8
Competing mRNA
(μg/ml)

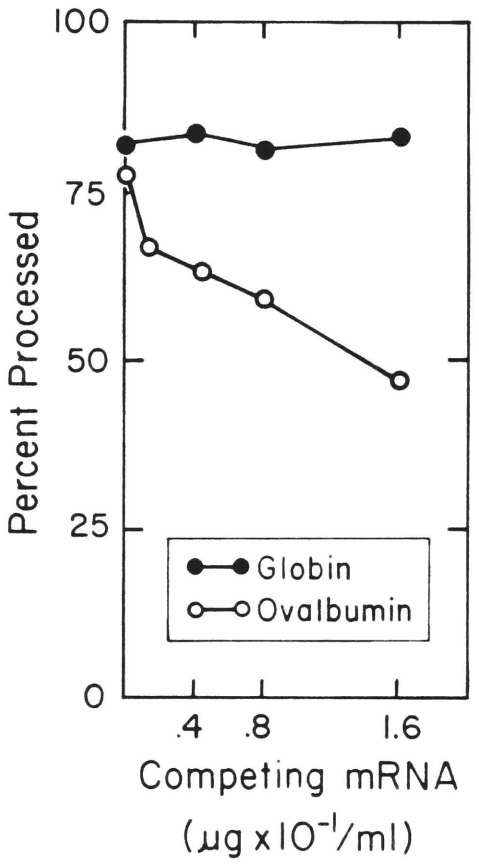

100
75
50
25
0
Percent Processed
Globin
Ovalbumin
.4
.8
1.6
Competing mRNA
(μg x 10⁻¹/ml)

Fig. 28 Competition for membranous receptors by nascent ovalbumin and nascent preprolactin. (a) A constant amount of ovalbumin mRNA (0.5 A_{260} units/ml) was translated in the presence of 10 A_{260}/ml of microsomal membranes, in the absence or presence of increasing amounts of an additional mRNA (open circles, prolactin mRNA; closed circles, globin mRNA). Translation products were analyzed and the radioactivity in gel slices containing Ov_o and Ov_1 was determined as described in Fig. 27. Percent of ovalbumin chains glycosylated (see Fig. 27) is plotted as a function of competing mRNA concentration. (b) As in (a) except that a constant amount of bovine pituitary prolactin mRNA (0.5 A_{260} units/ml) was translated in the presence of increasing amounts of either ovalbumin (open circles) or globin (closed circles) mRNA as the competing species of mRNA. Percent of prolactin chains processed (processed prolactin/ preprolactin + processed prolactin) is plotted as a function of competing mRNA concentration.

VI. Discussion

The results presented in Chapters II and IV present examples of the mounting evidence in favor of the signal hypothesis for secretory proteins. This has been reviewed more exhaustively(65) and will not be considered further here. Rather I will contrast what we know about secretory proteins with the evidence for a mechanism of membrane protein insertion, and indicate directions for future studies.

While detailed mechanistic studies await characterization of the membranous apparatus involved in segregation, the competition experiments presented here afford a preliminary statement on the mechanistic similarity of insertion and segregation. These results support a biosynthetic model involving a common set of early events in membrane assembly and protein secretion, wherein similar signal sequences recognize a common set of receptors to initiate the process of ribosome attachment, tunnel formation and initiation of chain transfer across the membrane. Subsequent to the onset of chain transfer, the events diverge; secretory chains such as prolactin are entirely transferred within the vesicle lumen while the transfer of transmembrane proteins such as G are arrested prior to completion of transfer, resulting in a polypeptide spanning the bilayer. In both cases, co-translational modification, e.g., cleavage of signals and glycosylation of suitable acceptor sites, proceeds during translation and probably, prior to cessation of transfer. In such a model, the information which arrests transfer - termed a "stop transfer" sequence (24) - would be expected to reside elsewhere in nascent G other than in the amino terminal sequence, which is cleaved. Such a "stop transfer" sequence might function by abrogating or altering the ribosome membrane junction or self-associated tunnel components, and results in retention of the carboxy terminal poly-

peptide region as a cytoplasmic domain. Earlier studies have firmly documented vast evolutionary conservation of the role of signal sequences in the transfer of chains across membranes (34). By corollary, it seems likely that the "stop transfer" role is a conserved function of a set of defined polypeptide sequences, adjacent to, or itself constituting, the region of the inserted polypeptide which actually spans the bilayer.

One implication of this model of early biosynthetic events is that information other than that involved in the initial biosynthetic disposition (i.e. on free versus bound ribosomes, and hence, cytosolic versus extracellular consignment of polypeptides) is required to consummate the later stages of subcellular sorting.

A primary goal for the coming period in this field is clearly delineated by the studies presented here: to understand in greater detail the mechanistic events in both secretion and membrane assembly.

A classical approach would involve the dissection and reconstitution of microsomal membranes in order to characterize invidudual components and to determine the steps involved in segregation and insertion. The assays for segregation as well as approaches involving competition, as presented here, should be useful ones to pursue.

Ideal probes would be proteolytic enzymes which would allow dissection of structural as well as functional receptor domains out of the plane of the bilayer, and affinity reagents such as signal peptide analogs which should permit purification of holoproteins engaged in specific functions.

Early attempts at both approaches to understand <u>specific</u> mechanistic features of this mechanism are already under way (66,67) and hold great promise for the near future. A second important area for future research

concerns signal pathology. The elucidation, at least in its general features of a novel machinery required in proper working order by all living cells has obvious implications for disease. In so far as cellular membranes are a likely first line of impact of environmental insults to the cell, such a complex mechanism is a likely early target within intracellular membranes. A variety of degenerative diseases might be excellent candidates for Signal Pathology. A degenerative rather than a genetic origin for such lesion would be expected since those individuals lacking properly functioning signal machinery would probably be aborted while those who develop lesions in specific organ sites later in life perhaps as a result of local or general environmental influences, would exhibit organ-specific pathology of degenerative diseases, with longer range delayed onset systemic implications.

VII. Materials and Methods

A. Materials

[^{25}S] methionine was from Amersham Searle, Arlington Heights, Ill. All [^{3}H] amino acids and [^{35}S] cystine as well as liquifluor scintillation cocktail were from New England Nuclear, Boston, MA. ConA Sepharose, protein A sepharose, and Sephadex-G-25 were from Pharmacia Fine Chemicals, Uppsala, Sweden, RCA (ricinus communis)-agarose was from PL Biochemicals, Inc., Milwaukee, WI. Trasylol (10,000 units per ml) was from FBA Pharmaceuticals, New York, NY. Tissue solubilizer and apomyoglobin were from Beckman Instruments, Palo Alto, CA. Trypsin, chymotrypsin and Proteinase K were from Boehringer Manneim, Germany. Other conventional buffers and reagents were generally obtained from Sigma Chemical Co., St. Louis, MO; or from CalBiochem-Behring Corp., La Jolla, CA.

B. Methods

1. Cell fractionation

a. Preparation of rough microsomes, detached polysomes and free polysomes from boving anterior pituitary -- Fresh bovine pituitaries were obtained from a local abattoir. The glands were heterogeneous with respect to their origin from animals either old or young, male or female and gonadectomized or not. The anterior portion of the pituitary was readily separated from the posterior part, yielding approximately 1 g of tissue per gland. Usually 20-50 grams of anterior pituitary were processed at a time. After mincing with scissors, the pieces were passed through a tissue press (stainless steel with 1 nm diameter perforations) and the resulting brei was homogenized in 2 vol of ice cold 0.25 M sucrose. The homogenate was centrifuged 10 min at 1000 x g to yield a postnuclear supernatant.

The postnuclear supernatant was centrifuged for 10 min at 12,000 x g in an angle rotor. Aliquots of the resulting postmitochondrial supernatant were layered on discontinuous sucrose gradients containing 1.5 ml each of 2.0, 1.5 and 1.0 M sucrose-TeaKM (TeaKM = 50 mM triethanolamine·HCl, pH 7.4, 50 mM KCl and 5 mM $MgCl_2$) and centrifuged for 20 hr at 4°C in a swinging bucket rotor at 190,000 x g_{av}. The pellet comprised the free ribosomes. The material banding above the 2.0 M sucrose layer (crude rough microsomes) was removed with a syringe and mixed with an equal volume of "top" fraction (material remaining above the 1.0 M sucrose layer and containing RNAse inhibitor). Aliquots of the diluted crude rough microsome fraction were layered over 1 ml of 1.3 M sucrose - TeaKM and centrifuged in an angle rotor for 30 min at 100,000 x g_{av} to yield a pellet of rough microsomes.

Other aliquots of the diluted crude rough microsomes were treated with sodium deoxycholate (final concentration of 1%) to solubilize the microsomal membrane, layered over 1 ml of 2.0 M sucrose-TeaKM and centrifuged in an angle rotor for 20 hr at 100,000 x g to yield a pellet of detached ribosomes.

b. Preparation of EDTA-stripped rough microsomes from dog pancreas -- Freshly obtained dog pancreas were rinsed with ice cold 0.25 M Sucrose, 0.15 M KCl, 0.01 M Tea, 0.005 $MgCl_2$-1 mM DTT, (STeaKMD) minced with a razor blade, passed through a tissue press and homogenized in 4 vol ice cold TeaKMD. The homogenate was centrifuged at 1000 x g for 10 min to yield a postnuclear supernatant. The postnuclear supernatant was centrifuged at 10,000 x g for 15 min to yield a postmitochondrial supernatant which was centrifuged at 100,000 x g for h hr over a cushion of 1.3 M Sucrose-TeaKMD. The resulting pellet of crude rough microsomes

was resuspended with 4 strokes homogenization by hand in TeaK and adjusted to a final concentration of 50 A_{260}/ml. An equal volume of 20 mM EDTA-Tea-K was added, mixed well and centrifuged through a cushion of 0.6 M sucrose-Tea for 2 hr at 1000k x g. The resulting pellet was frozen in liquid N_2 and stored at -80° until use, at which time it was thawed, resuspended by homogenization in 0.01 M Hepes-1 mM DTT 20% glycerol to a final concentration of 100 A_{260}/ml and distributed into small aliquots, frozen in liquid N_2 and stored at -80°.

2. mRNA purification

Frozen tissue was pulverized in liquid N_2 by mortar and pestle. The resulting frozen powder was added to a mixture of phenol-chloroform-isoamylalcohol-SDS buffer (50:50:1:100) where SDS buffer consists of 5% sodium dodecyl sulfate (SDS), 150 mM NaCl, 50 mM Tris pH 7.5 5 mM EDTA pH 7.0. The material was then subjected to full speed in a waring blender for 2 min, then centrifuged at 3000 rpm for 10 min in a Sorval $H\text{-}B_4$ rotor in glass bottles to separate aqueous and phenol phases. Each phase was reextracted separately with 1/2 volume of phenol-chloroform-isolamylalcohol (50:50:1) or SDS buffer, respectively.

The pooled aqueous phases were reextracted with 1/2 volume of phenol-chloroform-isoamylalcohol and centrifuged as before. The final aqueous phase was mixed with 2.3 volumes ice cold absolute ethanol and stored at -20°C for 24 hr. Total nucleic acid, precipitated by ethanol, was removed by centrifugation at 3000 x g/10 min followed by aspiration of the supernatant and air drying of the pellet. Total nucleic acid is extremely stable as an ethanol pellet and was stored up to 2 years at -20°C. The pellet was dissolved in sterile distilled water to a final concentration of approximately 20 A_{260} units per ml and lithium chloride

was added from a sterile stock solution of 12 M to a final concentration of 2.5 M. After incubation on ice for 8 hr the solution, now cloudly, was centrifuged at 6000 rpm for 1/2 hr to pellet single strand nucleic acid consisting essentially of RNA. This pellet was dissolved in sterile distilled water, NaCl adjusted to 0.15 M and precipitated with 2.3 volumes ice cold ethanol for 24 hr at -20°C. The resulting pellet after centrifugation, aspiration and air drying consisted of total cellular RNA. For most experiments this material was suitable for direct use. Where separation of mRNA from rRNA was desired chromatography on columns of oligo-dT cellulose was performed in SDS, by standard procedures (67).

3. Cell free protein synthesizing systems

a. Wheat Germ system (modified from ref. 68) - 2.0 g fresh wheat germ, obtained from commercial mills, was pulverized for 3 min by mortar and pestle at 4°C in three aliquots of 3.5 ml of extract in solution (90 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$). After centrifugation at 14,000 rpm (23,000 x g) for 10 min in a Sorval SS-4 rotor at 4°C the supernatant was adjusted to 20 mM Tris-Acetate pH 7.5 and 1 mM MgAc, recentrifuged and passed over a column (60 cm bed volume) of Sephadex G-25 equilibrated in 50 mM KCl, 4 mM Mercaptoethanol, 1 mM MgAc and 1 mM Tris-acetate pH 7.6. The cold volume absorbance peaic was collected in as small a volume as possible, centrifuged at 14,000 rpm for 10 min, divided into small aliquots, frozen in liquid N_2 and stored at -80°C. This was termed S-23.

b. Reticulocyte lysate translation system - Rabbits weighing approximately 2 kg were made anemic by five successive daily injections of phenylhydrazine (approx. 25 mg/animal) followed by a two-day rest period without injection. On the third day after the last injection, rabbits were bled by cardiac puncture and the reticulocytes lysed and prepared as described by Hunt and Jackson (69).

4. Cell free translation of mRNA

Conditions used for the wheat germ system were essentially as previously described (70). Briefly, in a 100 µl reaction mixture this consisted of 20-40 µl of S-23, 2.0 µl Hepes-KOH pH 7.5 at 20°C, 2.0 µmol KCl, 11.0 µmol KCH_3COO, 0.2 µmol $Mg(CH_3COOH)_2$, 8 nmol sperminc HCl pH 7.5, 0.2 µmol dithiothreitol, 0.1 µmol ATP, 0.01 µmol GTP, 0.8 µmol creatine phosphate, 4 µg creatine phosphouinase, 30 νCi [^{35}S] methionine (sp. act. 400 Ci/mmole), 2 nmol each of 19 unlabelled amino acids and 0.5 A_{260} total RNA. Modifications for translation in the presence of microsomal membranes or for synthesis with various other labelled amino acids, besides methionine, for radiosequencing, are described in the appropriate figure legends. Conditions used in the reticulocyte lysate system were those described by Shields and Blobel (71) based on the earlier procedure of Pelham and Jackson (72).

5. Post-translational assays

All post translational procedures e.g. proteolysis with trypsin and chymotrypsin, or proteinase K; sedimentation of microsomal membranes following in vitro translation; lectin-sepharose chromatography; immuno-precipitation of in vitro translation products (73), are detailed in the appropriate figure legends.

6. Analysis of translation products

Samples were prepared for polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) as described previously (22,71,74). Autoradiography (22) and fluorography (75) were also performed as described elsewhere.

7. Partial radiosequence determination on *in vitro* translation products

Incubation volumes for protein synthesis were scaled up in addition to [^{35}S] methionine or cysteine, they contained titrated amino acids at the highest available specific activity. Total translation products of various mRNA or immunoprecipitates of desired proteins (see Chapters II, III, IV) were subjected to polyacrylamide slab gel electrophoresis in sodium dodecyl sulfate. Gels were dried without fixing or staining and exposed to autoradiography. Desired proteins were excised from gels and electroeluted in the presence of 1 mg ovalbumin as a carrier, as previously described (34). To the eluted samples in a volume of 3 ml were added serially, 0.2 ml of 5 mg/ml sperm whale apomyoglobin, 0.1 ml of 10 mg/ml ovalbumin 0.4 ml of 4.0 M KCl and 3.5 ml of 50% trichloracetic acid. After vortexing, incubation on ice for 15 min, and aspiration of supernatants the resulting pellet was resuspended in 1 ml of water then precipitated with nine volumes of acidified acetone at -20^{o}C for 15 min. Resuspension, acetone precipitation and centrifugation was repeated and the resulting pellet dissolved in 50% heptafluorobutyric acid prior to loading into the Beckman 890C sequences running on a DMMA program. After each cycle of Edman degradation, thiazolinone derivatives were collected in mini vials, air dried and counted by standard double label procedures in 3 ml of toluene liquifluor. Repetitive yields were calculated (76) normalized to the first cycle.

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