PROTEOLYTIC PROCESSING AND COMPARTMENTALIZATION OF THE PRIMARY TRANSLATION PRODUCTS OF MAMMALIAN APOLIPOPROTEIN mRNAs

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I. INTRODUCTION

Lipoproteins are noncovalently linked complexes of lipids and proteins which are soluble in water. They are involved in the transport of lipids which, by themselves, would be insoluble in the aqueous extracellular environment. Several classes of plasma lipoprotein particles have been defined using density gradient ultracentrifugation as well as chromatographic and electrophoretic methods. Apolipoproteins are the protein components of these lipoprotein particles. The apolipoproteins and lipids represented in each of the five principal human plasma lipoprotein density classes are listed in Table 1.

Plasma lipoprotein particles are not static structures. Rather, their lipids and proteins are derived from a complex set of recombination and remodeling events that involve exchange of components between different density classes as well as transformation of resident lipids (see References 1 to 4 for reviews of our current understanding of these processes). The extracellular interconversions occur by enzymatic, carrier-mediated, and physicochemical processes. Although plasma lipoprotein transformations have been well documented, details of the early steps involved in the assembly of apolipoproteins and lipids into supramolecular complexes are largely unknown.

One approach to the problem of lipoprotein assembly is to analyze the molecular events involved in apolipoprotein synthesis and then interpret this information in light of what is known about the intracellular or extracellular compartments where lipids and apolipoproteins might first interact. Recently, molecular biological techniques have been used to obtain a number of insights about apolipoprotein biosynthesis. More specifically, the structures of the primary translation products of seven mammalian apolipoprotein mRNAs have been described during the past 3 years. Comparison of these amino acid sequences with the NH_2-terminal sequences of "mature" plasma lipoprotein-associated apolipoproteins revealed that the apolipoproteins are initially synthesized as larger precursors. These precursors contain

Table 1
COMPOSITION OF HUMAN PLASMA LIPOPROTEINS

	% Total lipoprotein mass						
		Lipids					
		Cholesterol					
	Apolipoproteins	Free	Esters	Triglycerides		Phospholipid	
Chylomicrons	2% ~90% B48	0.8	7.7	88	7.5	78.5%	Phosphatidylcholine
	10% CI					11.7	Sphingomyelin
	CII					4.2	Lysophosphatidylcholine
	CIII						
	AIV						
VLDL	10% 40% B100	7	12	53	18	59.7%	Lecithin
	30% CIII					14.8	Sphingomyelin
	10% CI					7.6	Polyglycerophosphatide + phosphatidic acid
	10% CII					4.6	Phosphatidylethanolamine
	5—10% E					3.6	Phosphatidylinositol
						1.5	Phosphatidylserine
LDL	21% >95% B100	8	35	9	22	63.7%	Phosphatidylcholine
	trace CIII					25.9	Sphingomyelin
						2.7	Lysophosphatidylcholine
						2.2	Phosphatidylethanolamine
						2.0	Polyglycerophosphatide + phosphatidic acid
						1.6	Phosphatidylinositol
						0.8	Phosphatidylserine
HDL_2	41% ~90% AI	5.4	16.2	4.5	29.5	73.9%	Phosphatidylcholine
	AII					14.5	Sphingomyelin
	10% AIV					3.3	Phosphatidylethanolamine
	CI					2.4	Phosphatidylinositol
	CII					2.2	Polyglycerophosphatide + phosphatidic acid
	CIII						
	D					2.0	Sphingomyelin
	E					0.9	Phosphatidylserine
HDL_3	55% 65% AI	2.9	11.7	4.1	22.5	77.1%	Phosphatidylcholine
	25% AII					9.2	Sphingomyelin
	5% CI					4.0	Lysophosphatidylcholine
	CII					2.5	Phosphatidylethanolamine
	CIII					2.4	Phosphatidylinositol
	AIV					2.0	Polyglycerophosphatide + phosphatidic acid
	D					0.6	Phosphatidylserine

Note: At least 50% of plasma apo AIV is not associated with any plasma lipoprotein density class and can be found in the density > 1.21 g/mℓ fraction.

From Scanu, A. M., Edelstein, C., and Shen, B., *Lipid Protein Interactions*, Vol. 1, Jost, P. C. and Griffith, O. H., Eds., John Wiley & Sons, New York, 1982, 259.

NH_2-terminal extensions of 18 to 26 amino acids which are removed prior to their integration into plasma lipoprotein particles.

This review focuses on two general proteolytic processing events which apolipoproteins undergo after initiation of translation of their mRNAs: (1) cotranslational removal of signal

peptides — a step which all known nascent apolipoproteins are subjected to during their translocation through the rough endoplasmic reticulum and (2) post-translational processing of propeptide segments — a step which probably involves only the two principal human HDL apolipoproteins, AI and AII. Information about the proteolytic processing of precursor apolipoproteins will be evaluated according to prevailing concepts of protein secretion. Specific emphasis given to the possible relationship between the processing of these proteins and a function which distinguishes this class of polypeptides — their incorporation into complex assemblies of lipid and protein.

II. COTRANSLATIONAL PROCESSING OF APOLIPOPROTEINS

A. Current Concepts of Preprotein Processing

The initial proteolytic processing which occurs during apolipoprotein biosynthesis is better understood after first reviewing the theories which have been proposed to explain how newly synthesized proteins enter the secretory pathway.

In 1975, Blobel and Dobberstein described the signal hypothesis.[5] The hypothesis was derived from a number of experimental observations made by a variety of investigators (reviewed in Reference 6). It represented an attempt to explain the mechanism by which presecretory proteins, synthesized from mRNA sequences restricted to the cytoplasm, could be translocated across a membrane bilayer and subsequently targeted to several intracellular compartments or exported from the cell. The central feature of the hypothesis was that protein passage across the endoplasmic reticular (ER) membrane occurred during translation (i.e., cotranslational translocation) and was mediated by a signal contained within the primary amino acid sequence of the growing peptide chain. In the intervening 10 years, several refinements of the signal hypothesis have been made, based largely on the work of Blobel and Walter and Dobberstein and Meyer.[7-15] Moreover, it has become clear that some nonsecretory proteins utilize the ER translocation apparatus.[16] These include lysosomal enzymes as well as integral membrane proteins destined to be incorporated into plasma, Golgi, ER, and sarcoplasmic reticular membranes.[16-20]

The signal hypothesis as currently described (Figure 1A) proposes that, following initiation of translation, elongation proceeds in the cytoplasm until a sufficient length of nascent peptide chain has been produced so that it can emerge from a groove in the large ribosomal subunit. The exposed NH_2-terminal "signal" sequence then interacts with a signal recognition particle, causing a temporary arrest of translation. The signal recognition particle (SRP) contains at least six different polypeptides and a small (7S) RNA molecule.[13] This translation-arrested complex subsequently becomes localized to a region of the rough endoplasmic reticulum (RER) where specific binding of SRP to a membrane receptor (the "docking" protein) occurs. Interaction of docking protein and SRP results in "reinitiation" of translation (Figure 1A). Strong experimental data support these aspects of the signal hypothesis, at least in eukaryotic cells.[7-21] The specific binding steps provide a mechanism for discriminating between proteins destined to be translocated across, or incorporated into, the ER membrane and those which remain in the cytoplasm.

The second stage of this process involves translocation of the growing peptide chain across the ER membrane. This event is still poorly understood. The signal hypothesis predicts that translocation is mediated by integral membrane proteins of the ER with the creation of a pore through the membrane bilayer.[5,21] However, an alternative view is that translocation occurs spontaneously after interaction of the signal peptide with the membrane bilayer. This latter idea has been expanded into the "membrane trigger hypothesis",[22,23] the "loop model",[24] and the "helical hairpin hypothesis".[25] These hypotheses were based largely on experimental results obtained during analysis of the mechanism of protein secretion in bacteria[22-24] or on theoretical calculations of signal peptide conformation.[25] According to these proposals, interaction of the hydrophobic signal peptide with membrane lipid or changes in signal

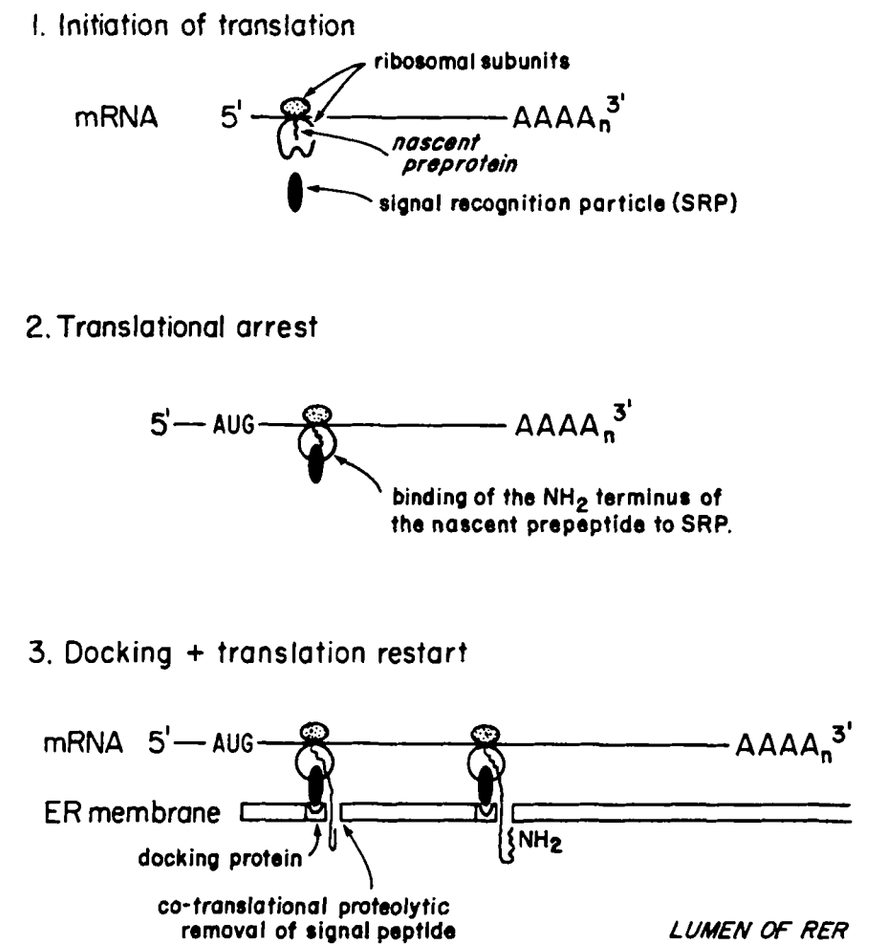


FIGURE 1A. The signal hypothesis. This general outline of the steps involved in the cotranslational translocation and proteolysis of nascent presecretory proteins is based on the work described in References 7 to 15.

peptide conformation due to the hydrophobic lipid environment of the ER generates the energy required for translocation. Current experimental data do not allow definitive conclusions to be made about which mechanism is correct.

The third event in export of most secretory proteins involves proteolytic removal of the signal peptide by a peptidase located on the luminal (noncytoplasmic) face of the ER (Figure 1A). Eukaryotic signal peptidase has not been purified or characterized in any detail. Most proteins utilizing the ER translocation apparatus undergo this proteolytic processing step. However, exceptions have been documented both for secreted proteins and for integral membrane proteins.[23,26-29] For example, ovalbumin (a secreted protein), opsin (a plasma membrane protein), and cytochrome P450 (an ER membrane protein) contain noncleavable amino acid sequences near their NH_2-termini, which interact with the ER translocation apparatus and allow cotranslational binding to it (presumably through SRP and docking protein). These examples of proteins with noncleavable signal peptide "equivalents" show that proteolytic processing is not an obligatory step in cotranslational translocation.

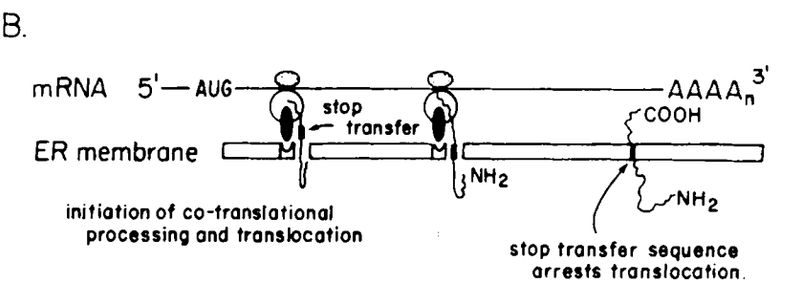


FIGURE 1B. Stop-transfer sequences arrest cotranslational translocation of integral membrane proteins.

Although secretory and integral membrane proteins share binding and signal peptide cleavage steps, membrane proteins do not complete the translocation process. Therefore, it has been suggested that "stop-transfer sequences" within integral membrane proteins arrest the translocation process[29,30] (see Figure 1B). These stop-transfer sequences include highly hydrophobic residues which, in the mature proteins, remain embedded within the membrane bilayer. However, adjacent hydrophilic sequences may also be required to arrest translocation.[31] This concept is of some importance in understanding the biosynthesis of apolipoproteins. Their known interactions with phospholipids and cholesterol are believed to occur via hydrophobic peptide domains which might resemble stop-transfer sequences.

B. Cotranslational Translocation of Apolipoproteins

The method generally used to analyze cotranslational translocation and signal peptide cleavage involves in vitro translation of mRNA sequences in the presence or absence of RER membranes.[5,32] These membranes contain signal peptidase activity. Signal peptide cleavage is assessed by comparing the NH_2-terminal sequences of the intact primary translation product and the protein generated after exposure to ER membranes. Translocation is examined by determining if the polypeptide synthesized in the presence of ER membranes is resistant to exogenous proteases (Figure 2). These proteases are added after translation is completed. Sequestration within the lumen of the ER (microsomal) vesicles protects the protein from the added proteases. Cotranslational insertion and integration in the ER membrane without translocation results in a protein which is fully or partly resistant to exogenous proteases even in the presence of low concentrations of nonionic detergents (see Figure 2).

Chan et al.[33] were the first to demonstrate that (avian) apolipoproteins VLDL-II and AI contain cleavable signal peptides. Subsequently, several groups have determined that mammalian apolipoproteins AI,[34-38] AII,[38,39] AIV,[40,41] CIII,[42] and E[43-45] all possess cleavable signal sequences. Based on an analysis of full-length cDNA clones, it appears that human apo CI[46] and CII[47,48] also contain NH_2-terminal extensions not represented in the mature plasma proteins. Identification of the COOH-terminal residues of the apo CI and CII signal peptides will require cotranslational cleavage assays. No information is currently available about the cotranslational processing of apo B or D.

These studies suggested that cotranslational proteolytic processing and translocation of apolipoproteins proceeded along pathways that were common to other secretory proteins. This proposal was examined in greater detail in cases of apo AI and AIV. Stoffel and coworkers[35] showed that SRPs are required for cotranslocational translocation of apo AI. In addition, SRP arrested translation of apo AI mRNA.[35] Studies with translocation-competent microsomes and exogenouses proteases[35-37,40,41] indicated that apo AI and AIV were completely transferred across ER membranes rather than being subjected to the arrested transfer characteristic of integral membrane proteins (compare panel 3 of Figure 2 with Figure 3).

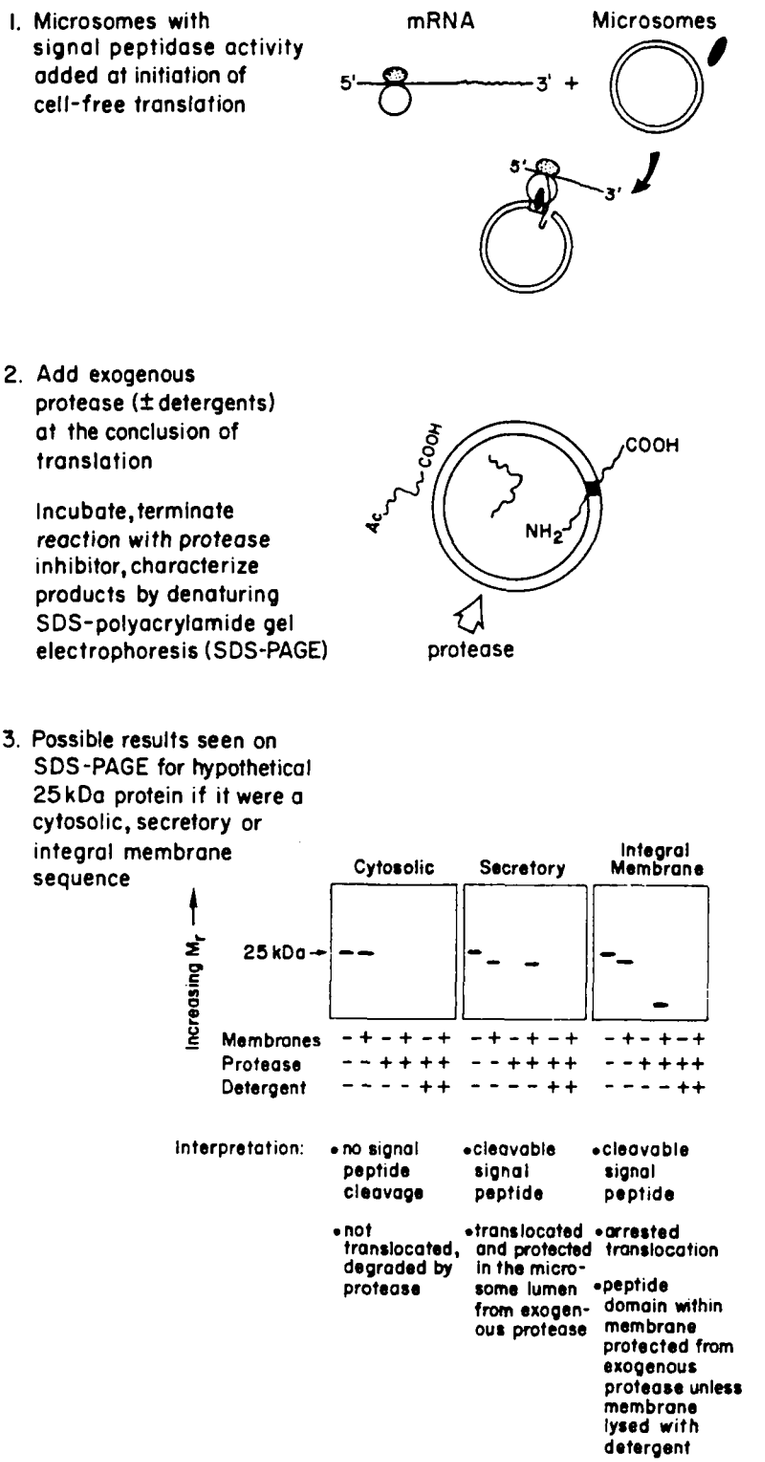


FIGURE 2. Cotranslational translocation assay.

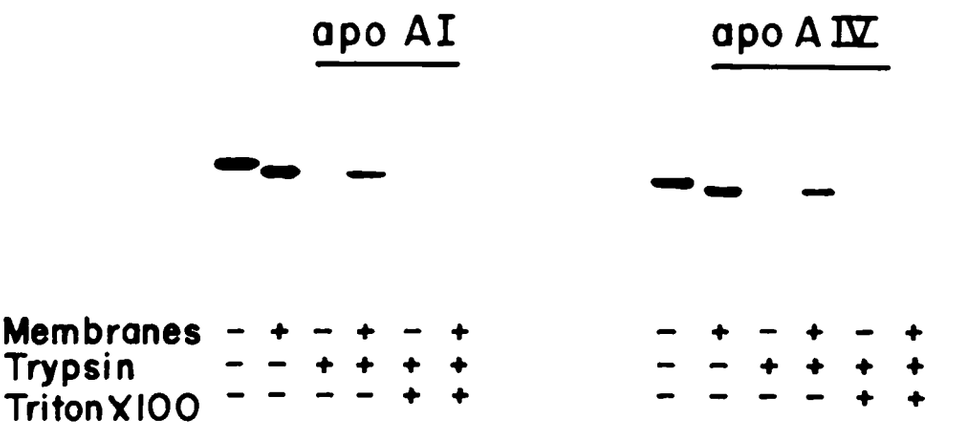


FIGURE 3. Cotranslational translocation and proteolysis of rat preproapo AI and preapo AIV. Ascites tumor cell lysates containing [^{35}S]methionine were programmed with rat small intestinal epithelial RNA.[36] Where indicated, microsomes prepared from ascites cells were added cotranslationally as described in Reference 32. Trypsin (1 μg/60 μl translation reaction mixture) was added post-translationally with or without Triton® X-100 (final concentration 0.1%). The solution was incubated for an additional 30 min at 37°C and apo AI and AIV isolated by immunoprecipitation with monospecific antisera in the presence (when appropriate) of soybean trypsin inhibitor. The denatured, immunoreactive proteins were subjected to electrophoresis through denaturing polyacrylamide slab gels (10% for apo AIV and 12.5% for apo AI) containing SDS. Fluorographs of the gels are shown.

Thus, the tandemly repeated amphipathic alpha helical segments found in these apolipoproteins (see References 49 to 52) do not seem to function as stop-transfer sequences. In view of the fact that apolipoproteins will spontaneously interact with synthetic vesicles composed of lipids similar to those found in the ER membrane, these translocation experiments could be interpreted as supporting the concept that a protein pore is present during transfer. Alternatively, the complete stop-transfer sequence of integral membrane proteins may not be present in these apolipoproteins.

C. Structures of Apolipoprotein Signal Peptides

Based on limited amino acid sequence data, Blobel and co-workers[5,53] originally predicted that signal peptides would have identical or very similar primary structures. This notion was quickly discarded after the signal sequences of proteins synthesized in the same tissue were found to differ.[54] Similarly, the signal segments of several orthologous* proteins[56] also had nonidentical sequences. These differences existed, yet cotranslational translocation and proteolytic processing of signal peptides apparently proceeded with high efficiency for all proteins. Therefore, Schechter and associates[57] suggested that the central core of hydrophobic amino acids represented the common signal involved in binding and translocation. This central hydrophobic core of 10 to 14 residues is found in all signal sequences.[58]

Inouye and co-workers[24] were among the first to suggest that signal peptides could be divided into three functional regions (Figure 4). The NH_2-terminal region is hydrophilic and usually positively charged. The hydrophobic central core is not interrupted by charged residues and has a very high probability of forming an extended alpha-helical or beta-sheet structure in aqueous solutions. This core region terminates with charged residues or those

* Fitch and Margoliash[55] have classified homologous sequences as being either orthologous or paralogous. Orthologous sequences reflect the phylogenetic branching order of the species in which they are found and generally have identical functions. Rat and human apolipoprotein AI would be considered orthologous proteins according to this scheme. Paralogous sequences arise from a gene duplication event that was fixed prior to speciation and generally have different functions. Alpha globin and myoglobin are paralogous polypeptides.

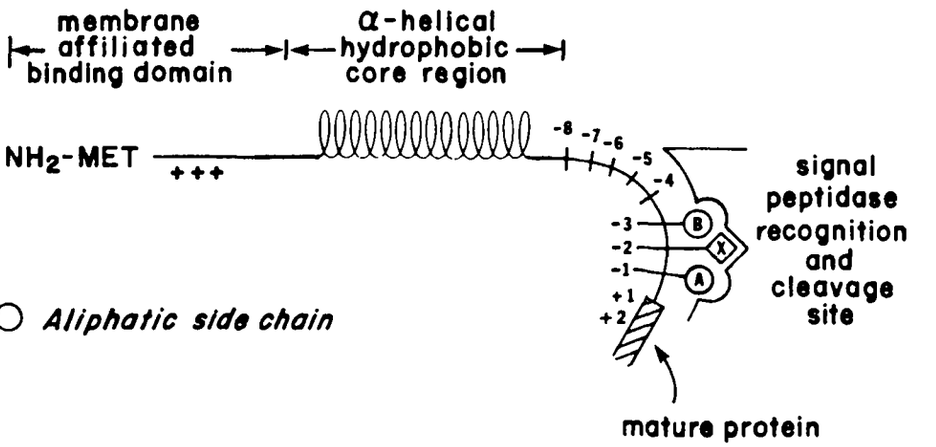


FIGURE 4. The general structure of eukaryotic signal peptides.[56,59,60] This regional model of prepeptides was first suggested by Inouye and co-workers.[24] The NH_2-terminus is hydrophilic and most frequently comprised of positively charged residues. The hydrophobic core of 12 to 14 residues is not interrupted by charged residues. Application of empirical rules for predicting secondary structure has indicated that this domain has a high probability of forming an extended alpha-helical or beta-sheet structure. The COOH-terminal region of the signal peptide terminates with charged residues or residues which are likely to form a beta bend. The signal peptidase recognition site is not composed of an obvious canonical sequence although the −1 and −3 positions (A and B in the figure) are typically occupied by amino acids with small side chains (alanine, serine, and glycine are most common).

likely to form a beta bend. The beta bend is followed by the signal peptidase cleavage site. Numerous studies have compared the primary structures of signal peptides as well as their predicted secondary structural characteristics.[56,58,59] All have shown significant agreement with this "regional" model and have furnished some interesting additional details.[56,59] In particular, the 6 to 8 amino acids proximal to the signal peptidase cleavage site (residues −1 to −8) appear to contain a more highly conserved primary structure than the rest of the signal sequence. The beta-bend region occurs between residues −4 and −7. This is followed by a triplet at positions −3 to −1 with the sequence A-X-B (Figure 4), where A and B are most often alanine residues or other neutral amino acids with small side chains (serine and glycine are also common). The −2 position (X in the "recognition" sequence) can apparently be any amino acid. Rules for predicting signal peptidase cleavage sites have been derived based upon these analyses.[56,59,60] Such rules are useful when a cDNA sequence has been determined and the precise size of the signal peptide is not known.

The structures of signal peptides present in the apolipoprotein AI, AII, AIV, CI, CII, CIII, and E primary translation products are shown in Table 2. With the exception of the apo CI and CII peptides, the precise site of cleavage by signal peptidase has been determined directly by amino acid sequence analyses of the intact and cotranslationally cleaved in vitro products. The boundaries of the basic NH_2-terminal region, the central hydrophobic core, as well as the beta-bend region with its associated peptidase "recognition" site have been demarcated in the table. It is clear that the apolipoprotein signal sequences generally mimic the pattern of organization seen in the prepeptides of other secretory proteins (Figure 4). Two exceptions to this statement are encountered in the signal peptides of human apo CI and rat apo E. The apo CI prepeptide contains a very long signal peptidase recognition site (see Table 2). The extended protease recognition site is unusual, but the biological significance of this structural "anomaly" is not known. In addition, a proline residue interrupts the hydrophobic core region of the rat apo E signal peptide.[61] The presence of a helix-breaking proline residue in this position (−8) is unexpected and atypical.

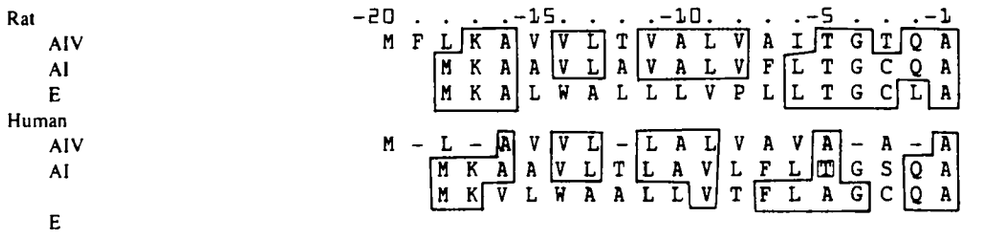
Table 2
NH_2-TERMINAL SEQUENCES OF THE PRIMARY TRANSLATION PRODUCTS OF MAMMALIAN APOLIPOPROTEIN mRNAs

	Signal peptide				
	Charged region	Hydrophobic core region	Peptidase cleavage site ↓	Pro peptide	Mature protein
Rat AI	M K	A A V L A V A L V F L	T G C Q A	W E F W Q Q	D E
Human AI	M K	A A V L T L A V L F L	T G S Q A	R H F W Q Q	D E
Human A-II	M K	L L A A T V L L L T I	C S L E G	A L V R R	Q A
Rat A-IV	M F L K	A V V L T V A L V A I	T G T Q A		E V
Human A-IV	M — L —	A V V L — L A L V A V	A — A — A		E V
Human C-I	M R L F L S L P	V L V V V L S I V L	E G P A P A Q G		T P
Human C-II	M G T R	L L P A L F L V L L V L	G F E V Q G		T Q
Rat C-III	M — — —	M L L — — A L — A L L A	— A — A		— E
Human C-III	M Q P R	V L L V V A L L A L L A	S A R A		S E
Rat E	M K	A L W A L L L V P L L T	G C L A		E G
Human E	M K	V L W A A L L V T F L A	G C Q A		K V

Note: The single letter code for amino acids is used: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine. " — " indicates that no amino acid assignment has been made at this position. Only the first two (NH_2-terminal) residues of the mature plasma proteins are shown.

Table 3

A. Signal Peptide Homologies Among Apolipoproteins AI, AIV, and E



B. Pairwise Comparisons of the Percent Amino Acid Sequence Identity Among the apo AI, AIV, and E prepeptides

		Rat		
		AIV	**AI**	**E**
Rat	AIV	—	67	28
	AI		—	44
	E			—

		Human		
		AIV	**AI**	**E**
Human	AIV	—	≥33	≥6
	AI		—	39
	E			—

		Human		
		AIV	**AI**	**E**
Rat	AIV	≥55	56	22
	AI	≥38	72	50
	E	≥17	50	67

Note: Signal peptides have been aligned at their cotranslational cleavage sites. A dashed line indicates that an amino acid assignment has not been made for that position. Calculations for panel B are based on data shown in panel A of this table.

Table 3 underscores one very unusual feature of some of the apolipoprotein signal sequences. The prepeptides of the three principal rat HDL apolipoproteins — AI, AIV, and E — are highly homologous.[36,51,61] For example, the signal peptides of rat apo AI and AIV are identical in 12 of 18 comparable positions. This amount of signal sequence homology (67%) among "different" proteins, even those which are synthesized in the same tissues, is extraordinary. Although the complete sequence of the human apo AIV signal segment is not known, similar degrees of homology appear to be present in human apo AI, AIV, and E (Table 3A and B). The −5 to −1 positions of the AI/AIV/E propeptides exhibit the greatest degree of sequence conservation.

Boguski et al.[51,52,62] have used a variety of computational methods to quantitatively describe the relationships between rat apolipoproteins AI, AIV, and E. These comparisons

suggested that their "mature" protein domains were derived from a common ancestral gene. This gene was most likely the result of *intra*genic duplications involving 33 and 66 nucleotide segments specifying oligopeptides with amphipathic alpha helical characteristics. *Inter*genic duplications probably then produced a primordial apo E gene and AI/AIV precursor. Subsequent evolution led to distinct apo AI and AIV genes. Further appreciation of the evolutionary, structural, and functional significance of the *prepeptide* homologies mentioned above may come once the patterns of apo AI, AIV, and E gene organization can be compared. For example, does the distribution of exons and introns in the apo AIV gene mimic that found in the apo AI or E genes? The structure of the human apo AIV gene has not been determined. However, the exons of the apo AI and E genes encode similar structural domains of their primary translation products.[63-65] The second exons of the apo AI and E genes both specify the first 14 amino acids of their respective prepeptides. Furthermore, the -1 to -4 residues of their signal peptides, as well as the NH_2-terminal region of the mature plasma proteins, are encoded by comparably positioned exons (exon 3). Finally, their repeated sequence domains are also contained within a single (fourth) exon. These observations raise the question of how the apo AI and E (and AIV) genes were assembled and whether the signal peptide homologies reflect the "later" addition of (common) exonic components to a primordial sequence block encoding their tandemly arrayed, amphipathic alpha helical docosapeptide repeats.

D. The Functional Domains of Apolipoprotein Signal Peptides

Two methods have been used to study prepeptide domains. In bacteria, mutations affecting the signal sequences of several secreted proteins have been identified and characterized (reviewed in Reference 60). Bacterial mutations have also been created by in vitro site-directed mutagenesis or by fusion of signal segments to other gene products (see References 66 and 67 for reviews). These studies have provided strong support for many of the concepts outlined above concerning the function of various regions of signal peptides. Studies in eukaryotes have generally been limited to a second technique — in vitro incorporation of amino acid analogs into primary translation products.[68] The newly synthesized cell-free products undergo conformational changes. The effects of these changes are monitored using cotranslational cleavage/translocation assays. Hortin and Boime[68] were the first to demonstrate that incorporation of β-hydroxyleucine into a secretory protein during translation could sufficiently alter its structure so as to prevent cotranslational translocation. Translocation was inhibited only for those proteins with a high percentage of leucines in their signal peptides. Inhibition was not a function of the leucine content of the *mature* portion of the molecule. Thus it appeared that prevention of translocation was due to substitution of a more polar amino acid (analog) in the central hydrophobic core region of the signal sequence. Further substantiation of this conclusion was provided when Walter and Blobel[8] showed that incorporation of β-hydroxyleucine into the signal peptide of bovine prolactin inhibited its binding to SRP.

Incorporation of β-hydroxyleucine into the primary translation product of rat apo AI mRNA does not prevent its normal cotranslational translocation.[36] This is related to the relative paucity of leucines in the apo AI signal peptide. Taken together with the work on analog incorporation into other signal segments, these data support the conclusion that the hydrophobic core of apolipoprotein prepeptides is essential for binding to SRP. Further studies using in vitro site-directed mutagenesis will be required to determine the functional importance of various regions (both conserved and nonconserved) of mammalian apolipoprotein signal peptides.

III. POST-TRANSLATIONAL PROCESSING OF APOLIPOPROTEINS

A. Current Concepts of Proprotein Processing

1. Intracellular Compartmentalization of Propolypeptide Cleavage Systems

Following cotranslational removal of signal peptides, secretory proteins are transferred from the ER to the Golgi complex.[69,70] The Golgi complex consists of a polarized stack of discoidal cisternae. These cisternae are bounded on so-called cis and trans faces by small vesicles.[71] The precise mechanism by which proteins are transferred from the RER to the Golgi is not known. One hypothesis postulates that vesicles bud from specialized regions of the RER. These vesicles contain proteins derived from the ER lumen and subsequently fuse with cis Golgi elements.[72]

Many believe that the ER-to-Golgi transport vesicles are "coated".[73,74] Membranes of coated vesicles contain a variety of peptides including clathrin, a large 180-kDa protein, and a smaller 35-kDa polypeptide. These two proteins are organized into a basic unit (a triskelion) which coats the vesicle membrane.[75] In fact, coated vesicles are thought to be generally responsible for intracellular transport of macromolecules (proteins and lipids) between organelles.

Although macromolecular sorting in the secretory pathway begins within the ER, coated vesicles and the Golgi apparatus provide additional mechanisms for insuring the accuracy of this process. Receptors present in the Golgi membrane participate in the editing process by binding to specific domains of polypeptides. An example of this is the mannose-6-phosphate receptor which binds to the oligosaccharides of hydrolases destined to be incorporated into lysosomes (see Section IV.E for a more detailed discussion). Additional selectivity in sorting/targeting may be imparted by proteins in the coated vesicle membrane. Rothman[76] has proposed that, after removal of the clathrin-containing triskelion, these proteins are able to interact with specific receptors contained on target organelle membranes.

Given these concepts of the organization of the secretory apparatus, where in the pathway does precursor protein processing occur? Post-translational proteolysis of proproteins *begins* approximately 10 to 20 min after synthesis has been completed and the folded polypeptide has been transported to the Golgi area.[77-80] A variety of experimental tools have been used to determine that polypeptide processing commences within the Golgi and continues in secretion granules formed from trans Golgi elements. For example, Steiner et al.[77,78] and Orci[81,82] employed antimycin A which blocks transport of proteins from the RER to the Golgi complex, and/or pulse chase experiments to demonstrate that proinsulin conversion begins in the Golgi. Orci[82] and Orci et al.[83] also reported that after transport from the RER to the Golgi complex radiolabeled *pro*insulin appears in clathrin-coated secretory granules at the trans face of the Golgi complex. More recently, Orci et al.[83] showed that monensin, a carboxylic ionophore, results in reduced proinsulin processing in pancreatic B cells and accumulation of radiolabeled proprotein in a clathrin-coated membrane compartment consisting of Golgi cisternae and secretory granules. They used these observations to suggest that normal proinsulin processing requires unimpeded transfer through this clathrin-coated membrane compartment related to the Golgi complex.[83] As the coated granules mature, they lose their clathrin coat.[82,83] Completion of proprotein cleavage may coincide with condensation of the reaction products and morphologic transformation of secretion vesicles to storage granules. The final step of protein export involves release of granule contents by exocytosis.[80]

2. Initial Post-Translational Processing of Propolypeptides by Endoproteases

Analysis of the precursors of a number of secretory proteins indicated that post-translational cleavage occurred at sites containing paired basic amino acids (e.g., arg-arg or lys-arg, see Figure 5). Little else distinguished the cleavage sites. For example, there appeared to be no other flanking amino acids which formed a consensus cleavage sequence. Furthermore,

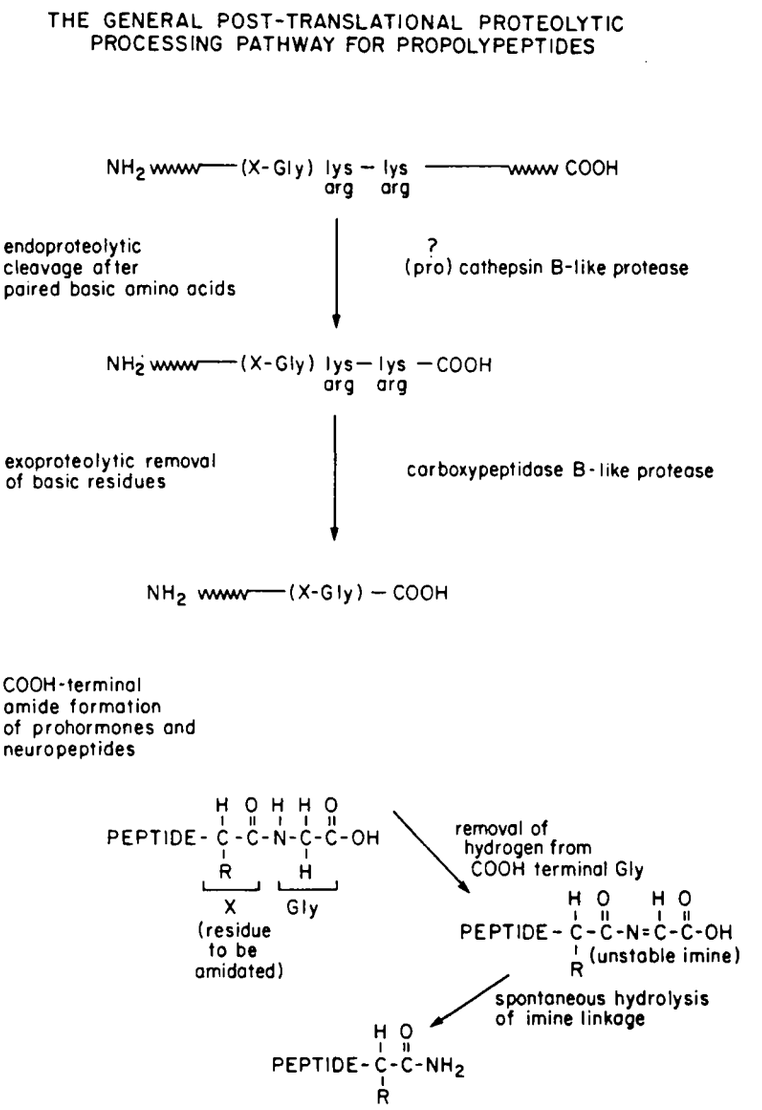


FIGURE 5. The general post-translational proteolytic processing pathway for propolypeptides. The dipeptide X-Gly is bounded by parentheses to indicate that it is not always found in this position of propolypeptides. Rather, it is a sequence commonly encountered in those prohormones and neural peptides which undergo COOH-terminal amidation after post-translational proteolysis. The mechanism shown for this amidation reaction was proposed by Bradbury et al.[117] and is adapted from Reference 113.

secondary structure predictions of the cleavage site did not disclose a typical configuration — leading to speculation that the only structural requirement for efficient cleavage is that the site be well exposed on the protein surface.[79,80] There are two additional pieces of evidence that paired amino acids are essential for efficient intracellular processing of these precursor proteins. Abnormal human proalbumins have been described in which substitution of a histidine or glutamine for one of the two arginine residues at the cleavage site markedly reduces cleavage efficiency.[84,85] Similarly, incorporation of canavanine, an arginine analog, or thialysine, into prohormone precursors blocks prosegment removal.[86]

Intracellular proteolytic processing of a protein precursor may involve cleavage at one or two sites to yield a single product (prototypes include proalbumin, proinsulin, and proparathyroid hormone). Alternatively, processing may require selection among many potential sites to generate one of several possible peptides — each with different biological functions. The proopiomelanocortin precursor is an example of the latter.[87] Differential processing of this protein in anterior and middle lobes of the pituitary can give rise to adrenocorticotropin or beta-melanocyte stimulating hormone, respectively.[88]

There are some exceptions to the rule that *intracellular* processing occurs after paired basic residues. The somatostatin,[89-92] vasopressin-neurophysin II,[93] cholecystokinin,[94] vasoactive intestinal peptide,[95] and canine pancreatic polypeptide[96] precursors are all processed after single basic (usually arginine) residues. However, cleavage after single basic residues is more typically an *extracellular* event and is frequently encountered during activation of zymogens (e.g., the clotting factors, see Reference 97). Intracellular proprotein proteolysis involving a leu-ala sequence can occur during processing of the human, porcine, and rat proinsulin C peptides,[98,99] while processing of the connecting peptide of prorelaxin involves cleavage of a leu-ser bond.[100]

The endoprotease responsible for post-translational proprotein processing after paired basic residues has not been fully characterized. Analysis of proinsulin, proglucagon, and prosomatostatin processing by granule fractions from anglerfish islets,[101] proopiomelanocortin cleavage by rat pituitary intermediate lobe granules,[102,103] as well as proalbumin conversion in rat liver lysosomes[104] has indicated that a thiol protease, similar but not identical to cathepsin B, may be responsible for propolypeptide conversion.[80] Steiner and co-workers[105-107] have used site-specific probes (^{125}I-Tyr-Ala-Lys-Arg chloromethyl ketone and ^{125}I-Tyr-Ala-norleucine-Arg chloromethyl ketone) to further characterize this thiol protease in various subcellular fractions of a rat insulinoma and in normal, partially purified rat islet granule preparations. These probes react with 25-, 31.5-, and 39-kDa proteins. Based on inhibitor studies and an analysis of pH optima, the 39- and 31.5-kDa proteins have been identified as cathepsin B-like proteases, while the 25-kDa polypeptide is probably cathepsin L or H.[107] Both molecular weight forms of the cathepsin B-like protease are present in secretion granules and lysosomes, while the 25-kDa form appears to be confined to lysosomes.[107] Docherty et al.[80,106,107] have made three recent observations that provide clues about how the thiol protease may interact with its proprotein substrate. First, they found that purified cathepsin B (M_r, 31.5 kDa) does not correctly cleave proinsulin. Second, they found that cathepsin B is initially synthesized as a larger 44-kDa precursor. Finally, they have presented evidence that the 39-kDa form represents a precursor of the 31.5-kDa protein and that this possible intermediate form binds the affinity probe containing two basic residues with higher efficiency than the affinity probe which contains a single basic residue. They therefore speculated that higher molecular weight "pro" cathepsin B precursors may, during the course of their own processing, be targeted to the same secretion granules as their proprotein substrates — allowing thiol protease and proprotein processing to occur in a coordinated manner. "Mature" cathepsin B on the other hand may segregate primarily into lysosomes. The fractional representation of "pro" vs. "mature" protease in an organelle may determine whether the enzyme expresses its specific endopeptidase as opposed to general degradative function.[80,107]

It is unclear whether the enzymatic activity responsible for cleavage after *paired* basic residues is also the same activity which mediates intracellular processing at those sites formed by a *single* basic amino acid. There is some indirect evidence that separate enzymes are involved. For instance, injection of mRNA preparations encoding the vasopressin-neurophysin II precursor into frog oocytes resulted in synthesis of a propolypeptide precursor with the following organization: NH_2-vasopressin (gly-lys-arg)-neurophysin II-(arg)-glycopeptide-COOH. However, this precursor was only processed at the single basic residue (arg) which

separates the COOH-terminal glycopeptide from neurophysin II. The lys-arg dipeptide which separates vasopressin and neurophysin II was not cleaved.[108] Similarly, the ability of cultured canine pancreatic F cells to process the pancreatic polypeptide precursor at a single basic residue was lost over time, while the capacity to process precursors after dibasic residues was retained.[96]

3. The Role of Exoproteases in Propolypeptide Processing

Studies on proinsulin processing have shown that following endoproteolytic cleavage an exopeptidase removes the COOH-terminal basic residues remaining in the reaction products (Figure 5). This activity has been characterized in insulin secretory granules[109] and is a metal-dependent carboxypeptidase B-like protease with an acidic pH optimum (5.5) and M_r of 54 kDa. A similar, if not identical, enzyme has been isolated from bovine adrenal medullary (chromaffin) granules and referred to as enkephalin convertase because of its apparent role in the generation of enkephalins from proenkephalin A.[110-112]

Carboxypeptidase-mediated removal of the COOH-terminal paired basic residues should result in polypeptides with a free carboxyl group (Figure 5). However, many biologically active peptides generated from precursors possess COOH-terminal alpha-amide groups.[113-115] Moreover, the alpha-amide is often essential for expression of the biological activity of these peptides. For example, deamidated forms of gastrin and cholecystokinin are inactive.[116] Structural analyses of this group of proteins have shown that a glycine residue is frequently situated proximal to the paired basic amino acids which form the initial thiol protease cleavage site.[113,114] In other words, precursor proteins which yield amidated peptides generally contain the sequence: NH_2-X-glycine-basic amino acid-basic amino acid-COOH, where X is the residue which is amidated (see Figure 5). Current evidence indicates that, while a variety of amino acids (X) can be amidated, those with charged side chains are less frequently modified.[113,117] Generation of terminal amides appears to be an event which follows the action of the carboxypeptidase and represents oxidative cleavage of this terminal glycine by an enzyme which requires molecular oxygen, ascorbic acid, and copper.[117,118] The presumed mechanism for COOH-terminal amide formation is shown in Figure 5.

4. Exceptions

While this scheme of propolypeptide processing is felt to be followed by most eukaryotic precursor polypeptides, another processing pathway has been recently described. This involves stepwise removal of dipeptides from the NH_2-terminus of protein precursors (Figure 6). A dipeptidylaminopeptidase has been found to catalyze the stepwise removal of the prosegment of promelittin.[119-121] This reaction commences at the NH_2-terminus of the propolypeptide and proceeds to completion at the Ala-Gly bond which separates the prosegment from the mature protein. The melittin propeptide consists of a repetitive sequence featuring an alanine or proline residue at even-numbered positions (emphasized by the boxes in Figure 6). The dipeptidylaminopeptidase has apparent specificity for paired amino acids which contain either a proline or alanine (or perhaps glycine) at the second position. The stepwise cleavage of dipeptides from the melittin prosegment is probably an *extracellular* rather than intracellular event.[121]

Stepwise activation of precursor proteins by a dipeptidylaminopeptidase has also recently been demonstrated in *Saccharomyces cerevisiae*.[122-124] The primary translation product of yeast alpha-mating factor mRNA contains an NH_2-terminal nonrepetitive domain followed by 2 to 4 tandemly repeated peptides that contain the alpha-mating factor (Figure 6). These repetitive sequences are separated by spacer oligopeptides with the general structure Lys-Arg-$[^{Glu}_{Asp}$-Ala$]_{2,3}$. Initial processing involves excision of the paired basic amino acids. The product released by this proteolytic step contains the alpha-mating factor domain plus an NH_2-terminal extension (Figure 6). This intermediate, which has reduced biological

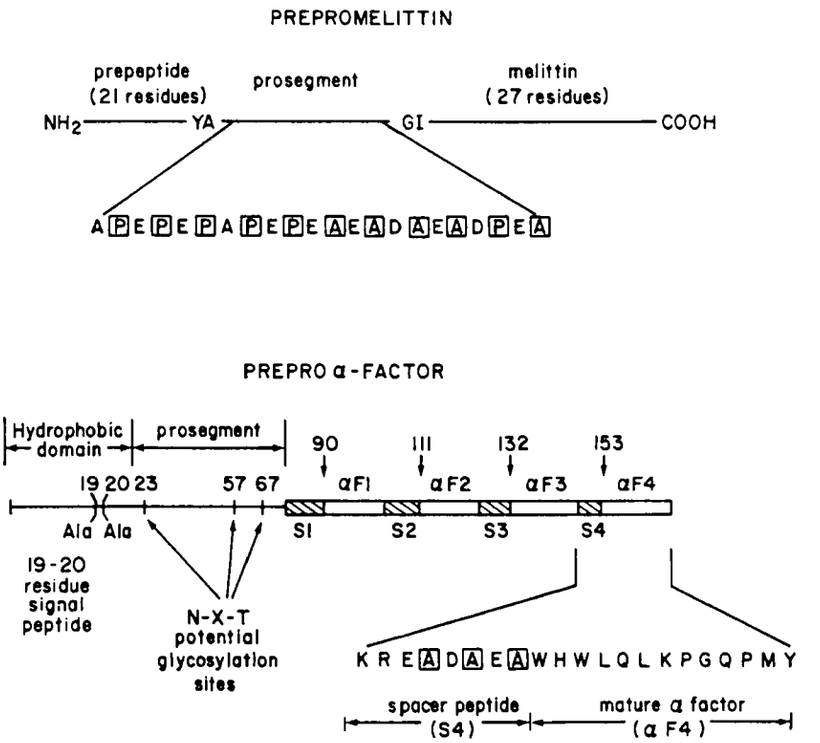


FIGURE 6. Alternative post-translational proteolytic processing pathways. Examples of precursor protein processing by dipeptidylaminopeptidases are shown. Kreil and co-workers have shown that the prosegment of honey bee melittin undergoes stepwise cleavage of dipeptides which terminate with either alanine or proline.[121] Most of the dipeptidylaminopeptidase is soluble and present in the venom sac.[97] The organization of the primary translation product of prepro α factor was deduced by Kurjan and Herskowitz[122] from an analysis of cloned DNA. The NH_2-terminus contains a hydrophobic domain which encompasses a signal sequence of 19 or 20 residues (the site of signal peptidase cleavage may be at ala_{19} or ala_{20}). An additional nonrepetitive domain contains three potential sites for N-linked glycosylation (indicated by the tripeptide asparagine-X-threonine). The COOH-terminus of the molecule contains four copies of the mature alpha mating factor (αF1-αF4) flanked by spacer peptides (S1-S4). Generation of the mature pherome involves initial cleavage of the spacer peptides after paired basic amino acids. This is followed by sequential removal of dipeptides (X-alanine) by a heat-stable, membrane-bound dipeptidylaminopeptidase.[124]

activity, is then converted to the mature active pherome by a heat-stable, membrane-associated, dipeptidylaminopeptidase.[97,124]

These last two examples illustrate that, while a general pathway for post-translational precursor protein processing has been identified in eukaryotes, variations exist.

B. Proapolipoprotein AII Processing

Apo AII is the second most abundant apolipoprotein in human plasma HDL, comprising about 25% of HDL protein mass. More than 90% of plasma apo AII is affiliated with lipoprotein particles. Human (plasma) apo AII consists of 2 identical 77 amino acid polypeptides that are covalently linked by a Cys_6-Cys_6 disulfide bridge.[125] This disulfide bridge apparently does not impose significant structural constraints on the protein: monomeric and dimeric apo AII exhibit similar circular dichroism spectra, similar surface activities at air-water interfaces, and similar thermal properties.[126,127] The function of apo AII is unclear. In vitro studies have suggested that apo AII can inhibit lecithin-cholesterol acyl transferase (LCAT), activate hepatic lipase, and participate in the conversion of HDL_2 to HDL_3.[128]

Table 4
POST-TRANSLATIONAL PROCESSING SITES IN APO AI AND AII

	Propeptide	Mature protein
Apo AI		
Rat	W E F W Q Q	D E P -[a]Q S . . . .
Human	R H F W Q Q	D E P P Q A . . . .
Apo AII		
Human	A L V R R ▲	Q A K E P C . . . .
	Post-translational processing site	

[a] Based upon optimal alignments of nucleotide and protein sequences, Boguski et al.[52] and Poncin et al.[153] determined that a proline residue has been deleted from this position in rat apo AI.

When apo AII is incubated with lipids, it forms lipid-protein complexes which are more heterogeneous in density and size than apo AI-containing particles. This suggests different roles for apo AI and AII in determining HDL structure.[129]

1. Definition of the Prosegment

The primary translation product of human liver and intestinal apo AII mRNA is 100-amino acids long.[38,39,47,130,131] This polypeptide contains a 23-residue NH_2-terminal extension that is not represented in mature plasma HDL particles (Table 2). In vitro cotranslational cleavage experiments indicated that the 23-amino acid NH_2-terminal extension contained an 18-residue segment which could be functionally described as a prepeptide.[39] The protein remaining after cotranslational removal of this 18-amino acid signal sequence contains 5 additional NH_2-terminal amino acids which are not present in plasma HDL-affiliated apo AII (Table 4). This pentapeptide segment — Ala-Leu-Val-Arg-Arg — resembles most propeptides, since it terminates with paired basic amino acid residues. The stable intracellular form of apo AII has recently been characterized in a human hepatoma-derived cell line known as Hep G2.[39] These cells synthesize and export at least 7 apolipoproteins: AI, AII, AIV, B, CII, CIII, and E.[41,132] Pulse-labeling studies showed that more than 90% of intracellular apo AII contains the NH_2-terminal pentapeptide prosegment.[39] These data therefore confirmed that the primary translation product of apo AII mRNA specified a prepropolypeptide consisting of an 18-amino acid signal sequence, a 5-amino acid prosegment, and a 77-amino acid mature protein.

2. Definition of the Compartment Where Proapo AII is Processed

Hep G2 cells have been utilized to study the processing of a variety of protein precursors including proalbumin[133] and procomplement factors.[134,135] Redman and co-workers[133] performed detailed pulse-chase experiments to show that proalbumin was correctly processed with high efficiency prior to export from these cells. Pulse-chase experiments have also been used to compare proapo AII and proalbumin processing by Hep G2 cells maintained for short periods in serum-free medium. The results[136] indicated that apo AII prosegment removal was primarily an *extracellular* event, in contrast to the predominant *intracellular* processing of proalbumin — even though in both cases cleavage occurred after paired arginine residues. Extracellular proapo AII conversion was relatively slow with a $T_{1/2}$ of 6 hr.[136]

3. Characterization of the Proapo AII Converting Enzyme in Hep G2 Cell Cultures[136]

Extracellular cleavage of proapo AII after *paired* basic residues represents a novel processing pathway. Extracellular processing could reflect secretion of one or more soluble

proteases by Hep G2 cells or direct interaction of the propolypeptide with the cell surface. When Hep G2 cells are subjected to a short pulse labeling with [^{3}H] amino acids and then removed by centrifugation, processing of radiolabeled proapo AII remaining in the supernatant (media) fraction is markedly reduced compared to control incubations containing cells. However, when unlabeled, serum-free media is harvested from overnight Hep G2 cell cultures and then added to [^{3}H] media obtained from parallel cultures after a short pulse labeling, efficient cleavage of proapo AII occurs. These two results not only provide further evidence that processing of proapo AII occurs in Hep G2 cell culture media, but implies that the protease(s) mediating prosegment removal is secreted by these cells. The converting activity in media is not blocked by the serine protease inhibitors phenylmethylsulfonylfluoride, aprotinin and furoyl saccharin, or by a metalloprotease inhibitor (*o*-phenanthroline). It is blocked by the thiolprotease inhibitors *p*-chloromercuribenzenesulfonic acid, leupeptin, antipain,and E-64.

More detailed inhibitor studies are necessary before a meaningful comparison can be made between the proapo AII processing enzyme in Hep G2 culture media and the intracellular propolypeptide-converting enzyme activity described in Section III.B.2. A thiol protease, which resembles lysosomal cathepsin B immunologically and catalytically, is exported from mouse mammary carcinomas and human breast tumors.[137,138] These proteases have a greater mass (39 to 40 kDa) than lysosomal cathepsins (25 to 35 kDa). Mort et al.[139] recently reported that human ovarian carcinomas secrete a single-chain 40-kDa thiol protease which reacts with antiserum to human liver cathepsin B. This larger cathepsin B-like protease can be activated following cleavage by pepsin to a 33-kDa form. These workers have proposed that the 40-kDa protein represents a (zymogen) precursor which is released by the neoplastic cells without being processed intracellularly and targeted to lysosomes.[139] The size of the thiol protease present in Hep G2 media has yet to be determined.

Although studies of proapo AII processing in Hep G2 cells document an extracellular site of cleavage, it is not known whether this occurs in vivo. These transformed cells recapitulate the in vivo proteolytic processing pathway followed by the other principal human plasma HDL apolipoprotein — apo I. This was established by comparing results obtained from cell and organ cultures[132,140] as well as by Edman degradation of apo AI isoforms purified from human thoracic duct lymph.[141] Similar types of analyses are needed to define the in vivo site of proapo AII processing. Isoform analyses may be particularly helpful for these studies since proapo AII is easily distinguished from mature apo II (the pI of proapo AII is 6.61 vs. 4.95 for the 77-amino acid plasma protein, Reference 136). In a recent study, Lackner et al.[142] detected proapo AII (isoforms) in human thoracic duct lymph and plasma, indicating that at least some of the propolypeptide is exported from hepatocytes and/or enterocytes in vivo.

C. Proapolipoprotein AI Processing

Apo AI is the principal apolipoprotein of human plasma HDL, comprising about 60% of its total protein mass. Apo AI is largely composed of tandemly repeated 22-amino acid segments. These docosapeptides are predicted to have amphipathic alpha-helical conformations — i.e., having both hydrophobic and hydrophilic faces.[49,50,143] It is this structural feature which is felt[143-146] to be responsible for two of the characteristic properties of apo AI — namely its ability to bind lipids as well as its capacity to activate LCAT — the enzyme which catalyzes esterification of cholesterol and conversion of phosphatidylcholine to lysolecithin.

1. Definition of the Prosegment

Edman degradation of the primary translation product of human intestinal[37] and liver apo AI mRNA[38] revealed that it contained a 24-amino acid NH_2-terminal extension which was

not represented in plasma HDL-affiliated apo AI (Table 2). This finding agreed with the predicted structure of the apo AI primary translation product derived from sequencing full-length cloned apo AI cDNAs (see References 47 and 147 to 150). In vitro cotranslational cleavage experiments employing ascites[37] and pancreatic[38] microsomes possessing signal peptidase activity (see Section II.B) indicated that the NH_2-terminal extension contained an 18-amino acid long prepeptide. Cotranslational removal of the signal segment produced a polypeptide with a hexapeptide, NH_2-terminal extension that had not been previously detected in plasma HDL-associated apo AI. This hexapeptide had no sequence homology with the apo AII prosegment (Table 4) and contained a COOH-terminal Gln-Gln dipeptide.

Pulse-labeling experiments with Hep G2 cells[37,150] indicated that the prosegment was present in greater than 95% of intracellular apo AI and that apo AI was exported from these cells without proteolytic processing of this hexapeptide domain. Similar analyses employing cell-free translation of *rat* intestinal apo AI mRNA,[36] as well as pulse labeling of cultured rat hepatocytes,[151] indicated that rat apo AI was initially synthesized as a precursor protein containing an 18-amino acid signal sequence plus a 6-amino acid propeptide and suggested that this prosegment was not removed prior to export.

Thus, the apo AI prosegment represented a second unusual apolipoprotein propeptide which differed from other prosegments in two respects: it did not terminate with paired basic amino acids and preliminary in vitro experiments indicated that processing was extra- rather than intracellular.

2. Evidence for In Vivo Extracellular Cleavage of Proapo AI

a. Studies in Rats

Sliwkowski and Windmueller[152] analyzed the biosynthesis, secretion, and metabolism of rat apo AI. To do this they defined the primary structure of the various isoforms of circulating apo AI. In humans, apo AI processing results in conversion of basic isoforms to relatively more acidic species.[37,132,140,141,150] The opposite is true for rat apo AI isoforms.[152] This difference is understandable after examining the structures of their respective propeptides (Table 4). The human prosegment contains two basic residues (arginine and histidine) which are replaced by a neutral and acidic amino acid (tryptophan and glutamic acid) in the rat sequence.[52,153] However, the COOH-terminal four residues of both propeptides are identical as are the NH_2-terminal three residues of the mature proteins. Therefore, the proapo AI cleavage sites are identical in these two species.

In vivo pulse-labeling studies indicated that 85% of the newly synthesized apo AI present in rat hepatocytes and enterocytes contained the propeptide.[152] This conclusion was based on Edman degradation of purified [^{3}H]-labeled apo AI isoforms. Autoperfused rat intestinal segments as well as isolated perfused livers exported proapo AI without significant proteolytic processing of the hexapeptide prosegment (i.e., proapo AI represented approximately 80% of labeled perfusate AI). The kinetics of (extracellular) proapo AI to apo AI conversion were defined using pulse-chase experiments. If one assumes that the plasma clearance rates of proapo AI and mature apo AI are similar, then in vivo conversion proceeds to 80% completion in the rat within 10 h. This value is close to the circulating half-life of rat apo AI.[154] These experiments appeared to provide a kinetic explanation for the observed steady-state fractional representation of proapo AI in rat plasma (0.29, Reference 152) and were consistent with the notion that liver and small intestine produce proapo AI, which is slowly processed to apo AI in the circulation.

Sliwkowski and Windmueller[152] made several other interesting observations during the course of their analysis of extracellular proapo AI processing in the rat. While heparinized blood (or plasma) was able to support accurate processing of proapo AI in vitro, this proteolysis was much less efficient than that noted in vivo. For example, after a 10 h in vitro incubation, 20% conversion occurred vs. the 80% proapo AI processing observed in

vivo during this same time interval. Moreover, virtually all of the in vitro processing which did take place occurred within 2 h. Very little additional processing was observed during longer incubations. In vitro conversion was blocked by EDTA and EGTA. Inhibition by these chelating agents was not reversed by added magnesium. Removal of the cellular elements in blood had no effect on the limited conversion observed in vitro. When [^{3}H]-proapo AI was recirculated through isolated livers for up to 5 h in the presence or absence of serum, no significant processing was found.

Several conclusions were made from the data.[152] First, a metal-dependent protease present in rat plasma appeared to participate in the conversion of proapo AI to apo AI. Second, this activity was unstable. Third, additional processing might require interaction of proapo AI with peripheral tissues. Finally, rat liver did not appear to contribute all the factors required for extracellular in vivo proapo AI proteolysis.

b. Studies in Humans

Experiments utilizing human fetal and adult intestinal and liver organ explants[140,155] as well as Hep G2 cells[37,132,150] indicated that apo AI was exported as a propolypeptide. Evidence that human apo AI was secreted as a proprotein in vivo was supplied by Brewer et al.[141] and Ghiselli et al.[156] These workers sequenced AI isoforms present in human lymph and plasma and found proapo AI was not only present in these extracellular compartments, but also was a major AI species in the density 1.006 g/mℓ fraction of lymph. The fractional (steady-state) representation of proapo AI in human plasma is significantly lower than in the rat (2% vs. ~30%, References 141, 152, and 157). These differences in relative proapo AI levels can be better understood as a result of the work of Bojanovski et al.[158] They purified proapo AI from lymph chylomicrons and mature apo AI from plasma HDL. The [^{125}I]-labeled AI species were then simultaneously infused into normolipidemic subjects. The results provided definitive proof that proapo AI was converted to mature AI in vivo: (1) proapo AI was completely converted to mature AI at a rate of 4 pools/day; (2) the average residence time of proapo AI was almost 30-fold less than mature apo AI (0.23 days compared to 6.5 days); (3) *mature* apo AI arising (in vivo) from proapo AI processing had a residence time that was identical to that of injected *mature* apo AI; and (4) the rate of proapo AI and mature apo AI *production* was similar in the volunteers.[158]

In humans, fat feeding is associated with an increase in the representation of proapo AI in lymph chylomicrons and VLDL.[156] A similar increase in rat plasma proapo AI concentration occurred after chronic exposure to a diet high in fat and cholesterol.[152] Curiously, in rats fed a fat-free diet supplemented with orotic acid, an even more marked increase in plasma proapo AI levels ensued with steady-state levels approaching 50% of total apo AI.[152] This latter diet was associated with a reduction in synthesis of hepatic VLDL and HDL and a concomitant lowering of plasma triglyceride and cholesterol levels. Taken together, these dietary manipulations suggested that the steady-state level of plasma proapo AI was not a simple function of the concentration of neutral lipids in plasma.[152]

3. Characterization of a Proapo AI- to Apo AI-Converting Activity in Human Plasma

The precise extracellular site of human proapo AI processing has not been identified. However, Edelstein et al.[159] found that an enzymatic activity capable of converting proapo AI to mature apo AI was associated with plasma HDL, VLDL, and lymph chylomicrons. Among these lipoprotein-density classes, plasma HDL contained the highest specific activity as well as amount of converting enzyme. When plasma samples were subjected to prolonged centrifugation (up to 48 h) at density 1.21 g/mℓ, some of the processing activity was found in the bottom fraction.[159] This may reflect displacement of the protease(s) from the lipoprotein surface by ultracentrifugual forces and/or high salt concentrations.

This plasma proapo AI- to AI-converting activity has been further characterized using a variety of inhibitors.[159,160,161] 1,10 *o*-Phenanthroline and EDTA inhibit the enzyme activity,

while calcium and magnesium ions activate it. This suggests that the plasma-converting activity is metal dependent. Its insensitivity to phenylmethylsulfonyl fluoride (PMSF) and diisopropylfluorophosphate (DFP) indicates that it is not a serine protease. These inhibitor studies clearly distinguish the plasma proapo I-converting activity from the thiol protease capable of processing proapo AII in Hep G2 cell cultures.[136]

The mechanism by which the plasma proapo AI-converting enzyme removes the prosegment has not been established. Specifically, it is not known if hexapeptide removal is a one-step process resulting from a unique cleavage of the Gln-Asp bond (see Table 4). Similarly, it is not possible to state whether the plasma proapo AI-converting activity is mediated by a single protease or whether the enzyme(s) is (are) endo- or exoprotease(s). Finally, the site of synthesis of the human proapo AI-converting enzyme(s) has yet to be defined.

A number of workers have isolated proteases from human plasma or serum which share some of the characteristics outlined for the proapo AI-converting activity. Sasaki et al.[162] partially purified two enzymes from serum which hydrolyzed the synthetic substrate succinyl-trialanine *p*-nitroanilide (suc-ala$_3$-*p*NA). This is a specific substrate for elastase, yet the enzymes did not cleave naturally occurring elastin. One of these enzymes was inhibited by EDTA and was affiliated with VLDL and LDL. Maeda et al.[163] have isolated two enzymes associated with HDL which have properties that are very similar to those reported by Sasaki and co-workers. One enzyme cleaves suc-ala$_3$-*p*NA to succinyl-dialanine and alanine *p*-nitroanilide. It was inhibited by dithiothreitol, EDTA, and 1,10 *o*-phenanthroline. The other enzyme cleaved ala-pNA to alanine and *p*-nitroaniline. It was inhibited by 1,10 *o*-phenanthroline and bestatin and was similar to aminopeptidase M. These enzyme preparations must obviously be tested for their capacity to remove the hexapeptide prosegment from proapo AI before any conclusions can be drawn about their identity with the plasma proapo AI-converting activity.

The idea that proapo AI processing may occur on the surface of lipoprotein particles is in certain respects quite appealing. It offers a mechanism for bringing enzyme and substrate together. In this fashion, the lipoprotein surface serves as an affinity matrix (e.g., similar to that utilized in the clotting factor cascade). However, the scheme does place certain physical-chemical constraints on both the enzyme and its substrate. Both must be able to interact with lipid surfaces. By inducing changes in the conformation of their constituent apolipoproteins, the lipoprotein surface may modulate the number of peptide domains available for proteolytic processing. Thus, the role of the lipoprotein surface may be restrictive or permissive in terms of regulating the accessibility of surface apolipoproteins to the action of proteolytic enzymes. This might explain why the plasma proapo AI-converting enzyme does not cleave apo CII. The NH_2-terminal sequence of human *plasma* apo CII contains an oligopeptide spanning residues +5 through +8 that precisely duplicates the post-translational processing site present in proapo AI (Gln-Gln-Asp-Glu).

IV. LIPOPROTEIN BIOGENESIS AND PROCESSING OF THE APOLIPOPROTEIN PRIMARY TRANSLATION PRODUCTS

In general terms, it appears that assembly of lipid-protein complexes within the secretory pathway involves a temporally and spatially ordered association of components.[164] Only the barest outlines of this multistep process are available. Before discussing this information, two general concepts will be reviewed: (1) the notion that presecretory proteins are transported through the secretory pathway by selective processes as opposed to bulk phase movements and (2) the fact that cellular lipids, with the exception of fatty acids and two mitochondrial phospholipids, are synthesized on the ER membrane.

A. Selective Transport Vs. Bulk Phase Movements of Proteins in the Secretory Pathway

Signal peptides provide selectivity (and polarity) to the process of transporting proteins *into* the secretory pathway. Is there any selectivity maintained while proteins undergo ves-

icular transport *within* the secretory apparatus? The answer appears to be "yes", but the mechanism is unknown. Fries and co-workers[165] used pulse-chase experiments and subcellular fractionation procedures to determine the time course for intracellular transport of four secreted proteins (albumin, prealbumin, serum retinol-binding protein, and transferrin) in isolated rat hepatocytes. They found that these proteins were transported from the ER to the Golgi complex at vastly different rates (the $t_{1/2}$ ranged from 14 to 137 min). On the other hand, transport from the Golgi complex to the media occurred at a similarly rapid rate for all the proteins ($t_{1/2}$ ~15 min). Therefore, rapidly secreted radiolabeled proteins (e.g., albumin) were present in higher amounts in the Golgi complex than in the ER fraction. The opposite was true with more slowly secreted proteins (e.g., transferrin). Analogous studies carried out with Hep G2 cells[166] also documented different transport rates for secretory proteins between the ER and Golgi complex.

These observations have led to the notion that transport of secretory proteins between these organelles is mediated by a selective process rather than through bulk phase transfers.[165-167] In addition, it has been proposed that this selectivity is conferred by membrane-bound receptors that are transported to the Golgi complex.[166,168] The affinity of a protein for a receptor would determine its rate of transport. Support for this idea of selective transport also comes from the observation that minor changes in the primary structure of a protein can block its export from cells. Wu et al.[169] have recently reported that a single amino acid substitution (glycine for arginine) in the variable region of a IgA λ2 immunoglobulin chain leads to a block in secretion. Extrapolating from this result, seemingly minor conformational alterations may have profound effects on transport rates.[165,169]

B. Synthesis of Lipids in the ER Membrane

The lipid constituents of membrane bilayers (i.e., phospholipids and cholesterol) are synthesized on the ER. In fact, with the exception of fatty acids and two mitochondrial phospholipids (phosphatidylglycerol and cardiolipin), cellular lipids synthesized in eukaryotic cells are derived from a series of reactions catalyzed by membrane-bound enzymes situated on the cytosolic face of the ER bilayer.[170,171] Lipid synthesis apparently is an asymmetric process. For example, the intermediates generated during phospholipid biosynthesis by the cytosolic-facing, membrane-associated enzymes appear to be initially "fixed" and then further modified on one side of the bilayer. Although lipid synthesis initially results in expansion of the cytosolic leaflet of the membrane bilayer, a mechanism exists (albeit undefined) for parallel expansion of the opposing monolayer.[170]

C. Biosynthesis of Nascent Lipoproteins — Avian HDL Biogenesis as a Model System

Analysis of the mechanism of intracellular lipoprotein assembly is complicated by several physiologic facts: (1) more than one type of lipoprotein particle may be exported from the same cell, (2) a given apolipoprotein species may be present in more than one class of lipoprotein,[172] and (3) exogenous lipoproteins are taken up and "reprocessed" by the very cells which synthesize them. For example, most of these "problems" are encountered in rat hepatocytes and enterocytes. In chickens, the situation is less complex. More than 90% of total plasma lipoproteins are in the HDL density class.[173] In addition, apo AI comprises more than 90% of HDL apoprotein content.[174,175] This lack of complexity has made the study of intracellular HDL assembly in chicken liver particularly attractive.

Pulse-labeling experiments combined with subcellular fractionation schemes have shown that apolipoprotein synthesis occurs on the RER of rat liver,[176-178] while lipid synthesis takes place on the smooth ER.[179-182] Banerjee and Redman[172] used these same techniques, as well as ultracentrifugal flotation analyses, in order to identify the intracellular compartment where avian liver apolipoprotein AI becomes affiliated with lipid. Radiolabeled apo AI present in fractions enriched for RER and smooth ER did not float. On the other hand, radiolabeled

apo AI present in the Golgi apparatus floated between density = 1.063 and 1.21 g/mℓ. Although Golgi HDL particles had the same density as serum HDL, they contained more phospholipid, proportionately less triglyceride, and relatively less protein than their extracellular homologues. Spherical as well as disk-shaped bilayer lipoprotein particles were represented in the Golgi fractions. The majority of spherical particles were associated with membrane tails. The origins and significance of these tails are unknown. In general, Golgi HDL particles were more heterogeneous and larger than plasma HDL particles. The intracellular transport time for avian HDL was quite rapid. Pulse-labeling studies indicated that assembly and export takes 20 to 30 min in the young avian liver.[172]

These data show that following cotranslational translocation and proteolysis *avian* apo AI does not acquire enough lipid to float at HDL densities until it passes through the proximal portion of the secretory pathway (i.e., the RER and smooth ER) and enters the Golgi complex. The compositional and morphological differences between Golgi and extracellular HDL also suggest that nascent HDL may not be fully assembled in the Golgi.[172] This conclusion is possible to make in the young avian liver because of the unique representation of HDL within the hepatocyte: lipoprotein heterogeneity cannot be simply ascribed to different classes of particles with varying apolipoprotein compositions.

D. Biogenesis of Mammalian HDL

Two classes of plasma lipoprotein particles are derived from mammalian hepatocytes — HDL and VLDL. The current view of mammalian VLDL biogenesis is that triglycerides, phospholipids, and apolipoproteins are assembled into VLDL in the smooth ER.[179-182] Nascent VLDL recovered from rat liver Golgi complex preparations resembles mature serum VLDL particles both morphologically and compositionally.[178,180,183,184]

Hamilton and co-workers[185] tried to define the physical-chemical properties of nascent HDL exported from *rat* hepatocytes. To accomplish this, they perfused rat livers in the presence or absence of an LCAT inhibitor (5,5′-dithionitrobenzoic acid). The rationale for using the LCAT inhibitor was to prevent extracellular remodeling of lipoprotein lipids and thereby gain a clearer view of the primary (unmodified) HDL particle released by these cells. When the inhibitor was included, discoidal HDL accumulated in the perfusate. Compared to their spherical plasma HDL homologues, these disc structures contained relatively little cholesteryl esters and triglycerides. Similar discoidal HDL particles were also noted in cholesterol-fed guinea pigs, as well as in patients with LCAT deficiency[186] or cholestasis.[187]

Unlike spherical plasma HDL, the principal apolipoprotein of discoidal HDL was apo E. In addition, the discoidal particle was a better substrate for LCAT than plasma HDL.[185] These observations led Hamilton et al. to propose that rat hepatocytes export a disk-shaped structure consisting of a phospholipid bilayer which contains cholesterol and resembles cell membranes. The apolipoproteins associated with this HDL particle (primarily apo E) are arrayed on the edge of the disk, i.e., the apolipoprotein is interposed between the hydrophobic edge of the disk and the (polar) aqueous extracellular environment. After LCAT binds to this structure (surface or edge), cholesteryl esters are generated and subsequently move to the hydrophobic "interior" of the bilayer. At the same time, surface polar lysolecithin molecules are transferred from the particle surface to albumin. The net result of these events is that an oily core forms, displacing the leaflets of the bilayer, and a pseudomicellar spherical HDL particle is created.[185]

A pivotal question unanswered by this scheme was whether HDL apolipoproteins were secreted from hepatocytes affiliated with this discoidal particle or whether they were exported as relatively free polypeptides which then "organized" lipids into a supramolecular disk-shaped complex. Recently, Goldberg and co-workers[188] addressed this problem in the perfused primate liver. These investigators found that apo AI and AII released from perfused baboon livers appeared, on the basis of their behavior during Sephacryl S-300 chromatog-

raphy, to be unaffiliated with HDL-like particles. Their elution profile suggested that they were "small" (free) polypeptides (i.e., they were not assembled into large macromolecular arrays). Similar observations have been made by Alpers et al. in the rat intestine.[189] They used Sepharose 6B and 10% agarose chromatography as well as KBr isopycnic density centrifugation to characterize the physical properties of intracellular and extracellular apo AI. The majority of (pro)apo AI recovered from jejunal enterocytes and the lamina propria did not elute in the position of either HDL, VLDL, or LDL. Most (up to 75%) of the AI isolated from these compartments appeared to be "free" and not incorporated into large lipid-rich particles. However, by the time apo AI had entered the mesenteric lympatics, its physiochemical properties had changed. Here, more than 70% eluted in the HDL region, and there was no demonstrable free apolipoprotein. By contrast, intracellular as well as extracellular apo B and CIII appeared to be affiliated with large quantities of lipid. Apo B and CIII isolated from enterocytes eluted with chylomicrons, VLDL, as well as LDL and HDL. In the lamina propria and mesenteric lymph, the pattern became less complex with the bulk of apo B located in the region of VLDL and chylomicrons.[189]

These observations suggest that assembly of mammalian HDL-destined apolipoproteins into lipid-rich particles *may* be largely an extracellular event.

E. Speculation

The information itemized above can be used to generate a number of hypotheses about the relationship between apolipoprotein processing and lipoprotein assembly.

1. Interaction of Apolipoproteins with Lipids may Provide a Mechanism for Selective Transport within the Secretory Pathway

Experiments using Hep G2 cells indicated that the two principal HDL apolipoproteins, AI and AII, are released into the medium at different rates.[136] Cells were pulse labeled for 15 min with a [^{3}H]amino acid and then subjected to a chase. [^{3}H]proapo AII was no longer detectable within these cells after a 20-min chase. The clearance time for [^{3}H]proapo AI was approximately six times longer (120 min). The rate of clearance of [^{3}H]albumin from Hep G2 cells was intermediate between the two apolipoproteins (60 min). These divergent secretory rates cannot be simply accounted for by differences in the size of the polypeptides. In other words, it is difficult, *a priori*, to attribute these differences *entirely* to the time required for the cotranslationally processed proproteins to fully *enter* the secretory pathway unless their mRNAs were translated at markedly different rates and/or their translocations were affected by interactions with the ER bilayer.

The fact that these three proteins were exported into the medium at different rates implied that their intracellular transport occurred by a selective process rather than by bulk phase movements. Different export rates for proapo AI and proapo AII suggest that they are segregated from one another within the secretory pathway and are a *prima facie* evidence that they are not assembled into (a common) nascent HDL particle within the proximal portion of the secretory pathway.

If there is segregation of these two proapolipoproteins and selective transport, what are the structural determinants which are responsible for this effect? Neither proprotein is known to undergo post-translational modifications such as sulfation, phosphorylation, or N-linked glycosylation. However, affiliation of lipids derived from the ER membrane (e.g., phospholipids or cholesterol) with the proapolipoproteins could potentially serve to modulate their transport into and out of the Golgi complex. These protein-lipid interactions could involve lipids released from the ER bilayer, or alternatively, lipids which remain embedded in the ER membrane. The bound lipids might themselves serve as the targeting signals. Alternatively, conformational changes induced in the apolipoproteins after binding of the lipids could be responsible for selective sorting of apolipoproteins in the secretory pathway.

This scheme represents a sorting mechanism for apolipoproteins which would be somewhat analogous to the mechanism for sorting lysosomal enzymes. Work by a number of investigators (reviewed in References 190 and 191) has shown that the terminal mannose-6-phosphate residues represented in mannose-rich oligosaccharide chains act as recognition markers (although they may not be the only ones) permitting sorting of lysosomal enzymes during their transport through the Golgi. Current views[192-195] are that mannose-6-phosphate-containing hydrolases bind to specific mannose-6-phosphate receptors present either in or on Golgi membranes. The bound enzyme is then transferred from the luminal face of the Golgi by a receptor-mediated process and subsequently presented to lysosomes by a "carrier" vesicle. Thus, the location of the mannose-6-phosphate receptor in the Golgi complex would correspond to the initial site of sorting of lysosomal enzymes from other secretory proteins. Brown and Farquhar[196] have recently found that this receptor is concentrated in cis Golgi cisternae. The site(s) of apolipoprotein sorting in the secretory pathway remain(s) to be defined.

2. Lack of Intracellular Proapo AII Proteolytic Processing May Indicate that Compartmentalization of this Apolipoprotein within the Secretory Apparatus is Distinct from Other Proproteins

Efficient and proper intracellular processing of proalbumin by Hep G2 cells indicates that they possess a protease which potentially could remove the prosegment of proapo AII within the secretory pathway. Why then is proapo AII processed extracellularly in this system? Dissimilar processing of proalbumin and proapo AII could be due to their different compartmentalization in the secretory pathway. In fact, this could be taken as the first evidence that there is either functional or physical segregation of an apolipoprotein from the "mainstream" of presecretory proteins during their intracellular transport.

Several mechanisms can be envisioned to account for the fact that proapo AII escapes processing in Hep G2 cells. In the simplest case, proapo AII may be targeted to different vesicles than other secretory products which are not affiliated with lipids. For example, does proapo AII enter a Golgi-related, clathrin-coated membrane compartment like proinsulin does? Proapo AII-containing vesicles may not possess cathepsin B-like proteases. Alternatively, the association of proapo AII with lipids may affect its ability to interact with an active cathepsin B-like protease. This could explain the lack of intracellular proapo AII processing in Hep G2 cells whether or not proapo AII were targeted to the same vesicle as other secretory proteins. It could also explain the lack of proteolytic processing even if proapo AII were segregated into unique vesicles which possessed the same protease activities as those present in vesicles containing other presecretory proteins (e.g., proalbumin). Another possibility is that intracellular processing of precursor thiol proteases is not "coordinated" with the binding of lipids to proapo AII — again the net result being that the active protease cannot interact with its proapolipoprotein substrate. Finally, the rapid transit of proapo AII through the (Hep G2) secretory pathway could be responsible for its lack of intracellular prosegment processing.

Intracellular proapo AII compartmentalization may be further defined in light of the recent work of Kelly and associates.[197-199] They analyzed secretion of proopiomelanocortin in a mouse pituitary cell line (AtT-20 cells). This precursor entered a secretory pathway where export was regulated by secretogogues. The same cell line contained another (operationally defined) pathway for protein secretion which was termed constitutive. Proteins present in the constitutive pathway (e.g., a membrane glycoprotein encoded by an endogenous type-C virus) were not stored and were secreted within minutes after leaving the Golgi complex, whether or not a secretogogue was present. Kelly has proposed that proteins of the regulated pathway may contain common signals for sorting that are recognized by the transport apparatus. This idea was supported by results obtained after transforming AtT-20 cells with

an SV40-pBR322 recombinant vector containing a full-length human preproinsulin cDNA.[199] Stably transformed cells correctly processed the precursor protein, stored it, and released it after stimulation with a secretagogue. The opposite result was obtained when fibroblast L cells were transformed with the same recombinant plasmid; in this case, the cells only secreted *pro*insulin and did so in a constitutive manner. Analogous experiments performed with proapo AII cDNA may yield insights about whether the proapolipoprotein and prohormones enter distinct or common pathways and could provide some clues concerning the mechanism by which proapo AII "escapes" intracellular post-translational proteolytic processing.

3. Proteolytic Processing of Proapo AII and AI May Be Important in Regulating the Assembly of Supramolecular Complexes of Lipid and Protein

Although no general biological function has been proven for propeptides, Docherty and Steiner[79] have suggested several possibilities. They include a role in the proper folding of precursor proteins (e.g., the C-peptide in proinsulin) and a role in compartmentalization and transport of proteins through the secretory pathway or as feedback signals affecting protein synthesis.

Very little information is available about the physiologic roles of the apo AI and AII prosegments. A modest amount of data has been generated about the effects of the prosegments on apolipoprotein AI and AII targeting to *mature* plasma lipoprotein particles. In vitro recombination experiments[159,200] have shown that proapo AI is preferentially bound to plasma HDL_2 and HDL_3 particles just like mature apo AI. Proapo AII is preferentially targeted to plasma HDL_3 — a pattern which is similar to that found with mature apo AII.[136] The binding of these proapolipoproteins is efficient. Greater than 90% of the propolypeptides are stably integrated into the HDL subclasses as judged by density gradient ultracentrifugation.[136,159] Thus, the prosegments do not seem to impose a restriction on the ability of the apolipoproteins to specifically and stably affiliate with mature plasma lipoproteins.

The presence or absence of the apo AI prosegment has no apparent effect on the ability of apo AI to activate lecithin:cholesterol acyltransferase.[201] Both the pro and mature forms of apo AI exhibit the same cofactor activity. In this respect, proapo AI does not appear to be a zymogen (i.e., a larger precursor of a secretory protein that is proteolytically processed after secretion — converting an inactive form to a functional sequence).

What then are the roles of the apo AI and AII prosegments and what is the significance of their extracellular proteolytic processing? The extracellular site and distinct proteolytic processing pathways for these two proapolipoproteins provide a potential mechanism for regulating the sequential assembly of protein and lipid into supramolecular complexes. A corollary to this idea is that the proapolipoproteins themselves possess the structural determinants for ordered assembly. At least two possible mechanisms relating processing to assembly may be envisioned. In the first, apolipoprotein incorporation into large macromolecular complexes is largely an extracellular event. Although it is probable that the proapolipoproteins are associated with some lipid ligand population in the secretory pathway, this ligand population may be far less complex than that encountered outside the cell. Despite the fact that the prosegments are small and may not have a significant conformational effect on the *entire* mature domain, they may have important regional effects. For example, residues 8 to 33 in mature apo AI have been shown by computer analysis[202] to contain amphipathic sequences with the most hydrophobic nonpolar face in the molecule. Proteolytic removal of the prosegment thus may have a permissive or restrictive effect on the ability of the mature apolipoprotein to bind lipids. If processing is a sequential process involving different enzyme systems situated in different extracellular compartments, then a potential pathway for ordered assembly of apo AI and AII into HDL might exist. Processing of proapo AII, for example, could permit subsequent assembly of more complex lipid-apolipoprotein arrays, which then

are in a position to accept other apolipoproteins, i.e., proapo AI. Further modification of this nascent (e.g., discoidal) lipoprotein could be effected by the growing population of apolipoproteins. Proapo AI (or its processed product) by activating LCAT, would mediate the transformation of discoidal HDL to spherical HDL. Such transformations may then allow subsequent remodeling and exchange reactions to proceed. Thus, proteolytic processing of the proapolipoproteins would be viewed as part of a cascade of interdependent lipid and protein transformations which ultimately result in the generation of "mature" lipoprotein particles.

A second mechanism relating processing to HDL assembly has the prosegments exerting their effects on lipoprotein assembly *within* the secretory pathway. The presence of a prosegment may permit or prohibit a set of interactions between these proteins and lipid ligands. This could result from a direct conformational effect of the prosegment on certain domains of the mature propolypeptide or could reflect a targeting function for the propeptides. For example, the prosegments may effect the segregation of the proapolipoproteins into certain compartments of the secretory pathway (e.g., sorting of the apolipoproteins within the Golgi complex) or the prosegments could be involved in the specific interaction of their apolipoproteins with lipid arrays in the secretory pathway.

Mechanisms one and two are not mutually exclusive. In both cases, they raise the question of how much is the *potential* of a cell to make lipoprotein particles regulated by its resident apolipoprotein population and how much by its lipid population?

V. CONCLUSIONS

The structures of the primary translation products of seven mammalian apolipoprotein mRNAs have been determined in the past 3 years. In addition, a considerable amount of information has accumulated concerning the co- and post-translational proteolytic processing pathways taken by these presecretory proteins. The data can be summarized as follows:

1. The organization of the apolipoprotein signal peptides is typical of eukaryotic prepeptides. However, an unusual degree of sequence conservation is present among the signal segments of apolipoproteins AI, AIV, and E, especially in the region comprising the signal peptidase recognition site. The structural, functional, and evolutionary significance of this observation is unclear at present, although information about the organization of their genes may shed light on its meaning.
2. In those apolipoprotein sequences studied, cotranslational translocation and proteolytic processing appear to be highly efficient and result in sequestration of the processed protein within the lumen of the ER. The mechanism by which these lipid-binding proteins avoid arrest during their translocation through the lipid bilayer of the ER membrane has yet to be defined.
3. The two principal human HDL-affiliated apolipoproteins, AI and AII, undergo post-translational proteolytic-processing, resulting in the removal of nonidentical propeptides. In the case of proapo AII, this cleavage occurs after paired basic amino acids, whereas proapo AI processing occurs after an unusual Gln-Gln dipeptide. The enzymes responsible for propeptide processing are distinct. The processing pathways for these proapolipoproteins provide a potential series of steps for regulating the ordered assembly of HDL constituents.

Identification of the initial sites of lipid-apolipoprotein interaction may provide clues about the relationship between the processing of apolipoprotein primary translation products and their assembly into lipid-protein complexes. This is a formidable task. Conventional cell fractionation schemes result in the release of lipids and proteins which could subsequently

combine — even if they are normally in noncommunicating intracellular compartments. Similarly, it may be difficult to restrict extracellular recombination events and thereby gain information about the "primary" exported lipid-protein complex. Site-directed, in vitro mutagenesis of cloned apolipoprotein DNAs represents a way of rearranging specific domains of the primary translation product. The functional consequences of these rearrangements (deletions and substitutions) may be evaluated by introducing wild-type or mutant apolipoprotein sequences into mammalian cells by gene-transfer techniques. This approach could, for example, be used to directly assess the role of the AI and AII prosegments in intracellular apolipoprotein compartmentalization and lipoprotein assembly.

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