commentary

Cubbing in proapolipoprotein maturation[1]

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In this issue of the *Journal of Lipid Research*, Francone and colleagues (1) have described an interesting HDL phenotype in a mouse deficient in procollagen proteinase enhancer 2 (PCPE2). Present in the plasma of the mice are preβ HDL containing as its major, if not only, apolipoprotein pro-apoA-I, accompanied by the accumulation of an enlarged HDL also containing predominantly pro-apoA-I. This highlights a potentially new actor in the regulation of HDL homeostasis. Recent genetic association studies have suggested that PCPE2 variation may be weakly associated with HDL levels.

The phenotype described in the Francone paper, while of great interest, is not readily understandable in all of its details. In addition, this study raises some fascinating evolutionary and physiological questions: *a*) How and where is the apoA-I proprotein converted to the mature protein? What is the biochemical mechanism for this conversion? *b*) What is the functional role of the secretion of apoA-I as a proprotein? *c*) Does the proprotein differ from the mature protein in its function and regulation of cholesterol homeostasis? *d*) What is the mechanism for the accumulation of an enlarged HDL containing pro-apoA-I? How does this HDL compare in function to HDL in which mature apoA-I is the predominant apoprotein?

PRO-APOA-I MATURATION (CLEAVAGE)

ApoA-I is the major protein of HDL. It is an amphipathic, lipid binding protein containing 243 amino acids. Its primary translation product includes an 18 amino acid signal peptide that directs the primary translation product into the endoplasmic reticulum and is removed cotranslationally followed by a hexapeptide propeptide that is removed extracellularly to yield the mature apoprotein. Other secreted proteins, including several other apoproteins, are also secreted with a prosequence. The precise function of the prosequence is not always clear. The suggestion is that the prosequence often allows the mature protein to assume the "correct" conformation in the appropriate biological environment. Several examples of this mechanism are well known, e.g., pro-insulin and procollagen.

Procollagen has additional sequences at both the N- and C-termini. They are cleaved by different proteinases prior to fibrillogenesis to form the mature collagen with the cleavage of the C-terminal prosequence being especially critical (2). It would be unproductive for fibrillogenesis to occur while the protein is being synthesized as the collagen fibers are major extracellular matrix components. The cleavage of the C-terminal procollagen sequence is accomplished by the tolloid metalloproteinase bone morphogenetic protein-1 (BMP-1) and enhanced by PCPE1, a close relative of PCPE2, with which it shares 43% amino acid homology (2–4). Both BMP-1 and the PCPE proteins are CUB domain containing proteins (C = complement components C1r/C1s; U = sea urchin protein Uegf; B = BMP-1). The CUB domains facilitate protein-protein interactions, in this case BMP-1 and PCPE1 to allow for the productive cleavage of the C-terminal prosequence from procollagen. The cleavage of pro-apoA-I is by a very similar process involving BMP-1 (5) and PCPE2 forming a complex with the proapoprotein, again via the CUB domains of the first two components (6). Binding studies suggest that the order of addition of these components to the complex may favor the association of pro-apoA-I with BMP-1 followed by the addition of PCPE2. Indeed, quite limited but detectable cleavage of pro-apoA-I may occur in the absence of PCPE2, which, as its name suggests, serves as an enhancer for the cleavage. It is not clear from the studies of Francone and colleagues whether the plasma of the PCPE2 knockout animals contains any mature apoA-I, as the authors did not have the reagents to make such a quantitative assessment. This might be accomplished using an anti-peptide antibody that selectively recognizes the proprotein as has been described previously (7).

In procollagen, BMP-1 and PCPE1 cleave Ala-Asp or Gly-Asp bonds (8). In the case of pro-apoA-I, BMP-1 in concert with PCPE2 removes the N-terminal hexapeptide WHVWQQ from the N-terminal aspartic acid residue of the mature mouse proprotein. The human prosequence is RHFWQQ, again upstream of the N-terminal aspartic acid residue. The proprotein sequence was first alluded to by Gordon and colleagues (9) almost 30 years ago when they were studying apolipoprotein mRNA in rat intestine. The

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sequence of the human proprotein hexapeptide was determined from lymph (10) or from the primary translation product in HepG2 cells a year later (11). The Gln dipeptide at the end of the prosequence is characteristic of the apoA-I prosequence found across fish, birds, and mammals (12). This pathway is distinct from that employed for the processing of proneurohormones like insulin, which involves endoproteolytic cleavage between dibasic residues Arg-Arg or Lys-Arg (13). The cleavage of the prohormones occurs within the secretory pathway. In contrast, the cleavage of the pro-apoA-I is an extracellular event either in the plasma or tissue fluid though a particular micro-environment is not excluded. All three components of the complex involved in the conversion of the proprotein to the mature apoA-I, pro-apoA-I, BMP-1, and PCPE2, are secreted proteins, but precisely where they associate is not clear. Pro-apoA-I is secreted from the intestine and liver. BMP-1, the procollagen C-terminal proteinase, likely has a similar tissue distribution as the procollagen N-terminal proteinase with expression mostly in tissues rich in collagen, like the tendon, skin, bone, and aorta and more limited expression in the kidney and liver (14). PCPE2 has a more limited expression than PCPE1 and is most abundantly expressed in the heart, the intestine, and to a lesser extent in the kidney, with very little expression in the liver. It is a heparin binding protein and is associated with the cell surface (4), raising the possibility that the cleavage of pro-apoA-I may occur on a cell surface. PCPE2 is also found on HDL (5), suggesting that it may associate with lipid associated pro-apoA-I.

SECRETION OF APOA-I EXCLUSIVELY AS THE PROPROTEIN

At steady state, about 7% of the total plasma apoA-I is the proprotein (7), suggesting that the extracellular conversion to the mature protein is not quantitatively efficient or that it takes place in a particular micro-environment. The corollary to the hypothesis that the cleavage of pro-apoA-I occurs extracellulary is that the mature protein should not be found intracellularly in producing cells (i.e., hepatocytes and enterocytes). Although this expectation appears to hold for most of the species studied, it is not the case for chicken hepatocytes, which in culture contained 83% mature apoA-I even in the absence of serum in the culture medium (15). The molecular basis for this unexpected result remains to be clarified. Several investigators have studied the possible function of the prosequence in the secretion of pro-apoA-I. Using an in vitro transcription/translation/microsomal membrane system, it was shown that, in the absence of the prohexapeptide, there was a reduced efficiency of cotranslational translocation into the membrane vesicles (16). McLeod and colleagues (17) also showed that, in the absence of the prosequence, transport from the endoplasmic reticulum to the Golgi apparatus was impaired. Mutation of the Gln residues at the end of the prosequence, thus preventing its cleavage, had no impact on the ability of the mutated protein to be secreted from transfected C127 cells (18).

It is widely thought that most of the lipidation of apoA-I to form HDL occurs extracellularly as a result of interaction with ABCA1. However, some lipidation, about 20%, occurs intracellularly, meaning that pro-apoA-I can be lipidated (19).This has also been studied in primary hepatocytes when the newly synthesized apoA-I is initially phospholipidated in the endoplasmic reticulum and, more significantly, in the Golgi. The former lipidation appears to be ABCA1 independent whereas the latter is dependent on the transporter function (20). The role of ABCA1 in the conversion of the proapolipoprotein to the mature protein is highlighted by the changing balance between the proprotein and the mature protein in Tangier disease, now known to be attributable to a deficiency of the ABCA1 transporter. In this disorder, the relative concentration of proprotein and mature protein markedly favors the former (21), but this is not attributable to a fundamental defect in the conversion machinery in Tangier disease (22, 23). Even though it has been shown that the free proprotein can be converted to the mature form, it is possible that in vivo, a particular lipidated form of the proapoprotein may be the preferred substrate for its maturation. It would be worth studying whether the Tangier phenotype with respect to the relative predominance of pro-apoA-I in the very small pool of circulating apoA-I could be established in mice lacking ABCA1 function. Timmins and colleagues (24) have shown that the selective knockout of hepatic ABCA1 is associated with a substantial decline in circulating HDL accompanied by hypercatabolism of apoA-I in the kidney. They did not examine the catabolism of pro-apoA-I in this model. It is not clear whether the hepatic knockout is sufficient to establish the model for pro-apoA-I catabolism or whether a global knockout would be more useful.

The fate of the hexapeptide removed from the proprotein has also not been examined. It is not clear whether it is stable after separation from the protein. If it is reasonably stable, its measurement in the urine, for example, could provide information on the combined rate of apoA-I synthesis in the liver and intestine and the antibody described by Barkia (7) could be used for such a study.

THE COMPARATIVE PROPERTIES OF THE PRO- AND MATURE APOA-I

The differences in these two proteins remains unclear. Both are capable of binding lipid, apparently to the same extent. They both self-associate and appear to activate LCAT similarly (25). In their paper, Francone and colleagues provide a potentially important difference by suggesting that when normalized for apoA-I concentration, preβ HDL containing pro-apoA-I is defective in ABCA1-dependent cholesterol efflux. They refer to the knockout HDL as "dysfunctional," though the precise basis for this dysfunction remains to be clarified. A potential caveat in interpreting these results is that the acceptors were probably constituted of mixtures of HDL particles that were not precisely defined. It will be necessary to undertake

experiments like this with more discretely defined acceptors containing either pro-apoA-I or mature apoA-I, probably using reconstituted "HDL" particles. However, two other studies have also suggested that pro-apoA-I is not as efficient in promoting ABCA1-dependent cholesterol efflux as is the mature apoprotein (5, 26). The interaction of the proprotein with the ABCA1 efflux mechanism needs to be systematically investigated. Mature apoprotein in the model of Vedhachalam et al. (27) interacts with ABCA1 and its "membrane product" at two stages and which of these is affected by the hexapeptide prosequence on the acceptor would be of interest. It would also be important to complement the cell culture findings with a study of reverse cholesterol transport in vivo (28).

ACCUMULATION OF ENLARGED HDL

In the face of the cholesterol efflux dysfunction just discussed, the accumulation of an enlarged HDL in the PCPE2-deficient mice observed by Francone et al. seems counterintuitive. The enlargement of HDL implies that ongoing LCAT activity is normal. One of the factors that influences LCAT activity in a large HDL is the apoprotein composition of the HDL. However, no change in apoprotein composition was noted in the enlarged HDL. This highlights what is becoming widely recognized; namely, that the function of HDL is more important from an atheroprotective point of view than its level in the plasma. Cholesterol efflux or reverse cholesterol transport is but one of the atheroprotective effects of HDL. In this model, it would clearly be of interest to explore some of the other anti-inflammatory properties of this HDL in contrast to normal HDL. Further studies of the function of reconstituted HDL containing predominantly pro-apoA-I are indicated.

But what accounts for the increased level of this large HDL? Not studied here is HDL clearance that depends on uptake in the liver, selective cholesterol influx into sterol using cells and tissues, and the release of apoA-I from intact HDL with resulting filtration in the kidney (29). The capacity of the apoA-I to be filtered may be a significant determinant and this could be influenced by the size of the particle containing the protein. For example, the interaction of the HDL with lipases may influence the release of the apoprotein. The filtration of mature and pro-apoA-I is likely to be similar though this should be tested. Another aspect of HDL structure that could influence its metabolism is the conformation of the proapoprotein-containing particle, which would need to be investigated by the crosslinking and mass spectrometry of the proteins in the HDL (see, for example, Ref. 30). Of particular interest in the context of the above discussion is the high affinity interaction of apoA-I in the kidney with cubilin, a CUB domain protein that has a coreceptor megalin, which does not bind the apoA-I (31, 32). This system participates in the catabolism of apoA-I. Again, the comparative interaction of pro-apoA-I with cubilin is worthy of study. Its differential interaction with the proprotein may play a role in the balance between the proprotein and the mature protein in Tangier disease.

OTHER PROAPOPROTEINS

The presence of an N-terminal extension of apoA-I is not unique to this protein among the apolipoproteins. ApoC-II, the activator of LPL, exists in the plasma in two isoforms, which differ by the absence or presence of the N-terminal hexapeptide (33, 34). This hexapeptide sequence is very homologous to that found in pro-apoA-I; TQQPQQ (human), AQQPQQ (monkey), and AHVPQQ (bovine), ending in diglutamine residues, with propeptide cleavage occurring between the terminal glutamine in the propeptide and the N-terminal aspartic acid residue of the mature protein, similar to apoA-I. Both forms of apoC-II appear to be equally effective in activating LPL.

Apoprotein A-II is also secreted as a proprotein. In this case, the prosequence is a pentapeptide, ALVRR, terminating with dibasic amino acids, which is removed extracellularly by a cathepsin B-like protease and thus is quite different in processing than that for pro-apoA-I (35, 36). Although it is provocative that at least three HDL apoproteins are secreted as proproteins, the biological significance of this is far from clear. HDL is different than the apoB-containing lipoproteins, in that most of the lipidation takes place extracellularly.

As with most provocative papers, more questions are raised than might be answered in the space of a single article. This is certainly the case for the paper by Francone and colleagues. As alluded to in the above discussion, there is much more to be done. In addition, if a similar model for the apoA-II maturation were developed, an additional set of questions would arise.

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