A Proteomic Approach Identifies Proteins in Hepatocytes That Bind Nascent Apolipoprotein B*

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The biogenesis of apolipoprotein B is quite complex in view of its huge size, hydrophobicity, obligate association with lipids such as cholesterol and triglycerides prior to secretion, and intracellular degradation of a substantial proportion of newly synthesized molecules. Multiple proteins likely serve roles as molecular chaperones to assist in folding, assembly with lipids, and regulation of the secretion of apolipoprotein B. In these studies, we developed a strategy to isolate proteins associated with apolipoprotein B in rat livers. The purification consisted of two stages: first, microsomes were prepared from rat liver and treated with chemical cross-linkers, and second, the solubilized proteins were co-immunoprecipitated with antibody against apolipoprotein B. We found that several proteins were cross-linked to apolipoprotein B. The proteins were digested with trypsin, and the released peptides were sequenced by tandem mass spectrometry. The sequences precisely matched 377 peptides in 99 unique proteins. We show that at least two of the identified proteins, ferritin heavy and light chains, can directly bind apolipoprotein B. These and possibly other proteins identified by this proteomic approach are novel candidates for proteins that affect apolipoprotein B during its biogenesis.

Apolipoprotein B (apoB)† is secreted with lipids including cholesterol esters, phospholipids, cholesterol, and triglycerides, as very low density lipoproteins (1–5). The secretion of cholesterol from the liver in humans is tied to the export of apoB into plasma. Hepatic regulation of apoB secretion is chiefly post-translational (6); secretion reflects the balance between assembly of this protein with lipids into a lipoprotein particle and intracellular degradation. Both of these competing processes appear to involve several other proteins.

The biogenesis of this large (molecular mass greater than 500 kDa) (7, 8), hydrophobic protein requires the participation of several known chaperone proteins including some that are particular to the specialized physiologic role of apoB. Evidence suggests that calnexin, calreticulin, BiP, Erp72, GRP94, and protein disulfide isomerase (PDI) all interact with apoB during its translocation and further biogenesis once it has entered the endoplasmic reticulum (ER) (9–11). These proteins also function in the proper folding and quality control of other secretory proteins. However, the assembly of apoB with lipids into a lipoprotein particle necessitates additional proteins that may be particular to apoB. The lack of solubility of apoB in an aqueous environment, such as the lumen of the ER, necessitates its co-translational association with lipids (12). This process is facilitated by microsomal triglyceride transport protein (MTP), which plays a crucial role in the initial assembly and regulation of secretion of apoB (13–15). In a second step that is sensitive to inhibition by brefeldin A, apoB is assembled with a full complement of lipids into a mature lipoprotein particle (16, 17). The protein that mediates this addition of bulk lipids is not known.

Molecules of apoB that do not complete the assembly process due to insufficient lipids being available, misfolding, or inhibition of necessary chaperone proteins are targeted for intracellular degradation. Several groups have shown that apoB can be marked for degradation by the ubiquitin-proteasome pathway even during its translocation into the ER (18–20). ApoB can also enter other routes of intracellular degradation in hepatic cells (21, 22). Remarkably, some molecules of ubiquitin-conjugated apoB can be rescued from degradation and secreted when hepatocytes are treated with lipids (23). The variety of pathways of degradation and possibility of rescue implies that several types of proteins might play roles in determining the fate of molecules of apoB.

To gain a more complete view of the assembly, degradation, and regulation of the secretion of apoB, we wished to identify proteins that contact apoB throughout its biogenesis in the hepatocyte. We employed a proteomic approach to isolate and identify all proteins that could be co-immunoprecipitated with apoB after treatment with a chemical cross-linker. We demonstrate that some of the novel proteins bind to apoB and therefore are candidates for roles in its biogenesis.

EXPERIMENTAL PROCEDURES

Reagents—Janet Sparks kindly provided us with a gift of polyclonal antibody against rat apoB; goat polyclonal antibody against human apoB was purchased from Chemicon. The monoclonal antibodies 1D1, Bsol1, and BsoI7 were kindly given by Yves Marcel. Protein G-agarose was purchased from Kirkegaard and Perry Laboratories. Silver staining kits were obtained from BioRad. The chemical cross-linkers dithiobis(succinimidyl propionate) (DSP), dicycimimidyl suberate (DSS), and dimethyl 3,3’-dithiodispropionimidate-2 HCl (DTBP) were obtained from Pierce. Colloidal Blue staining kits were purchased from Novex. The plasmid TyB2, ER2566 bacteria, and chitin beads were purchased from New England Biolabs. A kit for generating capped mRNA using SP6 polymerase was purchased from Ambion. Rabbit reticulocyte lysate

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1 The abbreviations used are: apoB, apolipoprotein B; PDI, protein disulfide isomerase; ER, endoplasmic reticulum; MTP, microsomal triglyceride transport protein; DSP, dithiobis(succinimidyl propionate); DSS, disuccinimidyl suberate; DTBP, dimethyl 3,3’-dithiodispropionimidate-2 HCl; DTT, dithiothreitol.
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was from Promega, and \[^{[35]}S\]methionine Tran\[^{35}S\]-label (specific activity >1000 Ci/mmol) was purchased from ICN. Ultrafree MC 0.22 \(\mu\)m filter units were purchased from Millipore.

**Preparation of Microsomes—**Livers averaging 4 g each from adult Sprague-Dawley rats of various ages were excised shortly after euthanasia using an ether-oxygen \(\mathrm{O}_2\) mixture followed by i.p. injection of pentobarbital (Hoechst). Liver samples were minced in HB and stored at \(-80^\circ\)C for 30 min before freezing at \(-20^\circ\)C. The liver was homogenized in HB using a motorized Teflon-coated Dounce homogenizer. Cross-linking—We used DSP and DTBP for cross-linking proteins in our samples. Both cross-linking reagents are cleavable, homobifunctional, and have similarly sized spacer arms, and permute membranes. However, unlike DSP, DTBP is readily soluble in aqueous solutions. DSP was dissolved in dimethyl sulfoxide (Me\(2\)SO) and added to the sample at a final concentration of 50 mM. DTBP was added directly to the sample at a final concentration of 100 mM. Chemical cross-linking was carried out in homogenization buffer. The reaction was incubated at room temperature for 30 min then stopped by adding Tris-HCl to 50 mM and incubating another 15 min. The cross-linked microsomes were collected by centrifugation at 90,000 rpm for 15 min at 4 °C in a Beckman TL-100 tabletop ultracentrifuge.

For some experiments, antibodies were conjugated to either cyanogen bromide-activated beads or cross-linked to protein G-agarose. Cyano- gen bromide-activated beads were washed with cold 1 M HCl for 30 min, followed by brief washes with water and then buffer A (0.1 M NaHCO\(_3\)/0.5 M NaCl (pH 8.5)). Antibody was incubated with the beads for 2 h at room temperature. Unretained groups were blocked with 1 M ethanolamine (pH 8.0) for 2 h at room temperature. The beads were washed extensively with buffer A followed by five washes with 0.1 M NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and then incubated in homogenization buffer. The reaction was incubated at room temperature for 30 min then stopped by adding Tris-HCl to 50 mM and incubating another 15 min. The cross-linked microsomes were collected by centrifugation at 90,000 rpm for 15 min at 4 °C in a Beckman TL-100 tabletop ultracentrifuge.

**Plasmid Constructions—**Plasmids encoding the ferritin fusion proteins and TyB2 itself were transfected into the ER2566 strain of *Escherichia coli*, and overnight cultures were prepared from single colonies. The next day, the culture was diluted 1:10, and after 1 h at 0.2 m isopropyl thiogalactopyranoside was added. After 5 h, the cells were collected by centrifugation at 5000 \(\times g\) for 10 min at 4 °C. The pellet was resuspended in phosphate-buffered saline and disrupted over the horn of a sonicator (Heat Systems-Ultrasonics, Inc.) for a total of 5 min using pulses of 1 min interrupted by cooling on ice. Sonication, the microsomes at 100 \(\mu\)g/ml in 50 mM Tris-HCl buffer, and aliquots were spun in a microcentrifuge at maximum speed for 5 min at 4 °C. The supernatants were pooled in a new tube and incubated with chitin beads on a rotator at room temperature for 7 min. The beads were collected by centrifugation at 500 \(\times g\) for 5 min at 4 °C and washed three times with phosphate-buffered saline. Aliquots of the fusion proteins bound to chitin beads were displayed by SDS-PAGE and quantitated by Coomassie staining followed by autoradiography. Coomassie staining was performed to verify that equivalent amounts of fusion proteins were used in the binding assays.
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FIG. 1. A, strategy for the purification of proteins associated with apoB. See "Experimental Procedures" for details. B, one polyclonal antibody against human apoB can efficiently immunoprecipitate rat apoB. Rat livers were homogenized and solubilized in TXSWB. Equal dilutions of antibody were added to aliquots of the homogenate, and immunoprecipitation of rat apoB was carried out. The washed immunoprecipitates were separated on an 8% polyacrylamide gel and transferred to Hybond C Super membrane overnight. The blot was probed with antibody against rat apoB and visualized via chemiluminescence. Bands for apoB100 and apoB48, both of which are synthesized in rat liver, are seen in some lanes. The antibodies used for immunoprecipitation of the aliquots is indicated in the labels above the blot: rat apoB100 and apoB48, monoclonal antibodies against human apoB; C, Chemicon polyclonal antibody against human apoB.

The prominent bands corresponding to heavy and light chains posed two problems. First, the increased amount of antibody bound to the solid matrix was unsuccessful. Attempts to elute apoB from the polyclonal antibody while leaving antibody bound to the solid matrix was unsuccessful.

Therefore, we cross-linked the antibodies to protein G-agarose and used the conjugated antibodies for immunoprecipitation. Antibodies were conjugated via coupling to cyanogen bromide-activated beads or by cross-linking antibodies on protein matrix were unsuccessful.

Conjugated antibodies, we compared the proteins immunoprecipitated from aliquots of un-cross-linked microsomes (Fig. 3, lane 1)
with microsomes treated with DTBP (Fig. 3, lane 2). The conjugated antibody co-immunoprecipitated several proteins that are not readily evident in the uncross-linked sample. Furthermore, the conjugated antibody co-immunoprecipitated a very similar pattern of proteins from cross-linked microsomes as did unconjugated antibodies (free in solution) that were collected subsequently on protein G-agarose (Fig. 3, compare lanes 2 and 3). The pattern and intensity of the bands is very similar regardless of whether the antibodies were conjugated. The lack of heavy and light chains from the conjugated antibody reveals other bands (Fig. 3, lane 2) and permits increasing the scale of the purification without overloading the gel with protein. Thus, conjugated antibody offers significant advantages without significantly altering the recovery of co-immunoprecipitated proteins.

**Purification and Sequencing of Proteins**—Using this approach to isolate proteins that co-immunoprecipitate with apoB, we purified sufficient masses of each band for protein sequencing by mass spectrometry. In the previous analytical scale experiments, silver staining was used to detect bands that in some cases were at the threshold of detection in the range of 1 ng. To ensure adequate recovery of peptides from the gel, we anticipated needing roughly 50–100 ng of each band. Microsomes were prepared from the livers of four adult rats, treated with DTBP, solubilized, and subjected to immunoprecipitation with conjugated antibody against apoB. After cleaving the cross-linker, the released proteins were separated in one lane by SDS-PAGE and visualized using a colloidal Coomassie stain. This staining kit has a reported sensitivity of 10 ng. The gel used for sequencing is shown in Fig. 4. In view of the cost of protein sequencing, we excised and pooled bands of similar migration into nine pools.

The excised bands were given to the Harvard Microchemistry Facility for sequencing. After the proteins were digested with trypsin in the gel, the released peptides were sequenced by microcapillary reverse-phase high pressure liquid chromatography nano-electrospray tandem mass spectrometry on a Finnigan LCQ quadrupole ion trap mass spectrometer. The peptide data was correlated with known sequences using the algorithm Sequest (24) and programs developed at the Harvard Microchemistry Facility (25). Finally, the peptide sequences were reviewed manually for fidelity and consensus with known proteins.

A total of 377 peptides were matched to 120 known data base entries and 99 unique proteins. The proteins identified in each pool of bands is shown in Fig. 5. The peptides ranged in length from seven to 31 amino acid residues. On average, the median lengths were nearly 14 amino acid residues. The chance of an exact match of two peptide sequences over 14 amino acids is about $1.6 \times 10^{-13}$. This calculation lends considerable confidence to the identity of the matched protein, and the presence of longer or multiple peptides corresponding to the same protein strengthens the identification further. Two contaminants did show up in our results. Despite an effort to handle the gel with washed gloves as little as possible, keratin was detected by this highly sensitive procedure in four of the nine pools of bands and accounted for 18 of the matches overall. This contamination also could have occurred during handling of the glass plates prior to pouring the gel. The other contaminant stems from the immunoglobulin chains that eluted from the beads probably in small amounts because they account for 22 of the 377 peptides. Thus, 40 of the 377 peptides could be the result of contamination. The rest of the matches appear to derive from the samples. Nearly all of the peptides correspond to proteins of molecular mass that match their group indicating that significant degradation did not occur. (Note that the range of apparent molecular masses of the matched proteins, estimated from where the bands were excised, is approximate especially for proteins of slower migration.) The principal exceptions are peptides from keratin at scattered places on the gel, uricase (also called urate oxidase), $\alpha$ and $\beta$ globin, one peptide matching a cytochrome P450 enzyme, and two peptides that match D1 and BiP found in pool 3 (in the 20-kDa range). There were 40 unique ribosomal proteins identified. Presumably, these proteins were cross-linked to chains of apoB that had not finished elongation; the isolation of translation elongation factor $\alpha$ also supports this idea. Most of the remaining proteins are hepatic enzymes and not structural proteins, although two forms of actin did occur. Cytochrome P450 enzymes accounted for 12 matches. Of the 38 remaining proteins, some are known to contact apoB during its biogenesis such as D1 and BiP. We next investigated whether some of these newly identified proteins can bind apoB.

**Ferritin Chains Can Bind ApoB Directly**—Proteins identified by cross-linking and co-immunoprecipitation could contact or...
Identification of Proteins That Bind ApoB

| Pool | Bands | Size, kDa | Protein | Database number | mw, kD | Peptides | Median length |
|------|-------|----------|---------|----------------|-------|----------|--------------|
| 1    | 1, 2, 3 | 15-17   | ribosomal protein S16, murine | 133810 | 16 | 7 | 11 |
|      |        |         | ribosomal protein S15, human | 107615 | 15 | 6 | 10 |
|      |        |         | ribosomal protein S19, rat | 133837 | 16 | 5 | 10 |
|      |        |         | glutathione S-transferase, rat | 121741 | 17 | 3 | 17 |
|      |        |         | ribosomal protein S14, Drosophila | 133771 | 16 | 2 | 13 |
|      |        |         | ribosomal protein L31, human | 1655596 | 14 | 2 | 13 |
|      |        |         | ribosomal protein L23, human | 4506605 | 15 | 2 | 13 |
|      |        |         | ribosomal protein S20, human | 4506697 | 13 | 2 | 12 |
|      |        |         | ribosomal protein S26, hamster | 266970 | 13 | 1 | 16 |
|      |        |         | ribosomal protein P1, rat | 133053 | 11 | 1 | 17 |
|      |        |         | ribosomal protein L22, human | 4506613 | 15 | 1 | 14 |
|      |        |         | ribosomal protein S23, human | 4506701 | 16 | 1 | 13 |
|      |        |         | IgG heavy chain precursor, cow | 108750 | 50 | 1 | 17 |
| 2    | 4, 5, 6 | 18      | ribosomal protein S18, rat | 70965 | 18 | 13 | 10 |
|      |        |         | ribosomal protein L12, rat | 132653 | 18 | 5 | 10 |
|      |        |         | ribosomal protein S10, rat | 133715 | 19 | 3 | 15 |
|      |        |         | ribosomal protein L28, rat | 132836 | 16 | 2 | 11 |
|      |        |         | ribosomal protein S11, rat | 71005 | 18 | 2 | 9 |
|      |        |         | ribosomal protein S25, rat | 71047 | 14 | 1 | 10 |
|      |        |         | ribosomal protein S23, rat | 543449 | 16 | 1 | 12 |
|      |        |         | ribosomal protein S13, rat | 70997 | 17 | 1 | 11 |
|      |        |         | ribosomal protein L35, rat | 71362 | 15 | 1 | 11 |
|      |        |         | ribosomal protein L27A, rat | 71190 | 17 | 1 | 12 |
|      |        |         | keratin 9, human | 1082558 | 62 | 1 | 12 |
|      |        |         | keratin 10, human | 71528 | 59 | 1 | 10 |
| 3    | 1a, 7, 8 | 10, 20  | ribosomal protein L1, rat | 71106 | 20 | 6 | 9 |
|      |        |         | ribosomal protein L21, human | 1112991 | 18 | 4 | 9 |
|      |        |         | ferritin heavy chain, rat | 111625 | 22 | 3 | 12 |
|      |        |         | ribosomal protein S21, rat | 133879 | 9 | 2 | 13 |
|      |        |         | keratin 2a, human | 4557703 | 66 | 2 | 15 |
|      |        |         | ribosomal protein L21, rat | 132775 | 18 | 1 | 15 |
|      |        |         | ribosomal protein S27, rat | 133902 | 9 | 1 | 14 |
|      |        |         | ribosomal protein L24, rat | 1083791 | 18 | 1 | 14 |
|      |        |         | ribosomal protein S27, human | 4506711 | 9 | 1 | 14 |
|      |        |         | keratin, human | 88054 | 67 | 1 | 31 |
|      |        |         | ferritin light chain | 6753914 | 20 | 1 | 17 |
|      |        |         | protein disulfide isomerase, rat | 91897 | 57 | 1 | 12 |
|      |        |         | GRP78 (BiP), human | 121567 | 78 | 1 | 15 |
|      |        |         | ribosomal protein L26, human | 4506621 | 17 | 1 | 10 |
|      |        |         | ribosomal protein L23A, rat | 132848 | 18 | 1 | 14 |
|      |        |         | ribosomal protein S18, rat | 70965 | 18 | 1 | 9 |
|      |        |         | keratin 9, human | 1082558 | 62 | 1 | 20 |
| 4    | 9, 10, 11 | 22-25  | Ig heavy chain C region, sheep | 109009 | frag | 4 | 16 |
|      |        |         | vesicle trafficking protein sec22b | 4759086 | 25 | 3 | 15 |
|      |        |         | ribosomal protein L13A, mouse | 136478 | 24 | 3 | 12 |
|      |        |         | ribosomal protein S9, rat | 1173286 | 22 | 2 | 10 |
|      |        |         | IgG lambda chain, sheep | 109030 | frag | 2 | 15 |
|      |        |         | GTP-binding protein, rat (ras-related) | 92022 | 23 | 1 | 14 |
|      |        |         | RAB1, human | 4758988 | 23 | 1 | 18 |
|      |        |         | 25-Ds, rat | 1518818 | 25 | 1 | 15 |
|      |        |         | ribosomal protein L9, rat | 133033 | 22 | 1 | 12 |
|      |        |         | ribosomal protein S7, rat | 279641 | 22 | 1 | 12 |
|      |        |         | IgY chain, cow | 1408167 | 18 | 1 | 15 |
| 5    | 12, 13, 14 | 27-31  | ribosomal protein S4, hamster | 1350996 | 30 | 20 | 9 |
|      |        |         | ribosomal protein L3, rat | 1083788 | 24 | 5 | 10 |
|      |        |         | urate oxidase, mouse | 6678509 | 35 | 3 | 10 |
|      |        |         | ribosomal protein L10A, rat | 1709974 | 25 | 2 | 12 |
|      |        |         | ribosomal protein S3A, Xenopus | 133940 | 27 | 2 | 15 |
|      |        |         | IgG lambda chain, sheep | 109030 | frag | 1 | 15 |
|      |        |         | Ig lambda chain, sheep | 2766644 | frag | 1 | 15 |
|      |        |         | Ig lambda chain, human | 186644 | 25 | 1 | 8 |
|      |        |         | Ig lambda chain, hamster | 497361 | frag | 1 | 8 |
|      |        |         | Ig lambda chain, human | 87890 | 25 | 1 | 8 |

**Fig. 5.** Table of proteins cross-linked to apoB identified by sequencing. The pooled bands from Fig. 4 were sequenced by mass spectrometry (see "Purification and Sequencing of Proteins" for details). The size range of the pooled bands is only an estimate. Proteins that were identified by exact peptide matches are listed with their accession number in the National Center for Biotechnology Information protein data base. If peptides matched proteins from different species, the first match encountered in the data base is shown. The molecular mass for each full-length protein in the data base was calculated from its amino acid sequence; some of the matched data base entries are for fragments (denoted frag). The number of matching peptides and their median length are also shown. Within each pool, the proteins are listed in descending order based on the number of matching peptides. In pool 3, band 1a (10 kDa) was included that is not readily visible in the scan of the preparative gel.
simply be located within about a 12-Å range of apoB. We investigated whether some of the proteins discovered can bind apoB directly. We chose to investigate the binding of apoB by ferritin heavy and light chains due to their interaction detected in other types of experiments.2 We found that small amounts of apoB can be co-immunoprecipitated from HepG2 cells in the absence of a cross-linker using antibodies against ferritin; at these levels of co-immunoprecipitation, however, it is difficult to rule out cross-reactivity of the antibodies with epitopes on apoB (data not shown). Furthermore, if binding is brief in cells, the association might not be detected easily without using a cross-linker.

To circumvent these issues, we investigated whether these

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2 S. Hevi and S. L. Chuck, unpublished observations.
candidates can bind to apoB directly \textit{in vitro}. We constructed plasmids encoding fusion proteins with ferritin heavy and light chains. First, we cloned the coding sequences for ferritin heavy and light chains by reverse transcription-PCR using mRNA from HepG2 cells. The PCR products were engineered into the plasmid TyB2 and verified by sequencing. The resulting plasmids encoded ferritin heavy or light chain fused to an intein and chitin-binding domain (Fig. 6A). These ferritin fusion proteins and the product of unmodified TyB2 (the multiple cloning site, intein, and chitin-binding domain) were expressed in \textit{E. coli} and purified by binding to chitin beads.

The binding assay was carried out using equal masses of the three proteins (bound to beads) and $[^{35}S]$methionine-labeled apoB29 (the amino-terminal 29\% of apoB) that was synthesized using rabbit reticulocyte lysate.apoB29 is a large protein (molecular mass, \sim 150 kDa) that still can be translated efficiently \textit{in vitro}. We found that both of the ferritin fusion proteins bound apoB29, with the fusion protein encoding ferritin heavy chain having the greatest affinity (Fig. 6B, lanes 1 and 2). In contrast, the intein and chitin-binding domain by themselves did not bind apoB29 well (Fig. 6B, lane 3). Furthermore, the lack of binding of globin or other unlabeled proteins in the lysate to the ferritin fusion proteins, as ascertained by Coomassie staining of the gel (data not shown), indicates that the binding of apoB by ferritin is specific. Thus, ferritin heavy and light chains not only can be cross-linked to apoB in microsomes prepared from rat livers but also can bind apoB directly. These proteins are candidates for roles in the biogenesis of apoB.

**DISCUSSION**

We developed a biochemical approach to purify proteins associated with apoB. This strategy has its advantages and its limitations. Proteins in microsomes derived from rat livers were treated with a chemical cross-linker. This step enables detection of proteins that might bind apoB too weakly to remain associated throughout the immunoprecipitation procedure. The downside of using a cross-linker is that proteins that are in close proximity (\textit{i.e.} within about 11.9 Å, the length of the spacer arm) but not actually in contact could become cross-linked to apoB. Thus, the use of a chemical cross-linker results in decreased specificity as the price for increased sensitivity. Relatively few proteins, however, are co-immunoprecipitated with apoB when the microsomes were not treated with a chemical cross-linker (see Fig. 3, \textit{lane 1}, and the few bands seen in Fig. 2, \textit{lane 1}). Therefore, we chose to use a cross-linker. In the second stage of purification, the microsomes were solubilized, and the proteins were co-immunoprecipitated with antibody against apoB. Polyclonal antibodies often cross-react with other proteins. However, the relative lack of proteins other than apoB in the uncross-linked sample attests to the specificity of the immunoprecipitation step (Fig. 3, \textit{lane 1}). The combination of microcapillary high pressure liquid chromatography and tandem mass spectrometry is a powerful and sensitive tool for protein sequencing. This sensitivity has its limitations, too. Even small amounts of contamination with human keratin from handling the gel, glass plates, or container for staining can be detected. Some proteins might have been masked by the large number of ribosomal proteins cross-linked to nascent apoB or diluted below detection by pooling bands. A few anticipated proteins, including MTP, calnexin, calreticulin, and GRP94, did not appear in the list of matches. Apparently, these proteins failed to be cross-linked to apoB or were recovered in inadequate amounts from co-immunoprecipitation, or too few peptides of suitable lengths were released from the gel or reverse phase high pressure liquid chromatography for detection. Furthermore, a peptide from rat MTP may have been unmatched by the algorithms used. The sequence for rat MTP is not currently in the database. MTP from humans, hamsters, and cows are highly homologous and about 85\% identical in sequence, and rat MTP might match MTP of other species to a similar degree. The stringent matching process used, however, requires identity over the entire tryptic peptide and not just homology. All of these limitations of our biochemical approach were accepted in view of the lack of a tractable genetic or \textit{in vitro} system to study the biogenesis of such a large and complex protein. Despite these shortcomings, this strategy did identify proteins that bind to apoB.

Many ribosomal proteins were cross-linked to presumably incompletely elongated chains of apoB. When lipid synthesis is limiting in HepG2 cells, apoB proteins that appear to be full-length can be incompletely synthesized and remain in functional association with the ribosome and translocation machinery in the ER membrane (26). Thus, it is not surprising that such a large number of ribosomal proteins were cross-linked to apoB in rat liver microsomes.

This biochemical approach yielded 50 proteins other than ribosomal proteins and contaminants. Some known chaperone proteins were identified, including members of the PDI family and BiP. Despite a lack of evidence for degradation, peptides matching PDI and BiP were found not only in their appropriate size range but also in pool 3. These single peptides may have
been cleaved from smaller proteins than these known chaperones that serve similar functions. One matched protein, fatty acyl co-A ligase (27), is an enzyme involved in triglyceride biosynthesis, which can affect the secretion of apoB (28–30). Fatty acyl co-A ligase and apolipoprotein B can be found in the ER, and both are enriched in the mitochondria-associated membrane fraction (31). Our data raise the possibility that apoB is in close contact with this enzyme that synthesizes lipids to be transported. An enzyme that converts cholesterol esters to free cholesterol, cholesterol ester hydrolase (32), was identified. This enzyme converts cholesterol to free fatty acids, which accounted for 39 peptides overall. However, this enzyme also found. Five matches are with uricase (urate oxidase), a metabolic enzyme, 11-

esters to free cholesterol, cholesterol ester hydrolase (32), was identified. This result may fit in with the growing body of evidence that the pathways for hepatic cholesterol metabolism and lipoprotein secretion intersect (2, 33). Another steroid metabolic enzyme, 11β hydroxysteroid dehydrogenase (34), was also found. Five matches are with uricase (urate oxidase), which accounted for 39 peptides overall. However, this enzyme cannot play a role in the biogenesis of apoB in man because there is not a human homologue (35). Similarly, L-gulonolactone oxidase, a key enzyme in ascorbic acid biosynthesis, is missing in humans rendering us susceptible to scurvy (36). Twelve different cytochrome P450 enzymes were isolated. As a family, these microsomal, heme-containing enzymes are involved in the conversion of metabolites for excretion and synthesis of steroid hormones, but they have not yet been implicated in the biogenesis of apoB. Many other proteins also were isolated including some that bind GTP, participate in vesicle trafficking, play a key role in the conversion of vitamin A to retinoic acid, and detoxify substances (e.g. glutathione-S-transferase, epoxide hydrolase, and cytochrome P450 enzymes). These proteins might be intracellular neighbors that never interact; alternatively, they might bind apoB and play novel roles in its biogenesis.

Our strategy did identify some novel proteins that can bind to apoB. We show that ferritin heavy and light chains can bind apoB. This result raises several questions. Do ferritin heavy and light chains play a role in the biogenesis of apoB? Where do these cytosolic proteins bind on apoB? Do the iron storage and lipoprotein secretion pathways intersect? What other proteins that were cross-linked to apoB likewise bind apoB? We are currently investigating these questions.

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