Communication

Intrahepatic Assembly of Very Low Density Lipoproteins

PHOSPHORYLATION OF SMALL MOLECULAR WEIGHT APOLIPOPROTEIN B*

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Roger A. Davis‡, Gail M. Clinton†,
Roy A. Borchardt‡, Monica Malone-McNeal‡,
Tina Tan‡, and Gerri R. Lattier‡

From the †Cell Biology Unit, Department of Physiology
and the ‡Department of Biochemistry, Louisiana State
University Medical School, New Orleans, Louisiana 70112

The possibility that apo-B is phosphorylated was examined using cultured rat hepatocytes. Rabbit antibody prepared against rat apo-B was found to specifically react with both large and small molecular weight apo-B (by electrophoresing assay and by immunoprecipitation of [32P]methionine-labeled proteins synthesized and secreted by hepatocytes). Following a 4-h incubation with [32P]orthophosphate, immunoprecipitation, and sodium dodecyl sulfate electrophoresis, an autoradiographic band corresponding to small molecular weight apo-B was obtained from cells and medium. Compared to the relative abundance of [32P] which was associated with secreted small molecular weight apo-B, there was little (if any) detected in large molecular weight apo-B. Addition of excess unlabeled apo-B (obtained from rat serum) totally competed with the specific antiserum for this radioactive protein, indicating it was antigenically related to apo-B. Moreover, isolation of the [32P]-labeled apo-B electrophoretic band, followed by acyldisulfide and phosphoamino acid analysis, showed that at least 20% of the [32P] originally associated with small molecular weight apo-B was in the form of phosphoserine. Control experiments ruled out the possible contamination of apo-B with phospholipid as well as the possibility that the phosphoserine produced by acid hydrolysis could have been derived from phosphatidylserine. To examine the relevance of these data to the in vivo state, rats were injected with [32P]orthophosphate. Immunoprecipitation of their livers followed by autoradiographic analysis showed the presence of [32P] in small molecular weight apo-B. These data show for the first time that small molecular weight apo-B is synthesized as a phosphoserine containing protein.

VLDL1 secretion requires a complex sequential series of intracellular events. These include apolipoprotein and lipid biosynthesis, association of the lipid and protein moieties into lipid-protein aggregates, condensation into secretory vesicles, and targeting to the sinusoidal surface membrane for secretion into plasma (1–3). The molecular interactions through which the nascent VLDL particle is assembled and subsequently vectorially shuttled through this multigranulare pathway are unknown.

Although there are numerous discrete apolipoproteins associated with VLDL, evidence suggests that apo-B in particular is essential for secreting triacylglycerol-rich lipoproteins. Apo-B exists in at least two molecular weight forms (4–7), both of which are initially secreted by rat liver as a component of VLDL (6–8). In humans, genetic deletion of plasma apo-B abolishes VLDL secretion (9). These results suggest that one or both molecular weight forms of apo-B is required for VLDL assembly. Because of technical problems in working with apo-B, such as self-association and the inability to maintain its solubility in aqueous solutions, little is known about its structure, sequence, and chemical composition. Moreover, as a result of insufficient chemical data, the unique molecular feature which affords apo-B the ability to direct VLDL assembly and secretion remains unknown.

In this report, we show that the small molecular weight form of apo-B is secreted by cultured rat hepatocytes in a form which contains phosphorylated serine residues, as evidenced by specific immunochromel analysis and phosphoamino acid analysis. In marked contrast, there is little or no phosphorylation of the large molecular weight form of apo-B. These novel findings may provide a clue to the molecular interactions through which apo-B containing lipoproteins are assembled, secreted, and metabolized.

MATERIALS AND METHODS

All culture reagents, rats, and chemicals were obtained from sources previously described (10). Radioactive chemicals were obtained from ICN Biochemicals, Irvine, Ca.

Antibody Preparation—An ultracentrifugation fraction (d < 1.21 g/ml) obtained from rat serum was delipidated according to the method of Cardin et al. (11). Individual molecular weight forms of apo-B were purified by gel filtration on Sepharose Cl-6B according to the method of Sparks and Marsh (12). Analysis of the fractions on SDS-PAGE showed that some contained only one band (silver stained) which corresponded in molecular weight to either large molecular weight apo-B or small molecular weight apo-B. Antibodies against the pure individual apo-B forms were raised in rabbits using procedures previously described (10).

Antibody specificity was assessed using the electroblotting technique of Towbin et al. (13) (see Fig. 1). Although antibodies raised against either apo-B form showed relatively the same titers and specificity, antibodies raised against the large molecular weight apo-B form were used for these studies.

Preparation of Hepatocytes—Hepatocytes obtained from male Sprague-Dawley sucrose-fed rats (to induce VLDL secretion) were plated after collagenase digestion using methods which have been described in detail (14). After incubating for 4 h with DME containing 20% calf serum, the medium was changed to serum-free DME. The serum-free medium was phosphate- and methionine-free.

Protein 32P- and 35S-labeling Studies—After incubation with [32P] orthophosphate (3 mCi/2 ml) or [35S]methionine (100 µCi/2 ml) (see legends), the medium was removed via suction and immediately diluted 2-fold with SDS containing buffer B as described by Faust et al.
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al. (15). Boiling buffer B was immediately added to the plastic culture dish containing the cells. Visualization via phase contrast microscopy showed that all of the cells were rapidly lysed and came off the dish. The solution containing the cells was diluted with buffer B without SDS in order that the final concentration became 0.15%. Separate aliquots of medium and cells were placed in plastic 1.5-ml tubes and an appropriate amount of rabbit anti-apo-B antisera (previously unlabeled or labeled to precipitate all of the apo-B contained in each sample) was added. The solutions were incubated at room temperature for 18 h after which protein A bound to Sepharose (Pharmacia) was added at a concentration previously shown to bind all of the rabbit immunoglobulin in each sample. After an additional 4-h incubation, the Sepharose pellets were isolated by centrifugation in a microfuge (Fisher Scientific) for 5 min. The Sepharose pellet was washed six times with buffer D (as described by Faust et al. (15)). A sample containing 100 μg of rat serum protein (d < 1.21 g/ml) was then added as a cold carrier. The samples were then boiled for 15 min in sample buffer containing 8 M urea, 2% SDS, 10% glycerol, 5 mM mercaptoethanol, and 10 mM Tris/glycine (pH 8.3). The samples were then subjected to SDS-PAGE using the system of Laemmli (16) except we used a 3–10% linear polyacrylamide gradient. Molecular weight standards run on the same gel permitted an accurate molecular weight determination. Gels containing Coomassie-labeled protein (obtained from rat plasma) (Fig. 1). We were unable to detect a discrete band for large molecular weight apo-B. Additional experiments show that the autoradiographic technique used is capable of detecting 5% of the radioactivity found associated with small molecular weight apo-B. Thus, if large molecular weight apo-B is secreted in a phosphorylated form, it contains less than 10% of the 32P which is associated with small molecular weight apo-B. It is possible that in other metabolic conditions or in other species the relative phosphorylation of the different molecular weight forms of apo-B might be different from that observed in these experiments.

Immunoprecipitation of the cell lysate also showed the presence of radioactivity corresponding to the small molecular weight form of apo-B (Fig. 1). This phenomenon was, in fact, due to apo-B, excess (50 μg) of unlabeled apo-B (obtained from rat plasma) was added to the cell lysate and the medium prior to adding the antiserum. The results showed the complete disappearance of the band corresponding to small molecular weight apo-B in the medium and cells (data not shown). In several but not all experiments, 32P-labeled proteins that had molecular weights less than the plasma apo-B standards were isolated from the cell lysate. The autoradiographed bands corresponding to these proteins did not disappear when excess unlabeled apo-B was added to the immunoprecipitation buffer, suggesting they are not antigenically related to apo-B. Additional studies showed that these bands also were precipitated with serum from nonimmunized rabbits and protein A bound to Sepharose suggesting that these proteins have some affinity for these reagents. Phosphoamino Acid Analysis of Apo-B—To define the covalent linkage of the 32P in apo-B, the radioactive (i.e. 32P-labeled) protein corresponding to small molecular weight apo-B

2 Based on our experimental finding that large molecular weight apo-B is secreted at a rate which is 30–50% of the rate of small molecular weight apo-B secretion (10).

Fig. 1. Incorporation of [35S]methionine and [32P]orthophosphate into apo-B by cultured rat hepatocytes. Four h after initially plating hepatocytes in DME + 20% calf serum, the culture medium was changed to serum-free DME, which was methionine- and phosphate-free (lanes 3–6). After an additional 4-h incubation with [35S]methionine (lanes 3 and 4) or [32P]orthophosphate (lanes 5 and 6), the medium and cells were harvested using SDS containing buffer and subjected to immunoprecipitation, SDS-PAGE, and fluorographic analysis (lanes 3 and 4) or autoradiographic analysis (lanes 5 and 6). Lane 1, Coomassie-stained SDS-PAGE gel of rat serum lipoproteins (d < 1.21 g/ml); lane 2, electroblot of antiserum used for immunoprecipitation reacted against rat serum lipoproteins (d < 1.21 g/ml); lane 3, two-day activated fluorogram of 35S-labeled culture medium; lane 4, two-day activated fluorogram of 32P-labeled cell lysate; lane 5, two day activated autoradiogram of 32P-labeled culture medium; lane 6, two day activated autoradiogram of 32P-labeled cell lysate.

RESULTS AND DISCUSSION

Specificity of Antiserum and Immunoprecipitation—The specificity of the antiserum was determined by electrophoresis and by immunoprecipitation of cells and medium and was labeled with [35S]methionine (Fig. 1). Both methods showed that the antiserum specifically reacted with both molecular weight forms of apo-B. We used this specific antiserum to examine the possibility that apo-B is phosphorylated. Using the same preparations of cells and experimental protocol as was used for the [35S]methionine studies, we examined the incorporation of [32P]orthophosphate into immunoprecipitable apo-B. The results show that 32P becomes associated with a secreted protein (i.e. isolated in the medium) which has similar migration (SDS-PAGE) to the small molecular weight form of apo-B (isolated from rat plasma) (Fig. 1). We were unable to detect a discrete...
B was cut out of the SDS-PAGE gels, eluted by electrophoresis into a dialysis bag, and subjected to acid hydrolysis and paper electrophoresis. The results show that small molecular weight apo-B contains phosphoserine as the only detectable phosphoamino acid (Fig. 2). There also was the presence of $^{32}$P phosphoamino acid (free inorganic phosphate). Since unincorporated $^{32}$P phosphoamino acid was removed by precipitation with trichloroacetic acid and washing, it is probable that the free phosphate was not present in the unhydrolyzed sample. It is more likely that the free phosphate was produced during the acid hydrolysis step. Quantitation of the amount of radioactivity in phosphoserine showed that a minimum of 20% of the $^{32}$P originally associated with small molecular weight apo-B was in the form of phosphoserine. The 20% recovery of phosphoserine from apo-B after acid hydrolysis is similar to the recoveries found for other phosphoserine-containing proteins (19, 20). We can not rule out the possibility that some of the $^{32}$P associated with apo-B and hydrolyzed to free phosphate may have been derived from phosphorus forms in addition to phosphoserine.

To rule out the possibility that $^{32}$P phosphoserine produced by acid hydrolysis of apo-B was derived from phosphotidylserine, three control experiments were performed. First, cells and medium were labeled with $^{32}$P orthophosphate using the same protocol as described in Fig. 1. The $^{32}$P-labeled lipids were quantitatively extracted (10). The $^{32}$P-labeled lipids (following solvent evaporation) were added to unlabeled medium and cells (they were incubated for 4 h in a manner similar to that described in Fig. 1). Immunoprecipitation, SDS-PAGE, autoradiography, and radioactivity quantitation ($\beta$-scintillation counting) failed to show any radioactivity in the apo-B SDS-PAGE band. Furthermore, acid hydrolysis of the $^{32}$P-labeled lipids also failed to yield $^{32}$P phosphoserine as assayed in Fig. 2. Secondly, $[3H]$glycerol was added to cells for 4 h to form $^3H$ phospholipids. Immunoprecipitation, SDS-PAGE, and radioactivity quantitation by $\beta$-scintillation counting also failed to show any radioactivity. The sensitivity of this assay was 10 ng of phospholipid. Finally, phosphatidylserine was subjected to the same procedure as was used to isolate $^{32}$P phosphoserine from the $^{32}$P-labeled apo-B (as in Fig. 2). Acid hydrolysis of phosphatidylserine (100 $\mu$g) failed to yield any detectable phosphoserine. The quantitative acid hydrolysis of phosphatidylserine produced only free serine (data not shown). This assay was capable of detecting 0.4% of the phosphoserine which could have been produced from 100 $\mu$g of phosphatidylserine. Since acid hydrolysis of $^{32}$P-labeled apo-B yielded 20% of the $^{32}$P in the form of phosphoserine (Fig. 2), these data rule out the possibility that phosphatidylserine was the source of the $^{32}$P phosphoserine.

In Vivo Demonstration of Apo-B Phosphorylation—To determine if apo-B is phosphorylated in vivo, sucrose-fed rats were injected with 10 mCi of $^{32}$P orthophosphate. After 3 h, rats were bled, the serum was separated into a total lipoprotein fraction ($d < 1.21$ g/ml) by ultracentrifugation. Analysis of the fraction ($d < 1.21$ g/ml) by SDS-PAGE (silver stained) clearly showed the presence of both molecular weight forms of apo-B (data not shown). Immuno precipitation of the livers of these rats showed the presence of $^{32}$P in small molecular weight apo-B (Fig. 3). It is unknown if some of the $^{32}$P-labeled small molecular weight apo-B obtained in the serum may have also been produced by the intestine.

Additional studies showed that incubation of VLDL (containing $^{32}$P-labeled small molecular weight apo-B) with fresh rat serum for 8 h caused a dramatic loss of $^{32}$P (258 ± 24 cpm before incubation; 78 ± 15 cpm after incubation; $n = 3$ in each group). These results indicate that rat serum contains a process which can, in fact, dephosphorylate small molecular weight apo-B.

Recent studies show that apo-A-I (21-22) and apo-A-II (23) are synthesized by the liver as propeptides. Pro-A-I peptide is normally cleaved in plasma by a protease (24). It is possible that the phosphopeptide group on small molecular weight apo-B may behave similar to the NH$_2$-terminal extension of a propeptide (i.e. after being secreted by the liver, phosphorylated apo-B is dephosphorylated).

Phosphorylation may be a general characteristic of a specific class of amphotrophic proteins. Several other amphotrophic proteins which associate with lipids to form lipid-protein aggregates in a manner similar to VLDL apo-B are phosphorylated. For example, avian liver secretes lipoproteins which act to transport lipid to the oviduct for egg development (25). Two of these proteins derived from vitellogenin are polyphosphorylated (26). Casein, a major protein in milk, is hydrophobic, excreted complexed with phospholipid and triacylglycerol, and is polyphosphorylated on serine and threonine residues (27). In addition, myelin basic protein, which is also a hydrophobic protein complexed with phospholipid, is also phosphorylated on serine and threonine residues (28). Staehelin and Arntzen (29) propose that phosphorylation of proteins in chloroplasts provide the molecular forces ne-
essary for lateral movement in membranes. A similar role for phosphorylation of apo-B is possible. Phosphorylation might serve to shuttle VLDL during its assembly and/or secretion.

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