A Protein Partially Expressed on the Surface of HepG2 Cells That Binds Lipoproteins Specifically Is Nucleolin†

Clay F. Semenkovich,‡,§ Richard E. Ostlund, Jr.,*,‡ Mark O. J. Olson,∥ and Joseph W. Yang‡
Metabolism Division, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri 63110, and Department of Biochemistry, University of Mississippi Medical Center, Jackson, Mississippi 39216
Received February 15, 1990; Revised Manuscript Received July 13, 1990

ABSTRACT: Nucleolin, a major nucleolar protein of rapidly growing eukaryotic cells, has been thought to be predominantly if not exclusively located in the nucleolus. Recent data however [Borer, R. A., Lehner, C. F., Eppenberger, H. M., & Nigg, N. A. (1989) *Cell 56*, 379-390] suggest that the protein shuttles constantly between the nucleus and cytoplasm. Ligand blotting studies of whole cell extracts of HepG2 cells identified, in addition to the LDL receptor, another LDL binding protein of M_r 109000. The 109-kDa protein was partially purified by HPLC and, like the LDL receptor, bound apoB- and apoE-containing lipoproteins but not HDL. However, unlike the LDL receptor, the 109-kDa protein bound lipoproteins in the presence of EDTA and reducing agents, had a lower affinity for lipoproteins than the LDL receptor, and did not react with two antibodies raised against the LDL receptor. The protein sequences of three separate peptides derived from the partially purified 109-kDa species were determined and were identical except for one residue to three separate regions of the published sequence of nucleolin. On immunoblot analysis the 109-kDa protein reacted with a nucleolin-specific antibody, and purified nucleolin reacted both with anti-109-kDa antibody and with LDL. When intact HepG2 cells were treated with Pronase before harvest, there was a 46% decrease in 109-kDa protein while recovery of actin, an intracellular protein, was unaffected. When intact HepG2 cells were surface iodinated and the proteins subjected to HPLC fractionation, the 109-kDa protein was found to be iodinated. On blots, the binding of ^{125}I-LDL to the 109-kDa protein was inhibited by anti-109-kDa antibody and by the nucleolin-specific antibody. In addition, anti-109-kDa antibody significantly decreased the specific binding of ^{125}I-LDL to intact HepG2 cells. These data suggest that the lipoprotein-binding 109-kDa protein is nucleolin or a nucleolin-like protein that is partially expressed on the cell surface.

Nucleolin, formerly called C23, is the major non-histone protein of the nucleolus in exponentially growing eukaryotic cells. The protein has been implicated in regulation of rRNA transcription and ribosome assembly (Jordan, 1987), although the exact role of nucleolin in these processes is unknown. Several characteristics of the protein have been defined. Nucleolin is predominantly associated with nascent preribosomal RNA (Herrera & Olson, 1986), binds DNA with a preference for nontranscribed spacer sequences of rDNA (Olson et al., 1983), is phosphorylated (Bourbon et al., 1983), and appears to be processed in association with the synthesis of preribosomal RNA (Bouche et al., 1984). Recently, the nucleotide sequences of nucleolin from three species were determined (Lapeyre et al., 1987; Bourbon et al., 1988a; Srivastava et al., 1989). The predicted amino acid sequences indicate that nucleolin contains at least three structurally distinct regions: (a) a highly charged region near the amino terminus; (b) a central region containing RNA binding domains; (c) a glycine-rich extended conformation region at the carboxy terminus.

Immunochemical studies have localized nucleolin predominantly to the nucleolus (Olson et al., 1981). However, Borer et al. (1989) have recently shown that nucleolin and another nucleolar protein shuttle constantly between the nucleus and cytoplasm.

In the current work, we have identified a 109-kDa protein in HepG2 cells that, on the basis of partial protein sequence and immunologic data, appears to be nucleolin. The HepG2 nucleolin or nucleolin-like protein unexpectedly binds apoB- and apoE-containing lipoproteins specifically and appears to be partially expressed on the cell surface.

Experimental Procedures

Materials. HepG2 cells (Knowles et al., 1980) were a gift from Dr. Barbara Knowles (The Wistar Institute, Philadelphia, PA). Cells were grown in MEM + fetal bovine serum and lipoprotein receptors were induced by incubation in MEM + 10% lipoprotein-deficient serum (LPDS) as described (Semenkovich et al., 1982). Rabbit IgG raised against the bovine LDL receptor and hybridoma cells producing IgG-C7 were gifts from Drs. Michael Brown and Joseph Goldstein, Dallas, TX. Antibodies to the Mono Q peak of the 109-kDa protein were raised in rabbits. Antiserum to rat Novikoff hepatoma nucleolin was prepared as described (Olson et al., 1981).

Lipoproteins and Lipoprotein Binding. Human LDL and apoE-3 were prepared as previously described (Semenkovich et al., 1985; Ostlund et al., 1982). ApoE-3 was radioiodinated to 4800 cpm/ng with Enzymobeads (Bio-Rad, Richmond, CA), reduced and alkylated, and incorporated into dimyristoylphosphatidylcholine (DMPC) vesicles in a mass ratio of 1:3.7. ^{125}I-LDL binding to cells and determination of receptor-specific dextran sulfate releasable surface-bound LDL were done as described (Semenkovich et al., 1982). Cells were routinely incubated for 48 h in 10% human LPDS before

† This work was supported by National Institutes of Health Grants R01-HL29229 (to R.E.O.) and R01-GM28349 (to M.O.J.O.) and by Physician-Scientist Award DK01762 (to C.F.S.). A portion of this work was presented at the 62nd Scientific Sessions of the American Heart Association, New Orleans, LA, 1989.
* Author for correspondence.
‡ Washington University School of Medicine.
§ Current address: Lipid Research, Department of Medicine, Washington University School of Medicine, St. Louis, MO 63110.
∥ University of Mississippi Medical Center.

0006-2960/90/0429-9708$02.50/0

harvest. ApoE-free high-density lipoprotein (HDL) was prepared as described by Weisgraber and Mahley (1986). For some experiments, high specific activity ^{125}I-LDL (2060 cpm/ng) was prepared by incubating 200 μg of LDL protein in 200 μL of 0.1 M sodium phosphate buffer (pH 7.4) with three Iodobeads (Pierce Chemical Co., Rockford, IL) and 1 mCi of radioiodide for 45 min at room temperature.

Western Blotting. Cells were homogenized in 1.6% Triton X-100 containing 5 M urea, 0.3 mM leupeptin, and 1.5 mM phenylmethanesulfonyl fluoride (PMSF), and the lysate was clarified by centrifugation at 100000*g* for 15 min (Semenkovich et al., 1985). Either cell lysates or column fractions were electrophoresed in 0.1% SDS–6% polyacrylamide gels (Laemmli, 1970) and electrophoretically transferred to nitrocellulose paper. After nonspecific binding sites were blocked by incubation with BSA/goat serum buffer, nitrocellulose strips were incubated either with nonradioactive LDL or directly with ^{125}I-LDL. The latter were washed extensively in 45 mM NaCl/25 mM Tris-HCl/2 mM $CaCl_2$ (pH 8.0) in the absence of detergent, then dried, and autoradiographed. Strips containing receptors bound to nonradioactive LDL were incubated with affinity-purified ^{125}I-goat-anti-LDL antibody, washed extensively in the presence of 0.05% NP-40, then dried, and autoradiographed. Immunoblots to determine reactivity toward antibodies to the LDL receptor and the 109-kDa protein were incubated for 1 h at 4 °C with 8 μg/mL IgG and then washed and incubated with the radioiodinated second antibody. Immunoblots to detect reactivity with anti-nucleolin antibodies were incubated with a 1:286 dilution of anti-nucleolin antiserum and then processed in the absence of detergents as above. In all cases control preimmune IgG or nonimmune serum was analyzed in parallel.

Partial Purification of 109-kDa Protein. The protein was found in the 100000*g* total membrane and nuclear fraction after homogenization in 0.15 M NaCl containing 10 mM Tris-HCl (pH 7.4). Therefore, detergent extracts of whole cells prepared as for western blotting were used as the starting material. A 0.5 × 5 cm Mono Q high-performance ion-exchange column (Pharmacia-LKB) was equilibrated with 82% A solution (20 mM Bis-Tris-HCl, pH 6.0, containing 1.0% Triton X-100 and 2.0 mM $CaCl_2$) and 18% B solution (A solution + 2.0 M NaCl). An extract of HepG2 cells containing 200–2000 μg of protein was applied, and the column was washed with 10 mL of buffer. Retained proteins were eluted with a 20-mL linear gradient of 18% B to 35% B solution. The 109-kDa protein bound to the column and was eluted with NaCl as described under Results.

Purification of Nucleolin. Nucleolin was purified by a technique different from that used to isolate the 109-kDa protein. Novikoff hepatoma nucleoli prepared as described by Rothblum et al. (1977) were used as the starting material. Nucleoli were extracted with low ionic strength buffers (Herrera & Olson, 1986) and the extracts stored in 10 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 1 mM PMSF, 1.5 μmol/mL 1-[N^{α}-(*trans*-epoxysuccinyl)-L-leucylamido]-4-guanidinobutane (E-64), 10 μg/mL leupeptin, 1 μM aprotinin, 1 μM pepstatin, and 30% glycerol at −80 °C before use. Nucleolin was purified by heparin–Sepharose chromatography as described by Caizergues-Ferrer et al. (1987). The final product was homogeneous as judged by two-dimensional isoelectric focusing–SDS polyacrylamide gel electrophoresis.

Protein Sequencing. Minigels of 6% polyacrylamide, 0.1 M sodium phosphate (pH 7.1), and 0.1% SDS were aged overnight and then prerun for 30 min with gel buffer containing 0.1 mM thioglycolate (Moos et al., 1988). Mono Q fractions were electrophoresed and transferred to Immobilon P poly(vinylidene difluoride) paper for 30 min in 10 mM Hepes (pH 7.0) containing 0.5 mM dithiothreitol. The blotted 109-kDa protein was stained with Coomassie blue, excised, and analyzed on an Applied Biosystems Model 470A gas-phase protein sequencer. For tryptic digestion samples were transferred to nitrocellulose paper, detected with Ponceau S, destained, and incubated with trypsin in situ, and the released peptides were isolated by reverse-phase HPLC as described by Aebersold et al. (1987).

Cell Surface Iodination. HepG2 cells were plated in 35 mm diameter culture dishes and grown as described above. The medium was removed and the cells were washed eight times with Puck's saline G and placed on ice for 30 min. One millicurie of ^{125}I and three washed Iodobeads were added, and the dish was gently rocked for 45 min. The cells were then washed, scraped from the plate, extracted, and mixed with nonradioactive carrier extracts, and the 109-kDa protein was isolated by HPLC.

Competition of Antibody for ^{125}I-LDL Binding to Intact HepG2 Cells. Cells were plated at 10000/well on microtiter dishes and incubated with 10% LPDS for 48 h. The medium was removed and replaced with 35 μL/well MEM buffered with 20 mM Hepes (pH 7.4) containing 10% LPDS and either 200 μg/mL preimmune IgG (control) or 200 μg/mL anti-109-kDa protein IgG. The cells were placed on ice for 1 h after which time the medium was replaced with fresh medium containing the same preimmune or immune IgG and 10 μg/mL high specific activity ^{125}I-LDL. After incubation on ice for an additional hour, the cells were washed and surface-bound ^{125}I-LDL was removed with dextran sulfate. Specific binding was calculated by subtracting the results of wells incubated with 1000 μg/mL unlabeled LDL. The results of four experiments using a total of 27 wells for both control and immune IgG were subjected to a two-way analysis of variance by experiment and presence of preimmune or immune IgG.

Results

Ligand blotting of whole cell extracts of HepG2 cells detected two distinct bands of lipoprotein binding (Figure 1, lane 2). In addition to the classic LDL receptor (M_r 140000, open arrowhead) previously characterized by our laboratory in HepG2 cells (Semenkovich & Ostlund, 1987), a second band of M_r 109000 was detected in HepG2 cells (closed arrowhead) after incubating nitrocellulose-immobilized proteins with ^{125}I-LDL. Binding of ^{125}I-LDL to the LDL receptor and the 109-kDa protein was abolished by the inclusion of a 75-fold excess of unlabeled LDL in the incubation buffer (lanes 3 and 4), suggesting that, like the 140-kDa LDL receptor, the 109-kDa protein binds human LDL specifically. However, unlike the LDL receptor, binding of LDL to the 109-kDa protein was not calcium sensitive (lanes 5–8). Fibroblast extracts (lanes 5 and 7) and partially purified 109-kDa HepG2 protein (see below) (lanes 6 and 8) were ligand blotted in the presence of calcium (lanes 5 and 6) or in the absence of calcium and the presence of 2 mM EDTA (lanes 7 and 8). In the absence of calcium, ^{125}I-LDL did not bind to the fibroblast LDL receptor (open arrowhead position, lane 7) but binding to the 109-kDa protein (closed arrowhead, lane 8) was unaffected. In some experiments, fibroblasts did express small amounts of 109-kDa material (lanes 5 and 7), but the low-level, inconsistent expression of 109-kDa material in fibroblasts made characterization difficult. The 109-kDa LDL binding was also unaffected by incubation with the reducing agent dithiothreitol before electrophoresis and ligand blotting, while binding to

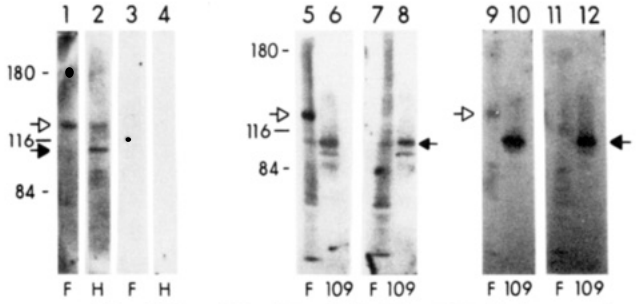

FIGURE 1: Ligand blots of HepG2 and fibroblast LDL binding proteins. Cells were incubated to induce LDL receptors; then whole cell extracts or HPLC fractions were prepared, electrophoresed in SDS–polyacrylamide gels, and transferred to nitrocellulose. F is fibroblast extract, H is HepG2 extract, and 109 is HPLC-fractionated 109-kDa protein. For lanes 1–4, strips were incubated with 20 μg/mL ^{125}I-LDL in the absence (lanes 1, 2) or presence (lanes 3, 4) of 1500 μg/mL unlabeled LDL. Lanes 5–12 were incubated with LDL and then with ^{125}I-anti-LDL IgG. For lanes 5–8, incubations with LDL were performed in the presence of 2 mM calcium (lanes 5, 6) or the absence of calcium and the presence of 2 mM EDTA (lanes 7, 8). For lanes 9–12, samples were incubated for 10 min at 37 °C in the absence (lanes 9, 10) or presence (lanes 11, 12) of 40 mM DTT before electrophoresis.

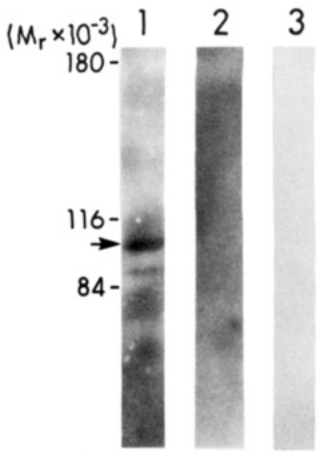

FIGURE 2: Binding of ^{125}I-apoE DMPC vesicles and ^{125}I-HDL to 109-kDa protein. The 109-kDa protein was electrophoresed and transferred to nitrocellulose paper. Lane 1, nitrocellulose strip incubated with ^{125}I-apoE associated with DMPC vesicles; lane 2, strip incubated with free ^{125}I-apoE unassociated with DMPC vesicles; lane 3, strip incubated with 20 μg/mL apoE-free HDL and then with ^{125}I-anti-apoA-1 antibody.

the fibroblast LDL receptor was abolished (lanes 9–12). However, LDL acetylation, which decreases the positive charge on apoB and disrupts interaction with the ligand binding region of the LDL receptor, caused similar decreases in LDL binding to the 109-kDa protein (28.0 ± 9.7% of control) and the fibroblast LDL receptor (15.7 ± 5.8% of control) when used in three ligand blotting experiments (blots not shown).

In addition to binding apoB-containing lipoproteins, the 109-kDa protein also bound apoE-containing lipoproteins. When the 109-kDa protein was partially purified as described below and blotted, the protein bound ^{125}I-apoE DMPC vesicles (Figure 2, lane 1). ApoE in the absence of phospholipid vesicles is unable to bind specifically to lipoprotein receptors (Innerarity et al., 1979); no bands were detected when ^{125}I-apoE in the absence of DMPC vesicles was used in blotting experiments (Figure 2, lane 2). Graham and Oram (1987) have detected in numerous tissues, including HepG2 cells, an HDL binding protein with a molecular weight of 110000, strikingly similar to that of the 109-kDa protein in the current study. However, the HDL binding protein, which may be the HDL receptor, does not bind *LDL*, and as shown in Figure 2, lane 3, the partially purified 109-kDa protein does not bind *HDL*.

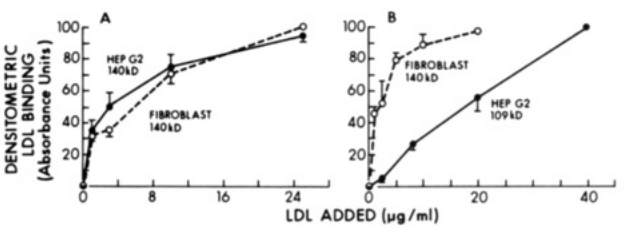

FIGURE 3: Dose–response experiments comparing the affinities of nitrocellulose-immobilized LDL receptor and 109-kDa protein for LDL by ligand blotting. Cells were grown to induce LDL receptors; then extracts were prepared and blotted on a series of nitrocellulose strips, followed by incubation with increasing concentrations of LDL. Results are expressed as densitometric absorbance units (mean ± SEM of five experiments for panel A and three experiments for panel B) following subtraction of nonspecific background binding at each LDL concentration. Note the different scales for the horizontal axes. (Panel A) Comparison of LDL binding by HepG2 and fibroblast LDL receptors. (Panel B) Comparison of LDL binding by HepG2 109-kDa protein and fibroblast LDL receptor.

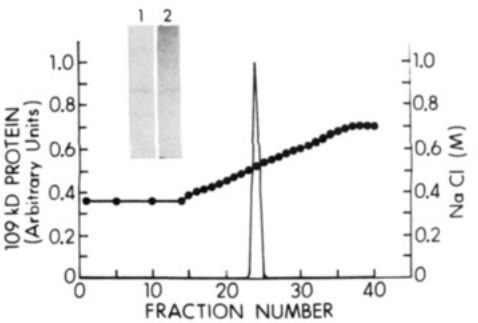

FIGURE 4: HPLC of the 109-kDa protein. A HepG2 cell lysate containing 3.8 mg of protein was applied to a 1-mL Mono Q column and eluted as described under Experimental Procedures. The fractions were subjected to ligand blotting and the 109-kDa band was scanned with a densitometer. Inset: lane 1, ligand blot of 280 ng of protein from the peak column fraction of the figure; lane 2, Coomassie blue stain of 3.9 μg of protein from the peak fraction.

Scatchard plots (not shown) derived from the *specific* binding of ^{125}I-LDL in dose–response experiments in intact cells were consistently curvilinear, suggesting more than one specific binding process. HepG2 cells had 10-fold lower affinity for LDL than fibroblasts (HepG2 K_m = 35.5 ± 9.1 μg/mL; fibroblast K_m = 3.2 ± 0.3 μg/mL). When dose–response experiments were performed by ligand blotting, the LDL receptor in both cell types (Figure 3, panel A) had high affinity for LDL (half-maximum binding = 2.5 μg/mL). However, the 109-kDa protein (Figure 3, panel B) had lower affinity for LDL (half-maximum binding = 18 μg/mL) than the LDL receptor.

The 109-kDa protein was partially purified by HPLC on a Mono Q anion-exchange column. The protein was acidic, requiring 0.533 ± 0.016 M NaCl (four experiments) for elution in a single peak at pH 6.0 (Figure 4). Blotting and protein staining indicated that the major protein component of the peak fraction was the 109-kDa protein (Figure 4, inset, lanes 1 and 2). The Mono Q column separates proteins on the basis of size and charge. To also eliminate protein–protein hydrophobic interactions that might result in copurification of minor proteins, additional HPLC separations were done in the presence of 8.0 M urea. Under these conditions, the 109-kDa protein required 0.356 ± 0.027 M NaCl (three experiments) for elution in a single peak. As was the case for the separations done in the absence of urea, 109-kDa protein, ligand blot activity, and nucleolin immunoblot activity (see

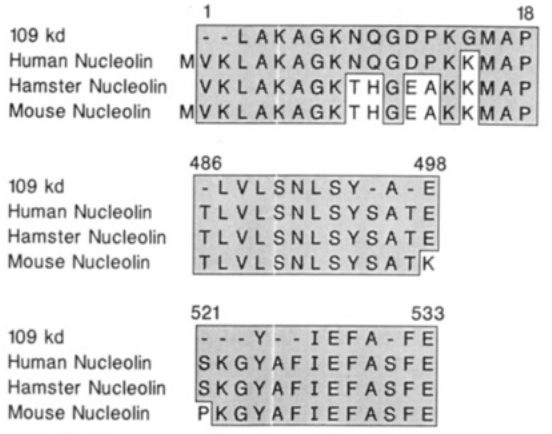

FIGURE 5: Protein sequence homology of the HepG2 109-kDa protein to nucleolin. Sources for deduced amino acid sequences are as follows: human nucleolin, Srivastava et al., 1989; hamster, Lapeyre et al., 1987; mouse, Bourbon et al., 1988a. Dashes indicate cycles for which the amino acid could not be unequivocally assigned. Numbers refer to the hamster nucleolin sequence.

below) also coeluted from the column in the presence of urea.

The predominant band after HPLC was cut from gels and sequenced directly as well as trypsin digested, and the products were sequenced. As determined by comparison with the sequencing control, β-lactoglobulin, the protein sequenced was the only protein present in sufficient quantity that could be sequenced. Amino acid sequences were determined for the amino terminus and two internal tryptic peptides from the HepG2 109-kDa protein (Figure 5). These sequences showed high homology to amino acid sequences predicted from nucleolin nucleotide sequences from human retina (Srivastava et al., 1989), Chinese hamster (Lapeyre et al., 1987), and mouse (Bourbon et al., 1988a).

The identity of the 109-kDa protein was further evaluated by immunoblotting (Figure 6). Immunoblotting using a polyclonal rabbit IgG raised against the LDL receptor (kindly provided by Drs. Michael Brown and Joseph Goldstein) showed the expected 140-kDa LDL receptor band in human fibroblasts (lane 2) and whole extracts of HepG2 cells (lane 1). However, the antibody did not recognize the 109-kDa protein in whole cell extracts of HepG2 cells (lane 1) nor did it recognize the partially purified 109-kDa protein (lane 3). In other experiments (not shown), a well-characterized anti-LDL receptor monoclonal antibody, IgG-C7, from the same laboratory, also failed to recognize the 109-kDa protein. However, a rabbit polyclonal antiserum raised against the partially purified 109-kDa protein detected both the partially purified 109-kDa protein (lane 8) and the purified rat nucleolin (lane 9) on immunoblots but did not recognize the fibroblast LDL receptor (lane 7). The 109-kDa protein was recognized by a rabbit polyclonal antibody raised against purified rat Novikoff hepatoma nucleolin (lane 10). Finally, purified rat nucleolin demonstrated LDL binding on ligand blots (lane 11). Thus, the HepG2 109-kDa protein appears to be immunologically distinct from the LDL receptor and shares protein sequence homology, immunologic determinants, and the property of LDL binding with the protein nucleolin.

Three lines of evidence suggest that the 109-kDa protein is partially expressed on the cell surface. First, the surface proteins of intact HepG2 cells were radioiodinated followed by HPLC separation with a Mono Q column and gel electrophoresis of the purified fractions. An iodinated species of M_r 109 000 was detected in the same fraction in which the HepG2 109-kDa protein eluted from the column (Figure 7). Second, incubation of intact HepG2 cells with 6 μM Pronase for 60 min at 37 °C caused a 46 ± 12% decrease in the 109-kDa protein (three experiments) as determined by scanning of ligand blots (not shown). In contrast, the intensity of actin, an intracellular protein detected by Coomassie blue staining on SDS gels, was 108 ± 6.9% of that of the control in treated cells. Third, treatment of intact cultured HepG2 cells with anti-109-kDa antiserum significantly decreased the specific binding of ^{125}I-LDL to the cell surface. Competition of antibody for ^{125}I-LDL binding to intact HepG2 cells was performed as described under Experimental Procedures. In the absence of anti-109-kDa antiserum, specific binding of ^{125}I-LDL to intact HepG2 cells was 274 ± 35 pg/well. In the presence of anti-109-kDa antiserum specific binding of ^{125}I-LDL to the cell surface was decreased by 29% to 195 ± 31 pg/well ($p = 0.002$ vs control). As expected, the majority of specific LDL binding was unaffected, consistent with LDL receptor activity.

In addition to inhibiting the binding of LDL to intact cells, anti-109-kDa antiserum inhibited the binding of ^{125}I-LDL to partially purified 109-kDa protein on blots (Figure 8, lanes 1 and 2). Antibody raised against purified rat nucleolin also inhibited the binding of ^{125}I-LDL to partially purified 109-kDa protein immobilized on nitrocellulose (Figure 8, lanes 3 and 4). These cross-inhibition results provide further evidence that the 109-kDa protein is nucleolin or nucleolin-like.

Discussion

We have identified a 109-kDa protein in HepG2 cells that binds LDL specifically but has LDL binding characteristics (low affinity, binding in the presence of EDTA and reducing agents), immunologic determinants, and a protein sequence distinct from that of the LDL receptor. Several features of

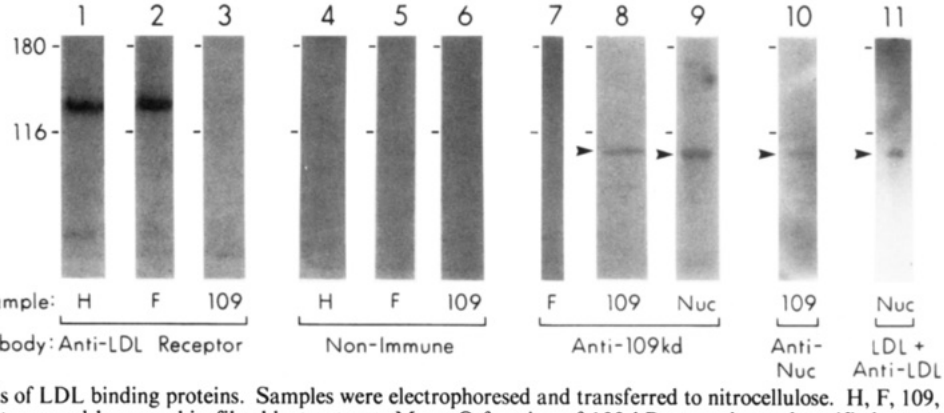

FIGURE 6: Immunoblots of LDL binding proteins. Samples were electrophoresed and transferred to nitrocellulose. H, F, 109, and Nuc represent the HepG2 cell extract, normal human skin fibroblast extract, Mono Q fraction of 109-kDa protein, and purified rat nucleolin. The blots were developed with polyclonal rabbit anti-LDL receptor IgG (lanes 1–3), control rabbit IgG (lanes 4–6), rabbit anti-109-kDa IgG (lanes 7–9), rabbit anti-rat nucleolin antiserum (lane 10), and LDL followed by anti-LDL antibody (lane 11).

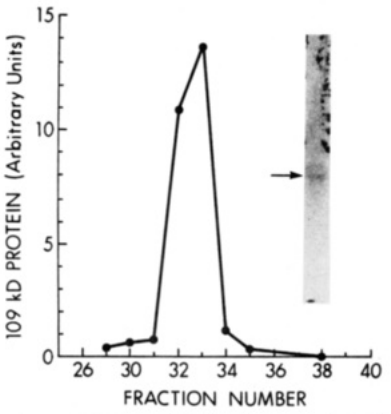

FIGURE 7: Densitometric scan of HPLC-separated, iodinated HepG2 cell surface protein. HepG2 cells grown in 35-mm wells were cooled to 4 °C for 30 min and then incubated with 1 mCi of ^{125}I in the presence of Iodobeads. Extracts were prepared, mixed with unlabeled HepG2 extract (4.5 mg of protein) as carrier, and applied to a Mono Q column. HPLC fractions were electrophoresed and autoradiographed. The autoradiograph was scanned with a densitometer horizontally at the 109-kDa level, and the results were plotted. Fractions 32 and 33 correspond to the same fractions in which the 109-kDa protein was detected by blotting and protein staining in parallel experiments. The inset shows the autoradiograph for fraction 32 of the cell surface iodination experiment with the 109-kDa position indicated by the arrow. The lane was loaded with 2.6 μg of protein.

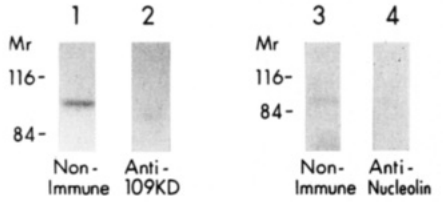

FIGURE 8: Competition of antibodies for ^{125}I-LDL binding to 109-kDa protein immobilized on nitrocellulose paper. The 109-kDa protein was electrophoresed and blotted to nitrocellulose paper in all lanes. The blots were blocked with albumin and incubated for 1 h at 4 °C in blot washing buffer containing 5 mg/mL BSA and either 250 μg/mL nonimmune IgG (lanes 1, 3) or 250 μg/mL immune IgG (lanes 2, 4). Then the blots were washed briefly, incubated with 10 μg/mL high specific activity ^{125}I-LDL for 1 h at 4 °C, washed, and subjected to autoradiography. Lane 2 received anti-109-kDa protein IgG and lane 4 anti-nucleolin IgG.

the 109-kDa protein suggest that it is closely related to or identical with nucleolin, a nucleolar protein until recently thought to be confined to the nucleolus. Finally, the 109-kDa protein appears to be partially expressed on the cell surface.

The current studies do *not* suggest that nucleolin is predominantly located on the cell surface in HepG2 cells. In fact, our studies are entirely consistent with the predominantly intracellular and intranuclear location of nucleolin. Instead, we speculate that a small subpopulation of this predominantly nucleolar protein in addition to shuttling constantly between the nucleus and cytoplasm (Borer et al., 1989) may also shuttle to the cell surface. Previous studies using biochemical and immunofluorescent techniques have also suggested that a minor amount of nucleolin is located on the surface of some cultured cells (Pfeifle & Anderer, 1983a,b). In other studies (Pfeifle et al., 1981), when intact cells were treated with trypsin inhibitor, the ability to phosphorylate surface nucleolin was gradually regained over 60 min. One interpretation of this experiment is that a small amount of nucleolin may be continually transported to the cell surface.

Lipids in general play important but mostly unexplained roles in the growth of cultured cells (Bettger & Ham, 1982). The current finding that nucleolin, a protein thought to regulate transcription of preribosomal RNA (Egyhazi et al., 1988), binds lipoproteins and is expressed on the cell surface provides a potential mechanism for extracellular regulation of nuclear events by lipids.

Several other investigators have detected more than one LDL binding protein by blotting techniques in several cell types including bovine adrenal (Kroon et al., 1984; Soutar et al., 1986), rat adrenal (Kroon et al., 1984), rat liver (Kroon et al., 1984; Cooper et al., 1987), and mouse 3T3 and J774 cells (Ellsworth et al., 1987). Although these additional proteins have usually been dismissed as the unglycosylated form of the LDL receptor or degraded LDL receptor, it is possible that some might represent nucleolin. Several lines of evidence also suggest that hepatocytes can interact with LDL by mechanisms other than that of the LDL receptor. Scatchard plots of specific binding of ^{125}I-LDL to liver membranes from rats (Windler et al., 1980) and dogs (Kovanen et al., 1981) are curvilinear with a low-affinity component. Rabbit liver (Kita et al., 1981) contains a low-affinity binding site for LDL that is not calcium dependent. Cultured human hepatocytes from patients with receptor-negative familial hypercholesterolemia bind and take up LDL by a low-affinity process (Hoeg et al., 1986a). Cultured human hepatocytes from normal subjects bind LDL with low affinity compared to human fibroblasts, and LDL binding is only partially inhibited by EDTA (Hoeg et al., 1986b). Since LDL binding to the 109-kDa protein appears to be calcium independent and of low affinity, we speculate that nucleolin or a nucleolin-like protein may account for some of the specific but low-affinity binding observed in hepatocytes.

As noted above, nucleolin has at least three structurally distinct regions. It is likely that the highly charged amino terminus is responsible for apoB and apoE binding. This domain contains three long stretches of negatively charged residues (Asp and Glu), which presumably bind to positively charged lysine and arginine residues on apoB and apoE. This hypothesis is supported by our finding that acetylation of LDL, a modification known to alter apoB charge and disrupt binding, causes similar decreases in LDL binding to the 109-kDa protein (nucleolin) and to the LDL receptor. However, nucleolin does not contain the cysteine-rich domains described for the LDL receptor (Yamamoto et al., 1984) or the LDL receptor related protein (Herz et al., 1988). Further, the carboxy-terminal region of nucleolin does not contain the sequence shown by Davis et al. (1987) to be critical for clustering of the LDL receptor in coated pits.

It is not known whether lipoproteins bound to nucleolin are internalized or whether nucleolin is subject to regulation by lipids or lipoproteins. It is tempting to speculate, however, that cholesterol may be capable of regulating nucleolin expression since a preliminary search of the hamster nucleolin promoter (Bourbon et al., 1988b) reveals the presence of several 7/8-bp matches to the sterol regulatory element (Osborne et al., 1988). Future studies of nucleolin expression may provide insight into a pathway that has the potential of directly linking extracellular and nuclear activities.

ACKNOWLEDGMENTS

We thank Dr. Larry Chan for encouragement and for critically reading the manuscript and Ms. Sally Tobola and Ms. Evon DuBose for secretarial expertise.

REFERENCES

Aebersold, R. H., Leavitt, J., Seavedra, K. A., Hood, L. E., & Kent, S. B. H. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6970–6974.

Bettger, W. J., & Ham, R. G. (1982) in *Cold Spring Harbor Conf. Cell Proliferation 9*, 61–64.

Borer, R. A., Lehner, C. F., Eppenberger, H. M., & Nigg, N. A. (1989) *Cell 56*, 379–390.
Bouche, G., Caizergues-Ferrer, M., Bugler, B., & Amalric, F. (1984) *Nucleic Acids Res. 12*, 3025–3035.
Bourbon, H., Bugler, B., Caizergues-Ferrer, M., & Amalric, F. (1983) *FEBS Lett. 155*, 218–222.
Bourbon, H.-M., Lapeyre, B., & Amalric, F. (1988a) *J. Mol. Biol. 200*, 627–638.
Bourbon, H.-M., Prudhomme, M., & Amalric, F. (1988b) *Gene 68*, 73–84.
Caizergues-Ferrer, M., Belenguer, P., Lapeyre, B., Amalric, F., Wallace, M. O., & Olson, M. O. J. (1987) *Biochemistry 26*, 7876–7883.
Cooper, A. D., Nutik, R., & Chen, J. (1987) *J. Lipid Res. 28*, 59–68.
Davis, C. G., van Driel, I. R., Russell, D. W., Brown, M. S., & Goldstein, J. L. (1987) *J. Cell Biol. 262*, 4075–4082.
Egyhazi, E., Pigon, A., Chang, J.-H., Ghaffari, S. H., Dressen, T. D., Wellman, S. E., Case, S. T., & Olson, M. O. J. (1988) *Exp. Cell Res. 178*, 264–272.
Ellsworth, J. L., Kraemer, F. B., & Cooper, A. D. (1987) *J. Biol. Chem. 262*, 2316–2325.
Herrera, A. H., & Olson, M. O. J. (1986) *Biochemistry 25*, 6258–6264.
Herz, J., Hamann, U., Rogne, S., Myklebost, O., Gausepohl, H., & Stanley, K. K. (1988) *EMBO J. 7*, 4119–4127.
Hoeg, J. M., Edge, S. B., Demosky, S. J., Jr., Starzl, T. E., Triche, T., Gregg, R. E., & Brewer, H. B., Jr. (1986a) *Biochim. Biophys. Acta 876*, 646–657.
Hoeg, J. M., Demosky, S. J., Jr., Lackner, K. J., Osborne, J. C., Jr., Oliver, C., & Brewer, H. B., Jr. (1986b) *Biochim. Biophys. Acta 876*, 13–21.
Innerarity, T. L., Pitas, R. E., & Mahley, R. W. (1979) *J. Biol. Chem. 254*, 4186–4190.
Jordan, G. (1987) *Nature 329*, 489–490.
Kita, T., Brown, M. S., Watanabe, Y., & Goldstein, J. L. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2268–2272.
Kovanen, P. T., Bilheimer, D. W., Goldstein, J. L., Jaramillo, J. J., & Brown, M. S. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1194–1198.
Kroon, P. A., Thompson, G. M., & Chao, Y.-S. (1984) *Biochem. J. 223*, 329–335.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lapeyre B., Bourbon, H., & Amalric, F. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1472–1476.
Moos, M., Jr., Nguyen, N. Y., & Liu, T.-Y. (1988) *J. Biol. Chem. 263*, 6005–6008.
Olson, M. O. J., & Thompson, B. A. (1983) *Biochemistry 22*, 3187–3193.
Olson, M. O. J., Guetzow, K., & Busch, H. (1981) *Exp. Cell Res. 135*, 259–265.
Olson, M. O. J., Rivers, Z. M., Thompson, B. A., Kao, W.-Y., & Case, S. T. (1983) *Biochemistry 22*, 3345–3351.
Osborne, T. F., Gil, G., Goldstein, J. L., & Brown, M. S. (1988) *J. Biol. Chem. 263*, 3380–3387.
Ostlund, R. E., Jr., Levy, R. A., Witzum, J. L., & Schonfeld, G. (1982) *J. Clin. Invest. 70*, 823–831.
Pfeifle, J., & Anderer, F. A. (1983a) *Biochem. Biophys. Res. Commun. 116*, 106–112.
Pfeifle, J., & Anderer, F. A. (1983b) *Biochim. Biophys. Acta 762*, 86–93.
Pfeifle, J., Hagmann, W., & Anderer, F. A. (1981) *Biochim. Biophys. Acta 670*, 274–284.
Rothblum, L. I., Mamrack, P. M., Kunkle, H. M., Olson, M. O. J., & Busch, H. (1977) *Biochemistry 16*, 4716–4721.
Semenkovich, C. F., & Ostlund, R. E., Jr. (1986) *J. Clin. Endocrinol. Metab. 62*, 1279–1287.
Semenkovich, C. F., & Ostlund, R. E., Jr. (1987) *Biochemistry 26*, 4987–4992.
Semenkovich, C. F., Ostlund, R. E., Jr., Levy, R. A., & Osa, S. R. (1982) *J. Biol. Chem. 257*, 12857–12865.
Semenkovich, C. F., Ostlund, R. E., Jr., Yang, J., & Reaban, M. E. (1985) *J. Lab. Clin. Med. 106*, 47–52.
Soutar, A., Harders-Spengel, K., Wade, D. P., & Knight, B. L. (1986) *J. Biol. Chem. 261*, 17127–17133.
Srivastava, M. (1989) *FEBS Lett. 250*, 99–105.
Weisgraber, K. H., & Mahley, R. W. (1986) *Methods Enzymol. 129*, 145–166.
Windler, E. E. T., Kovanen, P. T., Chao, Y.-S., Brown, M. S., Havel, R. J., & Goldstein, J. L. (1980) *J. Biol. Chem. 255*, 10464–10471.
Yamamoto, T., Davis, C. G., Brown, M. S., Schneider, W. J., Casey, M. L., Goldstein, J. L., & Russell, D. W. (1984) *Cell 39*, 27–38.