Regulation of the Ligand Binding Activity of the Human Very Low Density Lipoprotein Receptor by Protein Kinase C-dependent Phosphorylation*

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The very low density lipoprotein receptor (VLDL-R) binds and internalizes several ligands, including very low density lipoprotein (VLDL), urokinase-type plasminogen activator:plasminogen activator inhibitor type 1 complexes, lipoprotein lipase, and the 39-kDa receptor-associated protein that copurifies with the low density lipoprotein receptor-associated protein/α2-macroglobulin receptor. Although several agonists regulate VLDL-R mRNA and/or protein expression, post-transcriptional regulation of receptor activity has not been described. Here, we report that the ligand binding activity of the VLDL-R in THP-1 monocytic cells, endothelial cells, smooth muscle cells, and VLDL-R-transfected HEK 293 cells is diminished after treatment with phorbol 12-myristate 13-acetate. This response was blocked by inhibitors of protein kinase C (PK-C), including a specific inhibitor of the PK-C βII isoform, and was associated with phosphorylation of serine residues in the cytoplasmic domain of the receptor. Culture of endothelial cells in the presence of high glucose concentrations, which stimulate diacylglycerol synthesis and PK-C βII activation, also induced a PK-C-dependent loss of VLDL-R ligand binding activity. Taken together, these studies demonstrate that the ligand binding activity of the VLDL-R is regulated by PK-C-dependent phosphorylation and that hyperglycemia may diminish VLDL-R activity.

The very low density lipoprotein receptor (VLDL-R) is a member of the low density lipoprotein receptor (LDL-R) family, which encompasses several structurally related lipoproteins such as the LDL receptor-related protein/α2-macroglobulin receptor, gp330 (megalin), and apolipoprotein E receptor 2 (apoE receptor 2 among others). Members of this family are characterized by a cytoplasmic domain NPXY sequence that mediates ligand internalization through clathrin-coated pits and an extracellular domain comprised of cysteine-rich complement-type and epidermal growth factor precursor-like repeats that regulate ligand binding specificity. Except for the presence of an additional complement-type repeat in its extracellular domain, the domain structure of the VLDL-R is identical to that of the LDL-R (6–8). Despite this homology, however, the ligand binding specificity of these receptors differs. Though both receptors bind apoE-containing lipoproteins (6, 7, 9, 10), only the LDL receptor binds lipoproteins containing apoB-100 (6, 7), whereas the VLDL-R binds several additional ligands such as lipoprotein lipase (9), (11) urokinase-type plasminogen activator:plasminogen activator inhibitor type 1 (u-PA:PAI-1) complexes (11, 12), and Lp(a) (13). Finally, the 39-kDa receptor-associated protein (RAP), which co-purifies with (14, 15) and binds with high affinity to the LDL receptor-related protein/α2-macroglobulin receptor (16–18), binds tightly to the VLDL-R (19) but with only low affinity to the LDL-R (19, 20).

Differences in the tissue distribution of the LDL and VLDL receptors also suggest non-overlapping physiologic functions. Although the LDL-R is highly expressed in liver and promotes LDL uptake and clearance (21), the VLDL-R is most abundant in heart and skeletal muscle (22), suggesting involvement in the uptake of triglyceride-rich lipoproteins in tissues dependent on fatty acid metabolism (2, 7, 8). However, the broad ligand specificity of the VLDL-R as well as the lack of coordinate regulation between the VLDL-R and lipoprotein lipase suggests that this receptor may also function in other processes distinct from lipid metabolism (23). In this context, the VLDL-R regulates cell surface urokinase receptor expression and urokinase receptor-dependent cellular migration (24).

We previously reported that the VLDL-R is expressed by human arterial and venous endothelial and smooth muscle cells in vitro and in vivo (11, 25) as well as by macrophage-derived foam cells within atherosclerotic plaques (25). Nakazato et al. (26) detected VLDL-R mRNA in smooth muscle cells and macrophages within atherosclerotic aorta from cholesterol-fed NZW and Watanabe hereditary hyperlipidemic rabbits (26). The VLDL-R is also present in the endothelium of capillaries, small arterioles, and coronary arteries of mouse and bovine origin (27). These studies suggest a potential role for the VLDL receptor in (patho)physiological vascular processes such as atherosclerosis.
Unlike that of the LDL-R, the expression of the VLDL-R is not diminished by exogenous cholesterol (21, 28, 29). In choriocarcinoma cells, VLDL-R mRNA levels are regulated by insulin, 8-bromo-c-AMP, and clofibrate (30), whereas in HL-60 cells, 1,25-dihydroxy vitamin D3 stimulates receptor expression (31). In vivo, thyroid hormone (32) and granulocyte-macrophage colony stimulating factor (33) stimulate the expression of VLDL-R mRNA in skeletal muscle, whereas estradiol stimulates VLDL-R expression in myocardium (34). However, changes in mRNA levels may not reflect the expression of a functional VLDL-R protein and whether or not the activity or affinity of the VLDL-R may be regulated through post-transcriptional mechanisms has not been assessed. Here we report that the ligand binding affinity of the VLDL-R is diminished after protein kinase C (PK-C)-dependent phosphorylation.

**EXPERIMENTAL PROCEDURES**

Materials—Tissue culture medium and reagents were from Media-tech (Herndon, VA). Fetal bovine serum was from HyClone (Logan, Utah). The 39-kDa RAP, which co-purifies with the LDL receptor-related protein/α2-macroglobulin receptor (14, 15) and inhibits ligand binding to this (16–18) as well as to the VLDL-R (19), was expressed and purified as described (17). For selected studies, RAP was conjugated to cyanogen bromide-activated Sepharose 4B (8.9 mg/ml of packed beads) per the manufacturer’s protocol (Amersham Pharmacia Biotech) or labeled with 125I to a specific activity of 5.0 × 10^7 cpm/g using iodobeads. Affinity-purified anti-VLDL-R receptor antibodies, raised against a peptide corresponding to the C-terminal 20 amino acids of the human (35–37) mouse (38) and rabbit (6) VLDL-R, have been described (30). A rabbit antibody raised against a polypeptide encompassing the 160 N-terminal amino acids of the VLDL-R (25) was used for detection of a truncated VLDL-R mutant lacking the cytoplasmic domain. Anti-phosphoserine (IC8) and anti-phosphothreonine (1E11) monoclonal antibodies were from Biomol (Plymouth Meeting, PA), anti-phosphotyrosine antibodies were from Upstate Biotechnology (Lake Placid, NY), and anti-PK-C βII antibodies were from Santa Cruz Bio-technology (Santa Cruz, CA). Two-chain u-PA, PAI-1, and anti-PAI-1 antibodies were from American Diagnostica, Inc. (Greenwich, CT). Phorbol 12-myristate 13-acetate (PMA), potassium sulfate, streptavi-din-peroxidase, and peroxidase-conjugated secondary antibodies were from Sigma. Genistein, potato acid phosphatase, recombinant (Esche- richia coli) protein phosphatase 2B, protease inhibitors, and the PK-C inhibitors GF109203X and calphostin C were from Calbiochem. The involvement of the PK-C

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Measurement of VLDL Receptor mRNA—VLDL receptor mRNA was quantitated by Northern blotting. Total RNA was isolated using Trizol (Life Technologies, Inc.) and separated on a 1% denaturing agarose gel. RNA was transferred to nylon membranes in 20× SSC (1× SSC contains 0.15 mM NaCl, 0.015 mM sodium citrate, pH 7.0). The membrane was prehybridized at 42°C in a solution containing 50% formamide, 5× SSC, 5× Denharder’s solution, 1% SDS, and 100 μg/ml denatured salmon sperm DNA, and VLDL-R mRNA was detected by overnight hybridization at 42°C with a full-length VLDL-R cDNA (36) labeled with 32P (New England Amersham, Pharmacia Biotech). To ensure equal loading of RNA in all lanes, blots were stripped and reprobed with a 32P-labeled polymerase chain reaction product corresponding to nucleotides 384–684 of actin cDNA (42).

**Binding of VLDL-R Ligands to Control and PK-C-treated Cells—**To assess whether PK-C activation altered the expression of VLDL-R on intact cells, cell surface proteins were labeled using sulfo-succinimidyl 6-(biotinamido) hexanoyl (NHS-LC-biotin) (43) before and after exposure of cells to 1.5 × 10^7 M PMA for 2 h. VLDL-R were then ligand-precipitated from cell extracts using RAP-Sepharose 4B, separated by 7.5% SDS-PAGE, and transferred to PVDF. Biotinylated proteins were detected by chemiluminescence after incubation of membranes with streptavidin-peroxidase.

Two approaches were used to investigate the effect of PK-C activation on the binding of VLDL-R ligands to cells. First, the binding of 125I-RAP to control THP-1 cells or cells incubated for 60 min in the presence of 1.5 × 10^7 M PMA was assessed. 125I-RAP binding was measured by incubating increasing concentrations of 125I-RAP with control or PK-C-treated cells (2 × 10^6 cells/ml, 0.5 ml) for 2 h at 4°C in the absence or presence of a 100-fold excess of unlabeled RAP. Cells were washed and incubated with 100 μg/ml u-PA:PAI-1 (1:10) or 100 μg/ml u-PA through a 1-ml cushion of silicone oil (Fluid 500:Fluid 200, 7.5:1.5; Dow/Corning, Nye Lubricants, New Bedford, MA) and cell-associated ligand was determined by counting the cell pellets in a γ counter. Specific binding was defined as the difference between total and non-specific binding measured in the absence or presence of excess unlabeled ligand, respectively. Saturation isotherms were prepared from the binding data using non-linear regression and assuming one-site binding (Prism; Graph Pad Software, San Diego, CA) and used to determine the K_d and B_max. In selected experiments, the role of heparan sulfate proteoglycans in 125I-RAP binding was assessed by measuring binding in the presence of 100 μg/ml of proteamine sulfate (44) (Sigma).

In a second series of experiments, the binding of 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine (DiI)-labeled VLDL to control and PK-C-treated THP-1 cells was analyzed using flow cytometry. Human VLDL was purified and labeled with DiI as described (45,46). THP-1 cells (2 × 10^6 cells/ml) were washed and incubated for 2 h at 4°C with or without 25 μg/ml DiI-labeled VLDL. Cells were then washed again, fixed in 2% paraformaldehyde, and analyzed using a FACScan flow cytometer (Becton-Dickinson; excitation wavelength, 575 nm; emission wavelength, 575 nm; 488 nm) (47). Binding of DiI-VLDL was quantitated by subtracting the mean fluorescence intensity of control cells (autofluorescence) from that of cells incubated with DiI-VLDL. Binding specificity was confirmed by assessing the ability of a 100-fold excess of unlabeled VLDL or RAP to block the binding of DiI-VLDL to cells.

**Assessment of the Role of PK-C in Regulation of LIGAND BINDING AFFINITY by PMA—**The role of PK-C in regulation of VLDL-R binding was assessed by determining the role of the PK-C inhibitors GF109203X and calphostin C to block PMA-mediated inhibition of VLDL-R ligand binding using both ligand blotting and whole cell binding approaches.

The involvement of the PK-C βII isoform in mediating the effects of PMA was also assessed. First, we determined whether PK-C βII was translocated to the membrane fraction after exposure of cells to PMA.
Hyperglycemia-induced PKC- \( \beta \) phosphorylation was assessed by measuring incorporation of \( ^{32}P \) into mRNAs, and positive clones selected by culture in \( ^{32}P \)-labeled media.

Ligand binding of biotinylated cell surface proteins using RAP-ligand blotting, respectively. To determine whether \( ^{32}P \) was incorporated into serine, threonine, or tyrosine residues, immunoprecipitates from \( ^{32}P \)-labeled cells were separated by 7.5% SDS-PAGE, and the amount of VLDL-R protein was quantified using a PhosphorImager (model 445SI; Molecular Dynamics, Sunnyvale, CA). To assure that equal protein synthesis occurred under each of these conditions, a parallel experiment was performed in which aliquots of VLDL-R cells were labeled under identical conditions with \( ^{32}P \) methionine. To determine whether \( ^{32}P \) was incorporated into serine, threonine, or tyrosine residues, immunoprecipitates from \( ^{32}P \)-labeled cells were separated by 7.5% SDS-PAGE, and the amount of VLDL-R protein was quantified using a PhosphorImager (model 445SI; Molecular Dynamics, Sunnyvale, CA). To assure that equal protein synthesis occurred under each of these conditions, a parallel experiment was performed in which aliquots of VLDL-R cells were labeled under identical conditions with \( ^{32}P \) methionine.

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The preparation of a Truncated VLDL-R Mutant—To further assess the role of phosphorylation within the VLDL-R cytoplasmic domain on ligand binding activity, a truncated VLDL-R mutant was prepared. Briefly, the 3.2-kilobase VLDL-R cDNA was digested with AseI and BglII to yield 2438, 578, and 238 nucleotide fragments. The gel-purified 2438-, 578-, and 238-nucleotide fragments were recloned in pH-CMV-VLDL-TR in which the 238-nucleotide cDNA sequence encoding the VLDL-R cytoplasmic domain C-terminal to Leu-790 (proximal to all cytoplasmic domain serine, threonine or tyrosine residues) was deleted. Polymerase chain reaction of the coding region of the VLDL-R sequence encoding the VLDL-R cytoplasmic domain C-terminal to Leu-790 (proximal to all cytoplasmic domain serine, threonine or tyrosine residues) was deleted. Polymerase chain reaction of the coding region of this construct (sense primer 5'-CTAGTCAACAACTGAATGA-3', antisense primer 5'-TACCAGGCTTGATTCAACG-3') yielded a 415-nucleotide product, in comparison to a 656-nucleotide product from the wild-type construct. The DNA sequence of the mutant was confirmed by automated sequencing.

HEK 293 cells were transfected with wild type and mutant constructs, and positive clones selected by culture in 400 \( \mu \)g/ml G418. Ligand precipitation of biotinylated cell surface proteins using RAP-Sepharose confirmed that wild type and truncated VLDL-R were expressed as judged by the presence of labeling. Sixty minutes before the termination of labeling, either 1.5 \( \times \) \( 10^{-7} \) \( M \) PMA (final concentration), 1.5 \( \times \) \( 10^{-7} \) \( M \) PMA and either 10 \( \mu \)M GF109203X or 30 \( \mu \)M LY379196, or an equal volume of phosphate-buffered saline was added to the individual aliquots. Labeling was terminated by washing the cells in ice-cold phosphate-buffered saline, and cell pellets were immediately extracted in a buffer containing 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 \( \mu \)g/ml leupeptin, 50 mM \( \beta \)-phenylalanil-\( \beta \)-propyl-l-arginine chloromethyl ketone and 50 mM sodium fluoride, pH 7.4. Cell extracts were incubated overnight with 30 \( \mu \)l of rabbit anti-VLDL-R antibodies (0.66 mg/ml), and immune complexes were precipitated using 50 \( \mu \)l of protein A-Sepharose. Beads were collected by centrifugation and washed before elution of bound material in 50 \( \mu \)l of 1X Laemmli sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol) (49). The precipitated complexes were dissolved in a phosphate-buffered saline, and their ligand binding activity were assessed by immunoblotting and RAP microsomal binding, respectively.

RESULTS

Functional VLDL Receptor Expression Is Diminished after Exposure of Cells to PMA—We initially wished to determine the effect of monocyte differentiation, THP-1 cells were treated with PMA, which induced them to become adherent and acquire a macrophage-like phenotype (55). Contrary to expectations, the expression of both the \( \sim 100 \)- and \( \sim 130-kDa \) bands using the VLDL-R protein and its ligand binding activity were assessed by immunoblotting and RAP ligand binding, respectively.

To determine whether these responses were unique to transformed cell lines such as THP-1, we assessed the effects of PMA on the expression of VLDL-R by primary cultures of human dermal microvascular endothelial cells and human aortic smooth muscle cells. As observed with THP-1 cells, exposure to PMA completely blocked the ligand binding activity of VLDL-R from these primary cell cultures as well.

To assess these responses were limited to the binding of PMA, which is not a physiological extracellular VLDL-R ligand, we determined the effect of PMA on the binding of u-PA:PAI-1 complexes to the VLDL-R. PMA caused a marked decrease in the binding of these complexes as well (Fig. 2).

The Effects of PMA Reflect a Decrease in Affinity of the VLDL-R for Its Ligands—Exposure of THP-1 cells to PMA for 2 h did not affect their content of VLDL-R mRNA (Fig. 3, lanes 1 and 2). Similarly, immunoblotting studies revealed identical amounts of VLDL-R protein within extracts of PMA-treated and control cells (Figs. 2B and 4, upper panel), although markedly less RAP (Fig. 4, lower panel) or u-PA:PAI-1 complex (Fig. 2A) bound to VLDL-R in extracts from the PMA-treated cells. The observation that PMA did not affect the amount of VLDL-R within cell extracts, as determined by immunoblotting, but blocked ligand binding to the receptors suggested that it decreased ligand binding affinity.

PMA Inhibits VLDL-R Ligand Binding on Intact Cells—Two approaches were undertaken to assess the effects of PMA on ligand binding by VLDL receptors on intact cells. First, we measured incorporation of \( ^{32}P \) into the receptor. THP-1 cells (1 \( \times \) \( 10^{7} \) cells/ml) were pre-incubated in phosphate-free RPMI 1640 for 30 min and then divided into 4 aliquots that were each labeled for 3 h with 0.5 \( \mu \)Ci/ml \( ^{32}P \)orthophosphate. Sixty minutes before the termination of labeling, either 1.5 \( \times \) \( 10^{-7} \) \( M \) PMA (final concentration), 1.5 \( \times \) \( 10^{-7} \) \( M \) PMA and either 10 \( \mu \)M GF109203X or 30 \( \mu \)M LY379196, or a parallel experiment was performed in which aliquots of THP-1 cells were labeled under identical conditions with 0.2 \( \mu \)Ci/ml \( ^{32}P \)orthophosphate.
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compared the binding of \(^{125}\text{I}\)-RAP to control and PMA-treated THP-1 cells. \(^{125}\text{I}\)-RAP bound specifically to \(-31,400 \pm 1,038\) (S.E.) sites on control cells, with a \(K_d\) of \(-1.8 \pm 0.3\) nM, and to \(-40,110 \pm 12,700\) (S.E.) sites on PMA-treated cells, with a \(K_d\) of \(-22.9 \pm 4.5\) nM (Fig. 5A). Protamine sulfate did not affect the binding of \(^{125}\text{I}\)-RAP to either control or PMA-treated cells, suggesting that the observed binding was mediated by the VLDL receptor rather than glycosaminoglycans (56). Consistent with the similar \(B_{\text{max}}\) values determined for the binding of RAP to control and treated cells, PMA did not affect the amount of biotinylated VLDL-R that was immuno- or ligand-precipitated from cell surface-biotinylated THP-1 cells.

We also examined the effect of PMA on the binding of human VLDL to THP-1 cells. Dil-labeled VLDL (25 \(\mu\)g/ml) bound specifically to control THP-1 cells, as demonstrated by the ability of a 100-fold molar excess of unlabeled VLDL (Fig. 5B) or RAP (not shown) to block the binding of Dil-VLDL to cells by \(>90\%\). Exposure of THP-1 cells to PMA decreased the binding of Dil-VLDL by a similar extent as unlabeled VLDL (467.2 to 17.0 arbitrary fluorescence units), with this effect blocked by the PK-C inhibitor GF109203X (57) (Fig. 5C). Taken together, these studies demonstrate that exposure of THP-1 cells to PMA leads to inhibition of the binding of VLDL-R ligands without altering cell surface VLDL-R expression.

The Effects of PMA Depend upon PK-C Activation—Since PMA activates other protein kinases in addition to PK-C, we further assessed the role of PK-C in the inhibition of VLDL-R ligand binding in response to PMA. In contrast to the protein kinase A inhibitor H89 and the protein-tyrosine kinase inhibitor genistein (not shown), the PK-C inhibitor GF109203X (57) blocked the PMA-induced inhibition of \(u\)-PA:PAI-1 complex binding (Fig. 2A, lane 2), RAP (Fig. 4, upper panel, lane 4), and VLDL binding (Fig. 5C) to the VLDL-R. Similar results were observed with calphostin C, another specific PK-C inhibitor. Neither GF109203X (Fig. 3) nor calphostin C affected the levels of

FIG. 2. Effect of PMA on the binding of \(u\)-PA:PAI-1 complexes to VLDL-R. THP-1 cells were cultured for \(2\) h in the absence (lane 1 of each blot) or presence (lane 2 of each blot) of \(1.5 \times 10^{-7}\) M PMA, or PMA and GF109203X (lane 3 of each blot). Detergent extracts from cells incubated under each of these conditions were then divided into equal aliquots and analyzed by ligand blotting using anti-VLDL-R antibodies (B).

FIG. 3. Effect of PMA on the levels of VLDL-R mRNA. THP-1 cells were incubated for \(2\) h in medium containing either no additions (lane 1), \(1.5 \times 10^{-7}\) M PMA (lane 2), \(1.0 \mu\)M calphostin C (lane 3), or \(1.5 \times 10^{-7}\) M PMA and \(1.0 \mu\)M calphostin C (lane 4). Total cellular RNA was then isolated, and \(10 \mu\)g of RNA from cells treated under each of these conditions were analyzed by Northern blotting for VLDL-R mRNA (upper panel) or actin mRNA (lower panel).

FIG. 4. Effect of PMA on expression of functional VLDL-R and VLDL-R protein. THP-1 cells were incubated for \(2\) h in medium containing no additions (lane 1 of each blot), \(1.5 \times 10^{-7}\) M PMA (lane 2 of each blot), \(10.0 \mu\)M GF109203X (lane 3 of each blot), or \(1.5 \times 10^{-7}\) M PMA and \(10.0 \mu\)M GF109203X (lane 4 of each blot). Cell extracts were then prepared, and equal amounts of protein from each were separated by 7.5% SDS-PAGE and analyzed by ligand blotting (upper panel) or immunoblotting using anti-human VLDL-R antibodies (lower panel).

FIG. 5. Effect of PMA on binding of VLDL-R ligands to intact cells. A, the specific binding of \(^{125}\text{I}\)-RAP to THP-1 cells preincubated for \(2\) h in the absence (——) or presence (----) of \(1.5 \times 10^{-7}\) M PMA was measured as described under “Experimental Procedures.” Error bars represent S.E. of triplicate points from a single experiment representative of three performed. B, specificity of Dil-VLDL binding to THP-1 cells. Histograms represent control THP-1 cells not exposed to PMA or Dil-VLDL (red) and THP-1 cells incubated with Dil-VLDL in the absence (green) or presence (black) of a 100-fold excess of unlabeled VLDL. C, effect of PMA on binding of Dil-VLDL to THP-1 cells. Histograms represent control cells not exposed to PMA or Dil-VLDL (red), cells preincubated for \(2\) h in the absence (green) or presence (black) of \(1.5 \times 10^{-7}\) M PMA before incubation with \(25 \mu\)g/ml Dil-VLDL, or cells preincubated for \(2\) h in the presence of \(1.5 \times 10^{-7}\) M PMA and \(10 \mu\)M GF109203X (blue) before incubation with Dil-VLDL.
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FIG. 6. Involvement of PK-C \( \beta II \) in regulation of VLDL-R ligand binding affinity. A, subcellular localization of PK-C \( \beta II \) (105 kDa) before and after exposure of THP-1 cells to \( 1.5 \times 10^{-5} \) M PMA for 60 min. Cytosolic (upper panel) and membrane (lower panel) fractions were isolated before and at increasing times after exposure of cells to PMA. Proteins from these fractions were separated by 10% SDS-PAGE, transferred to PVDF, and detected by immunoblotting using PK-C \( \beta II \)-specific antibodies. B, inhibition of the PK-C-associated decrease in VLDL-R ligand binding by the PK-C \( \beta II \)-specific inhibitor LY379196. THP-1 cells were incubated for 2 h in the absence or presence of \( 1.5 \times 10^{-5} \) M PMA or in the presence of PMA and either GF109203X or LY379196. Cell extracts were then prepared and analyzed by RAP ligand blotting.

VLDL-R mRNA or protein (Fig. 2B, lane 3; Fig. 4, lower panel, lane 4).

We next assessed the specific involvement of the PK-C \( \beta II \) isoform in the decreased VLDL-R ligand binding occurring upon PK-C activation. PK-C \( \beta II \) is strongly expressed in failing human heart (58), a tissue in which we have observed that the VLDL-R is phosphorylated (59). Upon exposure of THP-1 cells to PMA, PK-C \( \beta II \) was rapidly translocated from the cytosolic to the cell membrane fraction (Fig. 6A). Moreover, as observed with GF109203X, a specific PK-C \( \beta II \) inhibitor, LY379196, blocked the PMA-induced decrease in RAP binding (Fig. 6B).

To further assess the role of PK-C \( \beta II \), HEK 293 cells were transfected with VLDL-R cDNA alone or co-transfected with VLDL-R and PK-C \( \beta II \) cDNA. As previously reported for bovine aortic endothelial cells (60) and mammary carcinoma cells (61, 62), VLDL-R-transfected HEK 293 cells expressed primarily the \( 105-kDa \) isoform of the receptor lacking exon 16, which binds ligands identically to the larger \( 130-kDa \) isoform (2, 62).

In contrast to cells transfected with the VLDL-R alone, co-transfected cells demonstrated a marked increase in the amount of PKC-\( \beta II \) in the membrane fraction (not shown). Moreover, ligand blot analysis revealed dramatically reduced binding of RAP to immobilized VLDL-R from the co-transfected cells, despite similar amounts of VLDL-R protein (Fig. 7, lanes 1 versus lane 5). Moreover, although the PK-C inhibitors GF109203X and LY379196 blocked the inhibitory effects of PMA on the ligand binding activity of the VLDL-R in singly transfected cells, these inhibitors not only blocked the effect of PMA but markedly stimulated VLDL-R ligand binding activity (compared with unstimulated cells) in the co-transfected cells (compare lanes 5 versus lanes 7 and 8). These results are consistent with the increased base-line activity of PK-C \( \beta II \) in the co-transfected cells.

The VLDL Receptor Is Phosphorylated on Serine Residues—

The results described above suggested that the VLDL-R was phosphorylated in a PK-C-dependent manner, leading to diminished ligand binding affinity. Therefore, we determined whether metabolically labeled THP-1 cells incorporated \(^{32}P\) from \\((^32P)\)orthophosphate into the receptor. In THP-1 cells, \(^{32}P\) was incorporated into both the \( 105- \) and \( 130-kDa \) isoforms of the VLDL-R, with approximately 3-fold more \(^{32}P\) incorporated after exposure to PMA. Incubation of GF109203X or LY379196 during metabolic labeling or treatment of the immunoprecipitated VLDL-R with protein phosphatase 2B reduced receptor phosphorylation to base-line levels (Fig. 8A). In control studies performed in parallel, PMA did not affect the levels of \(^{[35S]}\)methionine-labeled VLDL-R (Fig. 8C), suggesting that it did not influence receptor synthesis or degradation. Immunoblotting of immunoprecipitated VLDL-R using anti-VLDL-R antibodies confirmed that similar amounts of VLDL-R were immunoprecipitated from control and PMA-treated cells (Fig. 8D).

Incubation of PVDF-immobilized \(^{32}P\)-labeled VLDL-R with 1 kM KCl, which chemically dephosphorylates phosphoserine residues (50), released \(^{32}P\) from the receptor (Fig. 8B). The specificity of this approach was confirmed by the observation that immobilized phospho-MAP kinase (tyrosine-phosphorylated, from vascular endothelial cell growth factor-stimulated human dermal microvascular endothelial cells) was not dephosphorylated when subjected to identical treatment (not shown). Moreover, VLDL-R immunoprecipitated from extracts of PMA-treated THP-1 cells were recognized by anti-phosphoserine but not anti-phosphothreonine or anti-phosphotyrosine antibodies.

Direct Evidence for Modulation of VLDL Receptor Ligand Binding Affinity by Phosphorylation—Since the studies above demonstrated that the VLDL-R was phosphorylated in intact cells and that receptor phosphorylation diminished ligand binding affinity, we determined whether the affinity of the receptor for RAP was restored after enzymatic dephosphorylation. Treatment of extracts from PMA-treated THP-1 cells (which do not bind RAP) with potato acid phosphatase restored the binding of RAP to immobilized VLDL-R in a concentration-dependent manner (Fig. 9, lanes 3–5). An identical effect was observed after treatment of the phosphorylated receptor with protein phosphatase 2B.
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Additional evidence for a critical role for phosphorylation within the VLDL-R cytoplasmic domain in regulation of receptor ligand binding affinity was derived from study of a VLDL-R mutant lacking the cytoplasmic domain of the receptor. This mutant receptor was expressed in similar amount as the wild-type receptor (primarily as the −105-kDa isoform) on the surface of transfected HEK 293 cells, as determined by analysis of immunoprecipitated VLDL-R from cell surface-biotinylated cells. However, unlike the wild-type receptor, binding of RAP by the truncated receptor was unaffected after treatment of cells with PMA (Fig. 10). Taken together with the preceding studies, these experiments support the hypothesis that phosphorylation of serine residues within the cytoplasmic domain of the receptor plays an important role in regulating ligand binding affinity.

Activation of PK-C by Elevated Glucose Concentrations Leads to Inhibition of VLDL-R Ligand Binding Activity—Elevated glucose concentrations contribute to the pathogenesis of vascular disease in diabetes mellitus (52, 63). Culture of microvascular endothelial cells in the presence of high glucose or galactose stimulates the activation of PK-C by inducing de novo synthesis of diacylglycerol (51, 64). Treatment of diabetic rats with a specific PK-C βII inhibitor inhibits the development of vascular dysfunction that leads to retinal, renal, and neurologic disease (53, 54, 65).

To determine whether the ligand binding activity of the VLDL receptor was affected by high glucose, microvascular endothelial cells were incubated in the presence of various concentrations of glucose, galactose, or mannitol. Exposure of cells to 16.5 mM d-glucose or galactose caused a potent and rapid reduction in the binding of RAP by VLDL-R (Fig. 11, upper panel), whereas the level of VLDL-R detected by immunoblotting was unaffected (Fig. 11, lower panel). The effects of glucose and galactose were blocked by GF109203X and LY379196 (Fig. 11, upper panel, lanes 3 and 4 and 6 and 7, respectively), demonstrating an important role for PK-C, particularly the βII isoform, in this response. An identical concentration of mannitol, which does not induce diacylglycerol synthesis or PK-C βII activation in endothelial cells (51), did not affect the binding of RAP by VLDL-R.

**DISCUSSION**

These studies demonstrate that PK-C activation leads to phosphorylation of the VLDL-R and inhibition of its ligand binding activity in a number of cell types, including monocyte-derived THP-1 cells, human endothelial and vascular smooth muscle cells, and HEK 293 cells transfected with VLDL-R cDNA. PK-C-dependent phosphorylation of the VLDL-R leads to diminished binding not only of RAP but of other VLDL-R ligands such as u-PA:PAI-1 complexes and VLDL. The pathophysiologic relevance of this process is supported by the observation that glucose concentrations similar to those that induce PK-C activation in vitro and in vivo in experimental diabetes cause VLDL-R phosphorylation and loss of ligand binding activity in endothelial cells. Moreover, VLDL-R isolated from myocardium obtained from patients with advanced cardiomyopathies, a condition associated with PK-C βII activation in the heart (58), are also phosphorylated, whereas receptors from normal human myocardium are not.

VLDL-R phosphorylation was associated with a rapid loss of ligand binding activity, although levels of VLDL-R mRNA and protein and the expression of the VLDL-R on the cell surface were unaffected. Taken together, these observations suggest that phosphorylation results in diminished affinity of the receptor for its ligands. This conclusion is supported by direct radioligand binding studies in which [125I]RAP was found to

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bind to a similar number of sites on control and PMA-treated
THP-1 cells, although with ~13-fold lower affinity to the latter.
Furthermore, although the affinity of DiI-VLDL binding to
THP-1 cells was not determined directly, the complete disap-
pearance of binding after exposure of these cells to PMA sug-
gests that VLDL-R phosphorylation greatly diminishes the af-
finity of the receptor for apoE-containing ligands as well.

Several observations support the conclusion that phospha-
ylation of cytoplasmic domain serine residue(s) regulates the
ligand binding activity of the VLDL-R through a process in-
volving PK-C. First, the PMA-induced loss of ligand binding
activity was prevented by the PK-C inhibitors GF109203X (57),
calphostin C (66), and LY379196 but not by inhibitors of PK-A
or tyrosine kinases. Second, enzymatic dephosphorylation of
the VLDL-R restored its ligand binding activity. Third, 32P was
directly incorporated into the VLDL-R, with incorporation stimu-
lated by PMA. Fourth, chemical dephosphorylation of the
VLDL-R, as well as immunoblotting with specific monoclonal
antibodies, were consistent with the phosphorylation of serine
but not threonine or tyrosine residues within the VLDL-R
in response to PK-C activation. Finally, the ligand binding ac-
itivity of a mutant VLDL-R lacking the cytoplasmic domain
was unaffected by PMA.

The effects of phosphorylation on VLDL receptor activity
likely reflect a generalized mechanism through which phospha-
ylation within a receptor cytoplasmic domain regulates its
function by altering ligand binding affinity. For example, phos-
ylation of serine and threonine residues within the cyto-
plasmic domains of the insulin (67, 68) and epidermal growth
factor receptors (69–75) diminishes their affinity for insulin
and epidermal growth factor, respectively. With respect to the
epidermal growth factor receptor, these effects mimic those
involved in regulation of the receptor by heterologous ligands
(76) such as platelet-derived growth factor (77) and those in-
duced through a negative feedback pathway triggered by binding
of epidermal growth factor itself (78).

Regulation of the activity of LDL receptor family members
through phosphorylation is a novel concept of which few ex-
amples exist. Though Kishimoto et al. (79) observe that the LDL
receptor could be phosphorylated on Ser829 (79) by an LDL-
receptor kinase purified from bovine adrenal cortex (80), these
effects occurred only in a cell-free system, and incorporation of
[32P]orthophosphate into the LDL receptors of intact cells could
not be demonstrated (80). More recently, however, Bu et al. (81)
demonstrate that the LDL receptor-related protein α2-macro-
globulin receptor is phosphorylated in neuronal cells in re-
response to nerve growth factor, leading to enhanced expression
and endocytic activity. Our studies suggest that the cytoplas-
mic domain of the VLDL-R plays an important role in regulat-
ng receptor function, a hypothesis consistent with the com-
plete sequence conservation of this domain in the murine (38, 82),
rabbit (6), and human (35–37) receptors. Trommsdorff et al.
(83) also demonstrate a critical role for the cytoplasmic do-
mains of the VLDL-R and apoE receptor 2 in binding of the
cytoplasmic adaptor protein mammalian Disabled (mDab1), an
interaction critical for appropriate migration and layering of
neurons during development of the cerebellum and cerebral
cortex. Whether this process may be affected by VLDL-R phos-
phorylation has not been assessed.

Which serine residue(s) within the cytoplasmic domain of
the VLDL-R involved in regulation of ligand binding affinity is
uncertain, although preliminary studies suggest the involve-
ment of Ser-829.3 Interestingly, although the cytoplasmic do-
mains of the VLDL and LDL receptors are highly homologous
(49% matching residues), only 2 of the 6 serines within this
region of the VLDL-R align with any of the 4 serines in the
corresponding domain of the LDL-R, perhaps explaining the
different susceptibility of these receptors to phosphorylation in
intact cells.

In addition to hyperglycemia, PK-C activation may be induced
by several cytokines that affect the function of endothelial
and smooth muscle cells and monocytes (84–86). Mechanical
stimuli (87, 88) and hypoxia (89) also activate PK-C. The
pathophysiological consequences of excessive PK-C βII activa-
tion in myocardium, as occurs in diabetes (63, 65) and congestive
cardiomyopathies (58), is supported by transgenic mouse mod-
els in which myocardial PKC βII overexpression leads to a fatal
postnatal cardiomyopathy (90). Although our studies demon-
strate the involvement of PK-C βII in phosphorylation of the
VLDL-R, however, they do not exclude the possibility that
other PK-C isoforms such as PK-C δ may also mediate this
activity (91). Our studies must be considered in light of the fact
that homozygous disruption of the VLDL-R gene is not associated
with major phenotypic abnormalities (92). Although these re-
results demonstrate that the VLDL receptor does not play a
critical role in lipoprotein clearance in mice, they do not ex-
clude a role for this receptor in normal and/or pathophysiol-
ogical processes. The ability of the VLDL-R to mediate lipoprotein
uptake in vivo has been demonstrated by gene transfer studies
(93, 94), and the receptor may be involved in other processes in
addition to lipoprotein metabolism. Hence, a complementary
interpretation of the VLDL receptor knockout studies, based on
the findings presented here, is that in at least some tissues the
receptor may be phosphorylated and in a relatively inactive
state. If so, then genetic deletion of the receptor might have
relatively minor consequences. Further evaluation of this hy-
thesis will require in vivo examination of the function of
VLDL-R lacking specific serine residues involved in regulation
of receptor affinity.

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