Serine and Threonine Phosphorylation of the Low Density Lipoprotein Receptor-related Protein by Protein Kinase Ca Regulates Endocytosis and Association with Adaptor Molecules

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The low density lipoprotein receptor-related protein (LRP) is a large receptor that participates in endocytosis, signaling pathways, and phagocytosis of necrotic cells. Mechanisms that direct LRP to function in these distinct pathways likely involve its association with distinct cytoplasmic adaptor proteins. We tested the hypothesis that the association of various adaptor proteins with the LRP cytoplasmic domain is modulated by its phosphorylation state. Phosphoamino acid analysis of metabolically labeled LRP revealed that this receptor is phosphorylated at serine, threonine, and tyrosine residues within its cytoplasmic domain, whereas inhibitor studies identified protein kinase Ca (PKCa) as a kinase capable of phosphorylating LRP. Mutational analysis identified critical threonine and serine residues within the LRP cytoplasmic domain that are necessary for phosphorylation mediated by PKCa. Mutating these threonine and serine residues to alanines generated a receptor that was not phosphorylated and that was internalized more rapidly than wild-type LRP, revealing that phosphorylation reduces the association of LRP with adaptor molecules of the endocytic machinery. In contrast, serine and threonine phosphorylation was necessary for the interaction of LRP with Shc, an adaptor protein that participates in signaling events. Furthermore, serine and threonine phosphorylation increased the interaction of LRP with other adaptor proteins such as Dab-1 and CED-6/GULP. These results indicate that phosphorylation of LRP by PKCa modulates the endocytic and signaling function of LRP by modifying its association with adaptor proteins.

The low density lipoprotein receptor-related protein (LRP) is a large endocytic receptor that was initially identified as the molecule responsible for mediating the uptake of α2-macroglobulin-protease complexes and apoE-enriched lipoproteins (1–6). LRP is synthesized as a single chain 600-kDa precursor that is processed by furin into a 515-kDa heavy chain and an 85-kDa light chain (7). The heavy chain of LRP contains multiple clusters of cysteine-rich low density lipoprotein receptor class A repeats that function in ligand recognition, whereas the light chain contains multiple epidermal growth factor repeats, a transmembrane domain, and a cytoplasmic domain. In addition to α2-macroglobulin-protease complexes and apoE-containing lipoproteins, LRP also recognizes proteases such as tissue-type plasminogen activator (8) and matrix metalloproteinase-9 (9), serpin-enzyme complexes (10), and matrix proteins such as thrombospondin-1 (11). Upon binding to LRP, ligands are rapidly internalized via coated pit-mediated endocytosis and are subsequently degraded in lysosomes. LRP also plays a role in the phagocytosis of apoptotic cells (12). This may require association of LRP with a specific adaptor protein, CED-6/GULP, as recent data reveal that the ced-6/gulp gene in Drosophila is one of seven genes required for engulfment (13) and that the human homolog of CED-6/GULP binds to the second NPYX motif within the LRP cytoplasmic domain (14). Although its function as a cargo transporter is well established, evidence is accumulating indicating that LRP may also play important roles in modulating signaling events. Thus, LRP has been implicated as a component of the receptor complex for midkine, a heparin-binding growth factor with migration-promoting and survival-promoting activities (15), and has been suggested to be responsible for the effect that tissue-type plasminogen activator has on late phase long-term potentiation (16). Tissue-type plasminogen activator has also been shown to directly increase vascular permeability in the early stages of blood-brain barrier opening through an LRP-mediated cell signaling event (17). Another LRP ligand, activated α2-macroglobulin, mediates calcium influx specifically in neurons present in primary cultures of mouse cortex upon association with LRP (18). LRP is phosphorylated at Tyr63 within its cytoplasmic domain via platelet-derived growth factor (PDGF) receptor activation (19, 20) and modulates the functional activity of the PDGF receptor in atherosclerotic mouse models (21).

Mechanisms that direct LRP to function in cargo transport or in signaling pathways remain unknown, but likely involve the association of specific adaptor proteins with its cytoplasmic tail. This portion of LRP contains two NPYX motifs that are capable of interacting with cytoplasmic adaptor proteins harboring phosphotyrosine-binding (PTB) domains (22, 23). Phosphorylation of Tyr63, which is located within the terminal NPYX motif in the LRP cytoplasmic domain, provides a recog-
nition site for Shc (20, 24), an adaptor protein that participates in signaling pathways. In addition to tyrosine phosphorylation initiated by growth factor activation, the LRP cytoplasmic domain is also phosphorylated at serine residues by protein kinase A (25). At this time, however, the role of serine phosphorylation in LRP function remains unknown. Mutation of a critical serine residue in the cytoplasmic tail of an LRP mini-receptor slightly delays LRP-mediated endocytosis, suggesting a role for phosphorylation of LRP in endocytosis (25). In this study, we set out to investigate the hypothesis that phosphorylation of the cytoplasmic domain of LRP modulates its function by altering the affinity for cytoplasmic adaptor proteins. Our results reveal that serine and threonine phosphorylation of LRP modulates its phosphorylation at Tyr63, where a number of adaptor molecules bind, and modifies its association with adaptor proteins such as Shc, Dab-1 (Disabled-1), and CED-6/GULP.

EXPERIMENTAL PROCEDURES

Cell Lines, Proteins, Antibodies, and Reagents—Human embryonic lung fibroblasts (WI-38) and SV40-transformed African green monkey kidney fibroblast-like cell lines (COS-1) were obtained from American Type Culture Collection (Manassas, VA). LRP mini-receptor (NR4) was stably transfected into a hamster ovary cell line (CHO13-5-1) as described previously (26), and rat pulmonary artery smooth muscle cells (PAC-1) were obtained from Dr. Gene Liu. Human recombiant PKCα was purchased from EMD Biosciences (San Diego, CA). LRP was purified as described (2). Rabbit anti-LRP polyclonal antibody R2629, anti-LRP cytoplasmic tail C terminus antibody R704, monoclonal antibodies 5A6 and 8B8 recognizing the LRP light chain, and monoclonal antibody 8G1 recognizing the heavy chain were all prepared as described (4, 27). Anti-PKCα monoclonal antibody and polyclonal and monoclonal antibodies against Shc were obtained from Transduction Laboratories (San Diego, CA). Cells clonal antibody and polyclonal and monoclonal antibodies against Shc colonies were picked for both wild-type and mutant LRPs, and positive study, we also used minireceptor LRP (cloned into pcDNA3.1(H11002)) or full-length mutant LRP (cloned into pcDNA3.1(H11002)). LRP-null CHO13-5-1 cells were grown to 30% confluence in 150-mm plates, washed twice with Dulbecco’s modified Eagle’s medium (DMEM), preincubated in phosphate-free DMEM for 3–4 h, and labeled for 30 min to 1 h with 1 μCi/plate [32P]orthophosphate (Amersham Biosciences) with or without kinase inhibitors and PMA. Similarly, LRP-null CHO13-5-1 cells stably transfected with full-length wild-type or T16A/3S mutant LRP were grown for 4 h, labeled for 30 min to 1 h with 1 μCi/plate [32P]orthophosphate. Labeling was terminated by adding 5× SDS sample buffer containing β-mercaptoethanol, separated by 4–20% SDS-PAGE, transferred to nitrocellulose membrane stained with Ponceau S, exposed, and analyzed using a PhosphorImager (Amersham Biosciences).

In Vivo Phosphorylation and Phosphoamino Acid Analysis—PAC-1 cells or WI-38 fibroblasts were grown to 70–80% confluency in 150-mm plates, washed twice with Dulbecco’s modified Eagle’s medium (DMEM), preincubated in phosphate-free DMEM for 3–4 h, and labeled for 30 min to 1 h with 1 μCi/plate [32P]orthophosphate (Amersham Biosciences) with or without kinase inhibitors and PMA. Similarly, LRP-null CHO13-5-1 cells stably transfected with full-length wild-type or T16A/3S mutant LRP were grown for 60 min. The liquid hydrolysates were dried, resuspended in 10 μl 2 N HCl, and hydrolyzed with 6 N HCl at 110 °C for 60 min. The liquid hydrolysates were dried, resuspended in 10 μl of water, and spotted along with nonradioactive phosphoamino acid standard mixture on glass-backed cellulose TLC plates. One-dimen- sional electrophoresis was carried out using the Hunter thin-layer peptide mapping electrophoresis system (HTLE-7002, CBS Scientific, Del Mar, CA). After visualizing the phosphoamino acid standards with 0.25% ninhydrin in acetone, the TLC plates were exposed to Bio- imax-MS film with an intensifying screen at −70 °C to detect 32P incorporated into serine, threonine, or tyrosine residues, immunoprecipitates from 32P-labeled WI-38 fibroblasts were separated by 4–12% SDS-PAGE and transferred to polyvinylidene fluoride membranes, and the bands corresponding phospho- phosphorylated LRP was excised, washed, and hydrolyzed with 6 N HCl at 110 °C for 60 min. The liquid hydrolysates were dried, resuspended in 10 μl of water, and spotted along with nonradioactive phosphoamino acid standard mixture on glass-backed cellulose TLC plates. One-dimen- sional electrophoresis was carried out using the Hunter thin-layer peptide mapping electrophoresis system (HTLE-7002, CBS Scientific, Del Mar, CA). After visualizing the phosphoamino acid standards with 0.25% ninhydrin in acetone, the TLC plates were exposed to Bio- imax-MS film with an intensifying screen at −70 °C to detect 32P incorporated into serine, threonine, or tyrosine residues.

Enzyme-linked Immunoassays—Ligands (PKCs and bovine serum albumin (BSA) were added to the serum-free DMEM in 96-well polystyrene microtiter plates at 1 μg/ml in Tris-buffered saline (TBS; 50 mM Tris and 150 mM NaCl) with 2 mM CaCl2 overnight at 4 °C and blocked with 1% BSA in TBS and 2 mM CaCl2, for 1 h at room temperature. The wells were then incubated with increasing concentrations of purified human LRP, wild- type LRP cytoplasmic tail-GST fusion protein, or GST in TBS, 2 mM CaCl2, and 0.05% Tween 20. The bound radiolabeled antibody coated with antibody 8G1 (0.5 μg/ml) and horseradish peroxidase-conjugated goat anti-mouse IgG antibody, and bound wild-type LRP cytoplasmic tail-GST was detected using polyclonal antibody R704 (1 μg/ml) and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody. Wells were developed using tetramethylbenzidine peroxidase substrate (KPL, Gaithersburg, MD), and absorbance was measured at 450 nm.

Measurement of the Endocytic Rate Constant—LRP-null CHO13-5-1 cells stably transfected with full-length wild-type or T16A/3S mutant LRP were grown to 70% confluency in 6-well culture plates. The endo- cytic rate constant was measured by internalized/surface analysis as
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Rat pulmonary artery smooth muscle cells were grown to 70% confluence in M199 medium containing 10% fetal calf serum with antibiotics and glutamine in 150-mm culture plates. For treatment with PMA (150 nM), staurosporine (7 nM), or Go 6976 (10 nM), cell layers were washed and treated with 1% Nutridoma®NS (Roche Applied Science) in M199 medium for 1 h. Cell layers were twice washed with cold DPBS and lysed on ice in 1.0 ml of lysis buffer containing protease and phosphatase inhibitors as described above. After pre-clearing with rabbit IgG and protein G-Sepharose, the lysates were immunoprecipitated with antibody R2629 for 1 h at 4°C. Immunoprecipitates were washed three times with lysis buffer containing protease and phosphatase inhibitors and boiled in 2× nonreducing SDS-PAGE sample buffer for 10 min. Samples were separated by 4–12% SDS-PAGE and transferred to nitrocellulose membranes for immunoblot analysis. Membranes were blocked with 5% nonfat dry milk in TBS for 1 h at room temperature and incubated with antibody 5A6 (0.2–0.4 μg/ml) in 5% milk in TBS and 0.05% Tween 20 for 1 h. After 3 washes, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:10,000) in 5% milk in TBS and 0.1% Tween 20 for 1 h, washed three times, and developed with chemiluminescent reagent (Pierce). The bands were visualized with Biomax Light film (Eastman Kodak). For visualizing PKCα, membranes were stripped using a Reblot Western blotting recycling kit (Chemicon International, Inc., Temecula, CA) and probed with anti-PKCα monoclonal antibody as described above.

GH Pull-down Assay—GSH-Sepharose was prepared by coupling glutathione to epoxy-activated Sepharose. COS-1 cells were grown to 40% confluence in 150-mm plates and transfected with 10 μg of expression vector containing Myc-tagged wild-type or T16A/3S mutant LRP-β using FuGENE 6 in DMEM and 10% serum. 48 h after transfection, the cell layers were twice washed with cold DPBS, and lysates were prepared in lysis buffer containing protease and phosphatase inhibitors as described above. After pre-clearing with 75 μl of a 1:1 slurry of GSH-Sepharose overnight at 4°C, Immunoprecipitates were washed three times with lysis buffer containing protease and phosphatase inhibitors and boiled in 2× nonreducing SDS-PAGE sample buffer for 10 min. Samples were separated by 4–12% SDS-PAGE and transferred to nitrocellulose membranes for immunoblot analysis. Membranes were blocked with 5% nonfat dry milk in TBS for 1 h at room temperature and incubated with antibody 5A6 (0.2–0.4 μg/ml) in 5% milk in TBS and 0.05% Tween 20 for 1 h. After 3 washes, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:10,000) in 5% milk in TBS and 0.05% Tween 20 for 1 h, washed three times, and developed with chemiluminescent reagent (Pierce). The bands were visualized with Biomax Light film.

Transient Transfection of Shc, HA-GULP, and LRP-β in COS Cells for Co-immunoprecipitation Experiments—COS-1 cells were grown to 30% confluence in 150-mm plates and transfected with 7 μg of Shc or HA-GULP plasmid and 7.5 μg of empty vector (pGsc-Tag) or Myc-tagged wild-type, T16A/3S mutant, or NPYY/PAVα mutant LRP-β. 48 h after transfection, the cell layers were twice washed with cold DPBS, and lysates were prepared in lysis buffer containing protease and phosphatase inhibitors as described above. Cell lysates were pre-incubated with unlabeled immunogold-protein G complex for 2–3 h at 4°C. Shc- and LRP-β-expressing cell lysates were immunoprecipitated with either anti-Shc monoclonal antibody (5 μg/ml) or antibody 9E10 (10 μg/ml). Similarly, HA-GULP- and LRP-β-expressing cell lysates were immunoprecipitated with either anti-HA IgG polyclonal antibody (5 μg/ml) or anti-Myc IgG polyclonal antibody (5 μg/ml). Immunoprecipitates were washed three times with lysis buffer containing protease and phosphatase inhibitors and boiled in 2× nonreducing SDS-PAGE sample buffer for 10 min. Samples were separated by 4–12% SDS-PAGE and transferred to nitrocellulose membranes for immunoblot analysis.

RESULTS

LRP Is Phosphorylated at Serine, Threonine, and Tyrosine Residues—Previous studies have reported that LRP is phosphorylated at serine (25) and tyrosine (20) residues within its cytoplasmic domain. We sought to fully characterize the phosphorylation of LRP and to study the role this may play in LRP function. To this end, human WI-38 fibroblasts, which express high levels of LRP, were metabolically labeled by incubation with [32P]H3PO4 for 1 h at 37°C. Cell extracts were then subjected to immunoprecipitation with either affinity-purified anti-LRP IgG antibody R2629 or preimmune IgG as a control. The results of this experiment (Fig. 1A) demonstrate that a phosphorylated protein with an apparent mass of 85 kDa was immunoprecipitated by anti-LRP IgG antibody (lane 1), but not by preimmune IgG (lane 2). The phosphorylated band shown in lane 1 has an identical mobility on SDS-PAGE as the light chain of LRP as revealed by immunoblotting the immunoprecipitates with monoclonal antibody 5A6, which is specific for the LRP β-subunit (data not shown).

To identify the types of amino acid residues within the LRP cytoplasmic domain that are phosphorylated, LRP metabolically labeled with [32P]H3PO4 was subjected to immunoprecipitation and SDS-PAGE and then transferred to polyvinylidenefluoride membranes. The 32P-labeled 85-kDa β-subunit was next excised from the membranes and subjected to acid hydrolysis, and the phosphoamino acid content was determined by thin-layer cellulose electrophoresis. The results shown in Fig. 1B reveal that LRP was phosphorylated at serine, threonine, and tyrosine residues. Furthermore, the results reveal that under normal cell culture conditions, the extent of tyrosine phosphorylation was relatively low compared with the levels of serine and threonine phosphorylation.

Kinase Inhibitors Suggest That Protein Kinase A and PKCα Phosphorylate the LRP Cytoplasmic Domain—Our next experiments were aimed at identifying the kinase class responsible for LRP phosphorylation. WI-38 fibroblasts were incubated for 1 h at 37°C with [32P]H3PO4 in the presence of one of the following broad-spectrum serine/threonine kinase inhibitors: staurosporine, K-252a, H-89, and the tyrosine kinase inhibitor genistein. Following incubation, cell extracts were prepared, subjected to immunoprecipitation with anti-LRP IgG antibody, and analyzed by SDS-PAGE and autoradiography. The results of this experiment demonstrate that staurosporine and K-252a dramatically reduced the extent of LRP phosphorylation (Fig. 1C). As reported previously (25), H-89, the potent protein kinase A inhibitor, also reduced LRP phosphorylation. On the other hand, LRP phosphorylation was not noticeably affected by the tyrosine kinase inhibitor genistein, consistent with the data in Fig. 1B, indicating that only a small amount of tyrosine phosphorylation was detected on the LRP light chain. We also examined the phosphorylation of LRP in a rat pulmonary smooth muscle cell line and found that the selective PKCα inhibitor Ro-32-0432 significantly reduced the extent of LRP phosphorylation, implicating a role for PKCα activity in LRP phosphorylation (Fig. 1D).

PKCα Coprecipitates with LRP following PMA Treatment—Since inhibitor studies suggested that PKCα mediates LRP phosphorylation, we initiated experiments to determine whether PKCα can associate with LRP. For these experiments, a rat pulmonary smooth muscle cell line (PAC-1) was cultured in the absence (control) or presence of PMA. Following treatment, the cells were lysed, and LRP was immunoprecipitated.
The LRP/H925 were phosphorylated by PKC light chain confirmed that both serine and threonine residues are phosphorylated in the LRP β-subunit. Phosphoamino acid analysis of the LRP following incubation, LRP was analyzed by SDS-PAGE and autoradiography. The migration positions of standard phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) are indicated. P1 indicates free phosphate. C, shown are the effects of kinase inhibitors on LRP phosphorylation. WI-38 fibroblasts were incubated with [32P]orthophosphate (1 h at 37 °C) or presence of staurosporine (3 μM), H-89 (25 μM), genistein (50 μM), or K-252a (15 μM). Following incubation, cells were processed as described for A. D, rat pulmonary artery smooth muscle cells were preincubated in phosphate-free DMEM for 3 h; treated with PMA (300 nM), staurosporine (10 nM), or Ro-32-0432 (25 nM) for 15 min; and labeled with [32P]orthophosphate (1 mCi/plate) for 30 min. At the end of the incubation, cell layers were washed and lysed, and cell extracts were prepared and processed as described for A. The incorporation of [32P] into LRP was analyzed by phosphorimaging.

Having confirmed that the LRP cytoplasmic domain is a target for PKCα-mediated phosphorylation, we next set out to identify potential phosphorylation sites. The LRP cytoplasmic domain contains a consensus PKC phosphorylation site at Thr28 that was targeted for mutation (Fig. 3A). Another threonine (Thr16) that is upstream from the first NPXY motif was also targeted for mutation. The basis for selecting Thr16 as a potential phosphorylation site was derived from studies on another transmembrane protein, the amyloid precursor protein. The amyloid precursor protein contains a threonine residue (Thr665) that lies upstream from the NPXY motif in its cytoplasmic domain and is phosphorylated (31). Phosphorylation of this residue modulates the conformation of its cytoplasmic domain and its association with adaptor proteins that bind to the NPXY motif (32). Based on these prior studies, we selected Thr16 in the LRP cytoplasmic domain for mutation as well. However, we found that PKCα readily phosphorylated mutant molecules in which either Thr16 or Thr28 was replaced with alanine (T16A and T28A, respectively). Since we know that LRP contains phosphorylated serine residues and since prior work found that Ser76 is phosphorylated by protein kinase A (25), we mutated all three serine residues within the LRP cytoplasmic domain to alanines (S73A/S76A/S79A) and found that these mutations reduced the extent of phosphorylation only slightly. However, mutation of Thr16 as well as Ser76, Ser79, and Ser79 to alanine residues (designated T16A/3S) abolished phosphorylation of the LRP cytoplasmic domain mediated by PKCα. These results identify potential sites for phosphorylation of the LRP cytoplasmic domain by PKCα.

**LRP Serine/Threonine Phosphorylation Reduces the Endocytic Rate of LRP**—The previous experiments suggest that an LRP mutant in which Thr16 along with Ser73, Ser76, and Ser79 within the cytoplasmic domain are all changed to alanines might not be effectively phosphorylated in cells. If so, LRP carrying all of these

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**Fig. 1.** Serine, threonine, and tyrosine residues are phosphorylated in the LRP β-subunit in WI-38 fibroblasts metabolically labeled with [32P]orthophosphate in phosphate-free DMEM. Following labeling, cell extracts were subjected to immunoprecipitation with anti-LRP polyclonal antibody (lane 1) or non-immune IgG (lane 2). Immunoprecipitated proteins were subjected to 4–12% SDS-PAGE, transferred to polyvinylidene difluoride membrane, and analyzed by autoradiography. B, the metabolically labeled 85-kDa LRP β-subunit was excised, hydrolyzed with 6 N HCl at 110 °C for 1 h, subjected to thin-layer electrophoresis, and analyzed by autoradiography. The migration positions of standard phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) residues were indicated. P1 indicates free phosphate. C, shown are the effects of kinase inhibitors on LRP phosphorylation. WI-38 fibroblasts were incubated with [32P]orthophosphate (1 h at 37 °C) in the absence (Control) or presence of staurosporine (3 μM), H-89 (25 μM), genistein (50 μM), or K-252a (15 μM). Following incubation, cells were processed as described for A. D, rat pulmonary artery smooth muscle cells were preincubated in phosphate-free DMEM for 3 h; treated with PMA (300 nM), staurosporine (10 nM), or Ro-32-0432 (25 nM) for 15 min; and labeled with [32P]orthophosphate (1 mCi/plate) for 30 min. At the end of the incubation, cell layers were washed and lysed, and cell extracts were prepared and processed as described for A. The incorporation of [32P] into LRP was analyzed by phosphorimaging.
mutations might be a useful molecule for studying the consequence of serine/threonine phosphorylation on LRP function. To investigate this, LRP-deficient CHO13-5-1 cells were transfected with full-length wild-type LRP or full-length LRP containing all four of these mutations (T16A/3S) within its intracellular domain. Following metabolic labeling of the cells for 1 h with \(^{32}P\)orthophosphate, LRP was immunoprecipitated and analyzed by autoradiography. The results reveal that mutant LRP

**FIG. 2.** PKC\(\alpha\) associates with LRP as determined by coprecipitation analysis and solid-phase binding assays. A, rat pulmonary smooth muscle cells (PAC-1) were cultured in the absence (Control) or presence of 5 nM Go 6976 or 300 nM PMA. Following incubation, the cells were lysed, subjected to immunoprecipitation (IP) with anti-LRP polyclonal antibody, and analyzed for PKC\(\alpha\) content by immunoblot analysis (upper panel) or for LRP (lower panel). WB, Western blot. B, increasing concentrations of LRP were added to microtiter wells coated with human recombinant PKC\(\alpha\) (○) or BSA (■). Bound LRP was detected using anti-LRP monoclonal antibody 8G1 and horseradish peroxidase-conjugated goat anti-mouse IgG antibody. Each data point represents the average of triplicate values, and the solid curves represent the best fit to a binding isotherm determined by nonlinear regression analysis. C, increasing concentrations of the GST-fused LRP intracellular domain (GST-LRP-CD; ● and ○) or GST alone (■ and □) were incubated with microtiter wells coated with PKC\(\alpha\) (● and ■) or BSA (○ and □). Bound protein was detected using the C terminus-specific anti-LRP polyclonal antibody R704 and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody. Each data point represents the average of triplicate values, and the solid curves represent the best fit to a binding isotherm determined by nonlinear regression analysis.

**FIG. 3.** PKC\(\alpha\) phosphorlylates the LRP cytoplasmic tail expressed as a GST fusion protein. A, shown is a schematic diagram of the LRP cytoplasmic domain with key amino acids highlighted. For convenience, the first amino acid following the transmembrane domain is numbered 1 as suggested by Li et al. (29). B and C, shown are the results from phosphorylation analysis of wild-type (WT) and mutant (T28A, S73A/S76A/S79A (3S), T16A/3S, and T16A) LRP cytoplasmic domain molecules expressed as fusion proteins with GST. Phosphorylation was accomplished by incubating LRP cytoplasmic domains with human recombinant PKC\(\alpha\) at 37 °C for 15 min using the Biotrak PKC assay system (7.5 \(\mu\)g/reaction). Purified GST was used as a negative control. After 15 min, reactions were terminated by adding 5% reducing Laemmli sample buffer, separated by 4–20% SDS-PAGE, and transferred to nitrocellulose membrane. Membranes were stained with Ponceau S (B) and detected by phosphorimaging (C). D, for quantitative analysis, reactions were terminated by adding stop buffer containing orthophosphoric acid, spotted on phosphocellulose membrane, washed with 5% acetic acid, and counted. WT LRPCT, wild-type LRP cytoplasmic tail.
FIG. 4. T16A/3S mutant LRP is not effectively phosphorylated in Chinese hamster ovary cells or in COS-1 cells. A, LRP-null CHO13-5-1 cells were stably transfected with full-length wild-type (WT) or T16A/3S mutant LRP. Following treatment, the cells were metabolically labeled with [32P]orthophosphate (1 mCi/plate) for 1 h in phosphate-free medium and immunoprecipitated with anti-LRP antibody R2629. Immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The incorporation of 32P into LRP was analyzed using a PhosphorImager (upper panel), and total LRP was detected by immunoblotting with anti-LRP monoclonal antibody 5A6 (lower panel). B, COS-1 cells were transiently transfected with empty vector (Vector), Myc-tagged wild-type LRP-β (WT), or the T16A/3S mutant were immunoprecipitated with anti-Myc monoclonal antibody 9E10. Immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The incorporation of 32P into LRP-β was analyzed using phosphorimager (upper panel), and total LRP was detected by immunoblotting with anti-Myc polyclonal antibody and by chemiluminescence (lower panel).

FIG. 5. T16A/3S mutant LRP is internalized more rapidly than wild-type LRP. CHO13-5-1 cell lines stably transfected with either wild-type (WT; ○) or mutant (○) LRP were grown to 70% confluency. The endocytic rate constant was measured by incubating the cells with 125I-labeled antibody 8G1 (1.5 μg/ml) at 37 °C for the indicated time periods. Following incubation, the cells were placed on ice, and the amount of ligand bound to the surface (B) and internalized (I) was measured. Non-specific binding and internalization were determined by incubating parallel cultures with 50-fold excess unlabeled antibody 8G1 and were subtracted from the total to determine specific binding and internalization.

was not effectively phosphorylated in these cell lines compared with wild-type LRP (Fig. 4A). Similar results were obtained when COS-1 cells were transiently transfected with LRP-β and a mutant version of this minireceptor containing the T16A/3S mutations (Fig. 4B). LRP-β is an LRP minireceptor that represents the 85-kDa β-subunit of LRP containing the cytoplasmic domain, the transmembrane domain, and a portion of the ectodomain and is fully functional in endocytosis (20).

We next wanted to determine whether the LRP mutant is internalized at the same rate as the wild-type molecule. To measure this, we compared the endocytic rate constants for full-length wild-type and mutant LRPs using 125I-labeled antibody 8G1 as the ligand. The results reveal that the LRP mutant has a higher endocytic rate constant ($k_e = 0.192 \text{ min}^{-1}$) than the wild-type molecule ($k_e = 0.144 \text{ min}^{-1}$), revealing that lack of serine and threonine phosphorylation does not inhibit receptor internalization (Fig. 5). In fact, the data suggest that phosphorylation of the LRP cytoplasmic domain generates a molecule in which the rate of internalization is decreased by 25%.

LRP Serine/Threonine Phosphorylation Is Required for Optimal Association with Shc—The LRP cytoplasmic domain is phosphorylated at Tyr63 by PDGFR receptor-β activation (20). This residue is located within the second NPXY motif of the LRP cytoplasmic domain and is a site where a number of cytoplasmic adaptor proteins bind. Phosphorylation of Tyr63 within this motif generates a docking site for Shc (20, 24, 33), an adaptor protein that contains a PTB domain that recognizes phosphorylated tyrosine residues in the context of NPXY motifs.

Previous experiments have shown that co-transfection of COS-1 cells with Shc and LRP-β results in increased phospho-
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The trafficking and functional properties of receptor tyrosine kinases (34-36) and G-protein-coupled receptors (35, 37, 38) are regulated by phosphorylation of their cytoplasmic domains. In contrast to our understanding of how phosphorylation modulates the function of these signaling receptors, the role of phosphorylation in the function of endocytic receptors is much less defined. Thus, although the low density lipoprotein receptor (39, 40) and LRP (25) are phosphorylated at serine residues within their cytoplasmic domains, the functional significance of this phosphorylation has not been understood until now. LRP functions in endocytosis, in the phagocytosis of necrotic cells, and in modulating signaling pathways. Presumably, these varied activities are regulated by association of LRP with distinct cytoplasmic adaptor proteins (22, 23). We hypothesized that phosphorylation of the LRP cytoplasmic domain may modulate its interaction with adaptor proteins; and to test this hypothesis, we set out to investigate LRP phosphorylation in more detail.

Our initial studies employed inhibitors to identify the kinase class responsible for phosphorylating the LRP cytoplasmic domain. This work revealed that inhibitors of protein kinase A and PKCα reduced the extent of LRP phosphorylation. Protein kinase A was previously reported to mediate phosphorylation of the LRP light chain (25); and in this study, in vitro experiments demonstrated that PKCα readily phosphorylated the cytoplasmic domain of LRP. Furthermore, our experiments indicated that PKCα coprecipitated with LRP in smooth muscle cells treated with PMA. PKCα is a ubiquitously expressed serine/threonine kinase, and plays an important role in cellular proliferation, apoptosis, differentiation and motility. Upon activation, PKCα translocates to the membrane and associates with numerous receptors, including syndecan-4 (41, 42) as well as integrins (43, 44). Inhibition of PKCα activity or expression of a PKCα dominant-negative mutant suppresses focal adhesion formation and cell migration mediated by α5β3 integrin in cooperation with syndecan-4 in a human metastatic melanoma cell line (43). Furthermore, in a squamous carcinoma cell line, activation of PKCα and the subsequent phosphorylation of the β3 integrin subunit at serine residues are required for mobilization of α5β3 integrin from hemidesmosomes and its redistribution to cell protrusions (44). The association of PKCα with the LRP cytoplasmic domain and its subsequent phosphorylation of LRP indicate a new role for this kinase in modulating LRP function.

Phosphoamino acid analysis of metabolically labeled LRP revealed that serine, threonine, and tyrosine residues are phosphorylated. Mutational analysis demonstrated that conversion of Thr63 to alanine with Ser73, Ser76, and Ser79 to alanine generated an LRP molecule in which the extent of phosphorylation was greatly diminished. By comparing the properties of mutant LRP with that of the wild-type receptor, we gained insight into the contribution of serine and threonine phosphorylation to LRP function. First, we observed that mutant LRP was internalized 25% more rapidly than the wild-type phosphorylated receptor. This was confirmed by measurements of the endocytic rate constant, a reliable measure of receptor activity and func-
tion. Thus, serine and threonine phosphorylation of the LRP cytoplasmic domain appears to diminish its association with adaptors of the endocytic machinery, which, in the case of LRP, is the adaptor protein complex AP-2 (45). AP-2 is a multifunctional heterotetramer that contains α, β2, μ2, and σ2 subunits. The μ2-subunit interacts with the cytoplasmic domain of membrane-bound receptors containing a variety of tyrosine-based internalization motifs, although the motifs present in the LRP cytoplasmic tail do not conform exactly to the preferred internalization motifs, although the motifs present in the LRP cytoplasmic domain of the LRP minireceptor is phosphorylated at Tyr63 (20). In 38 fibroblasts and smooth muscle cells, tyrosine phosphorylation of LRP is mediated by PDGF receptor-β activation (20) and also occurs at Tyr63. This residue is located within the terminal NPXY motif of LRP, which is a binding site for a number of adaptor proteins, including Fe65, Dab-1, Shc, and CED-6/GULP (14, 22, 23). In addition to binding adaptor proteins that are involved in a variety of cellular processes, Tyr63 is also a component of the YXXL internalization sequence in LRP (29).

We noted that impaired serine and threonine phosphorylation of LRP dramatically impacted its ability to bind Shc. Shc is an adaptor protein that is involved in signal transduction by protein-tyrosine kinases (46) and contains a C-terminal Src homology-2 domain that binds with weak affinity to several phosphorylated tyrosine residues on the PDGF receptor (47) and a PTB domain that recognizes phosphorylated LRP (20, 24, 25). Phosphorylation of Shc at Tyr317 allows for Grb2 binding (48), thereby activating the Ras pathway, whereas phosphorylation of Shc at Tyr239 and Tyr240 initiates a second signaling pathway that is related to the reduction in tyrosine phosphorylation noted in mutant LRP or to a preference of these adaptor proteins for phosphorylated serine/threonine residues. Our studies reveal that impairment of serine/threonine phosphorylation inhibits the interaction of LRP with cytoplasmic adaptor proteins such as Dab-1 and CED-6/GULP, which are involved in signaling pathways and phagocytosis, respectively. Serine and threonine phosphorylation also modulates the internalization rate of LRP by reducing the endocytic rate constant by 25%. Thus, phosphorylation of LRP represents a molecular mechanism to switch LRP function from that of an endocytic receptor to that of a signaling receptor by modulating the class and type of adaptor proteins that associate with LRP.

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Serine and Threonine Phosphorylation of the Low Density Lipoprotein Receptor-related Protein by Protein Kinase C α Regulates Endocytosis and Association with Adaptor Molecules

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