Localization of Low Density Lipoprotein Receptor-related Protein 1 to Caveolae in 3T3-L1 Adipocytes in Response to Insulin Treatment*

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The insulin-induced translocation of low density lipoprotein receptor-related protein 1 (LRP1) from intracellular membranes to the cell surface in 3T3-L1 adipocytes was differentiation-dependent and did not occur in 3T3-L1 fibroblasts. Prompted by findings that the plasma membrane of 3T3-L1 adipocytes was rich in caveolae, we determined whether LRP1 became caveolae-associated upon insulin stimulation. The caveolae domain was isolated by the well characterized detergent solubilization and sucrose density ultracentrifugation methodology. Under basal conditions, only a trace amount of LRP1 was caveolae-associated despite the markedly elevated caveolin-1 and caveolae after adipocytic cell differentiation. Upon insulin treatment, the amount of LRP1 associated with caveolae was increased by 4-fold within 10 min, which was blocked completely by pretreatment with wortmannin prior to insulin. The caveolar localization of LRP1 in adipocytes was specific to insulin; treatment with platelet-derived growth factor-bb isoform did not promote but rather decreased caveolar localization of LRP1 below basal levels. The insulin-induced caveolar localization of LRP1 was also observed in 3T3-L1 fibroblasts where translocation of LRP1 from intracellular membranes to the cell surface was absent, suggesting that association of LRP1 with caveolae was achieved, at least in part, through lateral transmigration along the plane of plasma membranes. Immunocytochemistry studies revealed partial co-localization of LRP1 (either endogenous LRP1 or an epitope-tagged minireceptor) with caveolin-1 in cells treated with insulin, which was confirmed by co-immunoprecipitation of LRP1 with caveolin-1 in cells treated with insulin but not platelet-derived growth factor-bb. These results suggest that the localization of LRP1 to caveolae responds selectively to extracellular signals.

The low density lipoprotein receptor-related protein 1 (LRP1) is a large type I membrane protein (4525 amino acids) and belongs to the low density lipoprotein receptor (LDLR) gene family (1, 2). Mammalian LRP1 is abundantly expressed in adipocytes, neurons, smooth muscle cells, fibroblasts, and adipocytes. LRP1 is cleaved post-translationally by the endopeptidase furin in the distal Golgi, and the resulting extracellular α-chain (515 kDa) and transmembrane β-chain (85 kDa) form a non-covalent heterodimer (3). The α-chain is composed of three major structural modules, namely class A ligand-binding (complement-type) repeats, the epidermal growth factor (EGF) precursor-type repeats, and YWTD β-propellers. The ligand-binding repeats are dispersed throughout the LRP1 extracellular domain in four clusters denoted I to IV, of which clusters II and IV possess the ability to bind a variety of structurally unrelated ligands (4, 5). The cytoplasmic tail of the LRP1 β-chain contains motifs resembling those required for receptor-mediated endocytosis through clathrin-coated pits (e.g. NPXY and YXXL) that are found in other LDLR family members (6, 7). In addition, the cytoplasmic tail of the LRP1 β-chain also contains amino acid sequences that act as a binding site for adaptor proteins (8). Most of the LRP1-interacting proteins contain a protein interaction domain, phosphotyrosine binding domain, or PDZ domain and function in cell adhesion, trafficking, and signal transduction (9). The endocytic function of LRP1 in the uptake and degradation of triglyceride-rich lipoproteins containing apolipoprotein E has been documented by studies using different experimental model systems (10–14). However, the broad spectrum of LRP1 ligands (e.g. α₄-macroglobulin, various protease/protease inhibitor complexes, and certain growth factors) (15–18) and the lethality of gene inactivation in mice (19) suggest that LRP1 participates in biological processes other than lipoprotein metabolism.

Accumulating experimental data have indicated that LRP1 and some other LDLR family members are located within the lipid raft microdomains of the plasma membrane (20–22). Lipid rafts are lateral assemblies of sphingolipids and cholesterol that form a discrete liquid-ordered phase in the lipid bilayer (23), and they are defined as insoluble membranes isolated by nonionic detergents (e.g. Triton X-100) at 4 °C (24–26). Caveolae are a specialized type of lipid raft forming flask-related protein 1; PDGF-bb, bb isoform of platelet-derived growth factor; PDGR, PDGF receptor; LDLR, low density lipoprotein receptor; EGF, epidermal growth factor; PDGFR, PDGF receptor; EGFR, EGF receptor; apoER2, apolipoprotein E receptor 2; PI, phosphatidylinositol; DMEM, Dulbecco’s modified Eagle’s medium; TIR, transferrin receptor; HA, hemagglutinin; BSA, bovine serum albumin; PBS, phosphate-buffered saline; MES, 2-(N-morpholino)ethanesulfonic acid; HDM, high density microsomes; LDM, low density microsomes; PM, plasma membrane; PE, phosphatidylethanolamine; TGFRα, transforming growth factor receptor-α.
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**Materials**—Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were obtained from Invitrogen. Insulin, 3-isobutyl-1-methylxanthine, Wortmannin, and cholera toxin B peroxidase conjugate were obtained from Sigma. Dexamethasone and PDGF-bb were purified from bovine serum were obtained from Invitrogen. Insulin, 3-isobutyl-1-methylxanthine, Wortmannin, and cholera toxin B peroxidase conjugate were obtained from Sigma.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**3T3-L1 mouse fibroblasts (American Type Culture Collection, Manassas, VA) were propagated and differentiated into adipocytes as described previously (28). The 3T3-L1 adipocytes used for all experiments were 6–12 days post-differentiation. Greater than 95% of the cells were differentiated as determined by observation of lipid droplets under light microscopy (≥10 objective).

**Insulin, Wortmannin, and PDGF-bb treatment—**The 3T3-L1 fibroblasts and adipocytes were grown in serum-free DMEM containing 0.5% bovine serum albumin (BSA) for 12 h prior to experiments. Cells were incubated with serum-free media containing insulin (100 nM) or PDGF-bb (50 ng/ml) for 10 min at 37°C. In some experiments, cells were treated with wortmannin (100 nM) for 15 min prior to insulin treatment. After washing with ice-cold phosphate-buffered saline (PBS) three times, the cells were subjected to subcellular fractionation or were used for isolation of caveolae/fractions.

**Isolation of Caveolae—**Preparation of caveolae fractions was performed as described previously (24, 26) with minor modifications. All steps were carried out at 4°C. Briefly, 3T3-L1 adipocytes (18 × 10⁶ cells) were washed three times with ice-cold PBS, collected in 2 ml of MEM buffer (25 mM MES, pH 6.5, 150 mM NaCl) containing 1 mM Na₂VO₃, 1 mM NaF, and protease inhibitor mixture. Cells were homogenized by passing through a chilled ball-bearing homogenizer (20 strokes). The cell homogenates were mixed with Triton X-100 to a final concentration of 1% (v/v), incubated on ice for 30 min, and then added to an equal volume of sucrose solution (30%, w/v; prepared in MEM buffer) prior to loading into a 12-ml ultracentrifuge tube for SV410 rotor (Beckman Instruments). The resulting sample (4 ml) was overlaid with 3 ml of 50% sucrose and 3.6 ml of 5% sucrose (in MEM buffer) and centrifuged for 18 h, 39,000 rpm, at 4°C. After centrifugation, 12 fractions (900 µl each) were collected from the top of the tubes. An equal aliquot of each fraction was resuspended in SDS sample buffer, and proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and detected by immunoblotting with proper antibodies.

**Subcellular Fractionation—**The high density microsomes (HDM) and low density microsomes (LDM) were separated from the plasma membrane (PM) and the nucleus/mitochondrial membranes according to published methods (35, 36) with minor modifications (34). The resulting PM, HDM, LDM and nucleus/mitochondria pellet were resuspended in each (500 µg/ml) with horseradish peroxidase-conjugated cholera toxin B subunit, a ligand for ganglioside GM1 enriched in the plasma membrane of caveolae/lipid rafts (37).

**Immunoprecipitation—**Pooled caveolae fractions were resuspended and lysed in IPB buffer (25 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, and 1 mM CaCl₂ containing 1% Triton X-100, 60 mM octylglucoside, 1 mM Na₂VO₃, 1 mM NaF, and protease inhibitor mixture). Samples were incubated at room temperature for 0.5 h at 4°C with constant rocking. Cleared lysates were incubated with the specified antibodies at 4°C overnight. Protein A-Sepharose beads (40 µl/0.1 g/ml slurry) were added to the samples and incubated at 4°C for an additional 2 h. The beads were sedimented by centrifugation and washed once with IPB buffer containing 1% Triton X-100, twice with IPB buffer containing 0.5% Triton X-100, and twice with IPB buffer alone. The immunoprecipitated proteins were eluted using SDS sample buffer, resolved by SDS-PAGE, and detected by immunoblotting.

**Preparation of Adenovirus Vector and Infection into 3T3-L1 Fibroblasts and Adipocytes—**The adenoviral expression plasmid pAdCMV-LRP1-IV was constructed by using the pAdEasy vector system (Q-BIOGEN). The signal sequence of the pAdCMV-LRP1-IV was transfected into HEK-293 cells, and the resulting virus particles were purified by ultracentrifugation in a CsCl gradient. The virus stock was titered with HEK-293 cells, and plaque counts were counted on day 8. The virus particles were used to infect 3T3-L1 fibroblasts or adipocytes at 100 plaque-forming units/cell. After incubation at 37°C for 1.5 h with agitation at 15-min intervals, additional serum-free DMEM was added for an overnight incubation. Serum-free medium was changed to complete medium after 24 h of infection. The complete medium was maintained for 24 h and then replaced with serum-free DMEM for 16 h prior to insulin treatment.

**Immunocytochemistry—**The AdCMV-LRP1-IV-infected 3T3-L1 fibroblasts and adipocytes (plated on coverslips) were treated with or without insulin for 10 min at 37°C. After treatment, cells were washed with IPB buffer fixed with ice-cold PBS (1% paraformaldehyde for 30 min). The cells were washed three times with 100 mM glycine and blocked with 1% BSA for 1 h prior to probing with a monoclonal anti-HA antibody. Cells were washed with ice-cold PBS and permeabilized with 0.1% Triton X-100 for 5 min on ice. The cells were blocked with 1% BSA for 1 h prior to probing with a polyclonal anti-caveolin-1 antibody, followed by goat anti-rabbit IgG conjugated with Alexa Fluor™ 488 and goat anti-rabbit IgG conjugated with Alexa Fluor™ 594. All solutions were prepared in 1× PBS buffer, and all incubations and washes were performed at 4°C. After immunostaining, the coverslips were mounted onto a glass slide using SlowFade Light AntiFade kits were obtained from Molecular Probes.
**RESULTS**

**Insulin Stimulates LRPI Association with Caveolae in 3T3-L1 Adipocytes**—We (34) and others (40, 41) showed previously that insulin acutely stimulated the translocation of LRPI from an intracellular membrane compartment to the cell surface. In the present study, we characterized further the effect of insulin treatment on LRPI distribution between PM, HDM, and LDM by immunoblot analysis. Representative blots typical of three independent experiments are shown in Fig. 1. Under basal conditions, ~21% (+1.4%, n = 3) of the total LRPI in 3T3-L1 adipocytes was present in the plasma membrane fraction; 44% (+3.8%) and 14% (+0.3%) were associated with HDM and LDM, respectively, and the remainder 22% (+1.9%) was found in the combined nucleus/mitochondria fractions (data not shown). Insulin treatment (100 nM, 10 min) increased plasma membrane-associated LRPI by ~188% (+11.6%) of basal, accounting for 43% (+5.0%) of the total LRPI. The increased LRPI associated with plasma membrane was accompanied by a marked decrease in LDM-associated LRPI. This result, in agreement with our previous observation (34), confirms relocation of LRPI from LDM to plasma membrane in 3T3-L1 cells treated with insulin.

The abundance of caveolae on the plasma membrane of 3T3-L1 cells (32, 33) prompted us to determine whether LRPI was associated with caveolae upon insulin treatment. To this end, we compared the two commonly used protocols for caveolae isolation, namely the detergent solubilization (24, 26) and the detergent-free (42, 43) methods to separate caveolae/lipid rafts from non-caveolae fractions in preliminary experiments, and we found that the detergent solubilization and sucrose density ultracentrifugation method gave consistent and reproducible separations of the lipid-laden homogenate of 3T3-L1 adipocytes. After centrifugation, the protein marker caveolin-1 and the lipid marker ganglioside GM1 of caveolae were found predominantly in low density fractions 4–7 (which were at the interface between 5 and 30% sucrose densities), whereas the non-caveolae protein marker TfR presented at the bottom of the sucrose gradient (i.e. fractions 10–12) (Fig. 2A). Because it has been shown previously that caveolin-1 and GM1 reside predominantly on the plasma membrane (37), the fractions 4–7 of the sucrose gradient most likely represent caveolae domains of plasma membrane origin. A small proportion of caveolin-1 was present in fractions 8–10, which probably represented non-plasma membrane intracellular species (44–46). Different phospholipid compositions between caveolae and non-caveolae membranes have been reported previously; choline-containing lipids in caveolae fractions are rich in saturated fatty acyl chains and PE are rich in unsaturated fatty acyl chains (47, 48). Tandem mass spectrometry analysis showed that in 3T3-L1 adipocytes, sphingomyelin (SM1 and SM4) and phosphatidylcholine with saturated fatty acyl chains (e.g. 14:0–16:0, 16:0–16:0, and 14:0–18:0) were enriched in caveolae fractions (i.e. fractions 4–6), whereas unsaturated fatty acyl chains (e.g. 16:1–16:1, 16:0–18:1, 16:0–18:2, 18:0–18:2, and 18:1–18:1) were enriched in non-caveolae fractions. On the other hand, phosphatidylethanolamine containing unsaturated fatty acyl chains, particularly at the sn-2 position, were enriched in the caveolae fractions. Together, the distribution of protein and lipid markers provides evidence for the authenticity of fractions 4–6 being bona fide caveolae. Thus fractions 4–6 were considered as caveolae and fractions 10–12 were considered as non-caveolae membranes in the following studies.

Having ascertained that the detergent solubilization method was suitable for isolating caveolae from 3T3-L1 adipocytes, we determined whether LRPI became caveolae-associated in response to insulin treatment. Under basal conditions, the majority of LRPI was present at the bottom of the sucrose gradient (Fig. 3A, fractions 10–12). Semi-quantification of LRPI on immunoblots by scanning densitometry showed that only a small proportion of total LRPI (2.6 ± 0.6%, n = 3) was associated with caveolae (fractions 4–6). Upon insulin treatment, the proportion of total LRPI associated with caveolae (12 ± 2.8%, n = 3) was increased by 4-fold as compared with basal conditions. A portion of LRPI was located in fractions 7–9 that were neither caveolae nor non-caveolae membrane fractions. This fraction might represent intracellular membrane-associated LRPI because it co-fractionated with an intracellular pool of caveolin-1. The response of LRPI to insulin was not a nonspecific movement of membrane proteins, because TIR and PDGF-R remained in non-caveolae membranes in cells treated with insulin (Fig. 3, B and C). We (34) and others (49) have shown that insulin increased TIR cell surface presentation by 2-fold. However, the cell surface presentation of TIR was not...
associated with caveolae localization in 3T3-L1 adipocytes treated with insulin (Fig. 3B).

The translocation of GLUT4 from LDM to the cell surface is an extensively studied metabolic effect of insulin in 3T3-L1 adipocytes (50, 51). Previous studies showed that the magnitude of insulin-stimulated cell surface presentation of LRP1 (2-fold) (34) was less than that of GLUT4 (8-fold) (52). We compared the effect of insulin on caveolae localization between LRP1 and GLUT4, and we found that the magnitude of insulin-stimulated caveolae association of GLUT4 (2-fold) (Fig. 3D) was less than that of LRP1 (4-fold) (Fig. 3A). The relatively low increase in caveolae localization of GLUT4 upon insulin treatment has also been observed by others (53). The data shown in Fig. 1 and Fig. 3 together indicate that LRP1 exhibits a unique response to insulin in caveolae localization that is distinct from TfR, PDGFR, and GLUT4.

**Opposing Effects of Insulin and PDGF-bb on Caveolae Localization of LRP1—**To gain an insight into the mechanisms that are responsible for insulin-stimulated caveolae localization of LRP1, we compared the effect of insulin with that of PDGF-bb. It has been shown that PDGF-bb not only elicit signaling through phosphorylation of its cognate receptor tyrosine kinase PDGFR but also bind specifically to LRP1 (18). Moreover, activation of PDGFR is required for LRP1 phosphorylation in human fibroblasts (18, 21). Thus, the caveolae localization of LRP1 and PDGFR was also studied. PDGFR was associated
with non-caveola membranes under basal conditions (Fig. 4A, top panel). Treatment of the cells with PDGF-bb (50 ng/ml, 10 min) resulted in rapid receptor tyrosine phosphorylation, and the phosphorylated PDGFR migrated to caveola as detected by anti-phosphotyrosine antibody 4G10 (Fig. 4A, middle panel). This result agrees with previous findings that PDGF-bb causes tyrosine phosphorylation and caveola localization of the phosphorylated receptor (54). Insulin treatment did not stimulate PDGFR association with caveola (Fig. 4A, bottom panel) nor did it cause tyrosine phosphorylation of PDGFR (data not shown). Treatment with PDGF-bb did not stimulate but rather decreased LRP1 association with caveola to below basal levels (Fig. 4B, compare top two panels). The opposing effect on LRP1 caveola association between insulin and PDGF-bb is reminiscent of hormone selectivity in GLUT4 response: translocation of GLUT4 from LDM to plasma membrane in 3T3-L1 adipocytes has been shown to respond only to insulin but not PDGF-bb (55). The insulin-induced LRP1 association with caveola was sensitive to PI 3-kinase inhibition, because pretreatment with wortmannin (100 nM) for 15 min prior to insulin totally blocked the insulin effect (Fig. 4B, compare bottom two panels).

It has been shown that during 3T3-L1 cell differentiation, the expression of caveolin-1 and the amount of caveola on the plasma membrane are markedly elevated (32, 33). Thus, consideration was given to the possibility that increased caveola association of LRP1 in 3T3-L1 adipocytes might be attributable to the abundant expression of caveolin-1. To rule out that the insulin-stimulated caveola localization of LRP1 was concurrent with caveola formation, we determined the distribution of caveolin-1 between caveola and non-caveola membranes under conditions identical to those used for LRP1. Fig. 4C shows that the majority of caveolin-1 was found in fractions 4–6, and the distribution of caveolin-1 was static and largely unchanged under different treatment conditions (i.e. PDGF-bb, insulin, or wortmannin prior to insulin). These results suggest strongly that association or dissociation of LRP1 with caveola upon insulin or PDGF-bb treatment is unrelated to caveola-1 distribution in 3T3-L1 adipocytes. These results also rule out the possibility that the wortmannin-blocked caveola association of LRP1 is attributable to disassembly of caveola or redistribution of caveolin-1 upon PI 3-kinase inhibition.

To determine further the signaling events that associated with insulin-stimulated caveola localization of LRP1, we compared the effects of insulin and PDGF-bb on insulin receptor substrate 1 (IRS-1) phosphorylation. As expected, insulin treatment resulted in stimulation of IRS-1 tyrosine phosphorylation, whereas PDGF-bb did not (Fig. 4D). The majority of phosphorylated IRS-1 upon insulin treatment was not associated with caveola and remained in non-caveola fractions. Tyrosine phosphorylation of IRS-1 in 3T3-L1 adipocytes was minimally affected by wortmannin, in agreement with previous data (56) suggesting that IRS-1 phosphorylation occurs upstream of PI 3-kinase activation. Neither insulin nor PDGF-bb induced LRP1 β-chain tyrosine phosphorylation in differentiated 3T3-L1 adipocytes (data not shown). These results suggest that the PI 3-kinase pathway is involved in the insulin-stimulated caveola localization of LRP1 in differentiated 3T3-L1 adipocytes.

Insulin Stimulates LRP1 Association with Caveola in 3T3-L1 Fibroblasts—We next inquired into the origin of caveola-associated LRP1 in response to insulin treatment. The caveola-localized LRP1 may originate either from an intracellular pool through membrane translocation or else from a cell surface-associated pool through lateral transmigration along the plane of plasma membrane. To distinguish between these two possibilities, we examined the effect of insulin treatment on caveola localization of LRP1 using 3T3-L1 fibroblasts. In these cells, translocation of LRP1 from intracellular membranes to the cell surface was absent upon insulin treatment (Fig. 5A), probably because of the absence of an LDM-associated LRP1 pool. However, treatment of the fibroblasts with insulin did stimulate caveola localization of LRP1 (Fig. 5B, middle panel), although the level of caveola-associated LRP1 in fibroblasts was lower than that in adipocytes. The acute response of LRP1 to insulin treatment in fibroblasts was also sensitive to PI 3-kinase inhibition (Fig. 5B, bottom panel). Analysis of caveolin-1 showed that under basal conditions caveolin-1 was largely associated with non-caveola fractions, and a considerable amount of caveolin-1 remained in non-caveola fractions even after insulin treatment (Fig. 5C).

The insulin-stimulated caveolin-1 association with caveola could be prevented by wortmannin treatment (Fig. 5C, bottom panel). Thus, distribution of caveolin-1 and its association with caveola in fibroblasts are significantly different from those in adipocytes. However, as was found in adipocytes, the insulin-induced phosphorylation of IRS-1 was insensitive to wortmannin treatment (Fig. 5D). Because there is no translocation of LRP1 from intracellular membranes to the cell surface in fibroblasts, these results shown in Fig. 5 suggest that caveola association of LRP1 in 3T3-L1 cells is achieved through lateral transmigration of LRP1 along the plane of the plasma membrane.

Immunocytochemistry Studies of LRP1 and Caveolin-1—We attempted to visualize LRP1 and caveolin-1 located within caveola by using immunofluorescence microscopy. To this end, we first performed double immunofluorescent studies with...
3T3-L1 fibroblasts. Because the epitopes for anti-LRP1 β-chain or anti-caveolin-1 antibody reside on the cytosolic side of the PM, the experiments were performed with cells under permeabilizing conditions. Under basal (Fig. 6A, panels a and b) and insulin-treated (data not shown) conditions, a fraction of LRP1 was co-localized with caveolin-1 on the edge of the cells. However, extensive staining of intracellular LRP1 and caveolin-1 occurred under the permeabilizing conditions (Fig. 6A, panels a and b).
Insulin Stimulates Caveolae Localization of LRP1

**Fig. 6. Immunocytochemistry analysis of LRP1 and caveolin-1.** A, double immunofluorescence staining of LRP1 β-chain and caveolin-1 in permeabilized 3T3-L1 fibroblasts under basal conditions using a polyclonal anti-LRP1 β-chain antibody and a monoclonal anti-caveolin-1 antibody. Alexa Fluor 594 (goat anti-rabbit) and Alexa Fluor 488 (goat antimouse) were used to detect LRP1 (green) and caveolin-1 (red), respectively. The boxed areas in the merged images (panel a) are shown at higher magnification (×2.3) (panels b–d). B, double immunofluorescence staining of LRP1-IV-HA and caveolin-1 in transfected 3T3-L1 fibroblasts (panels a–h) and adipocytes (panels i–p). The LRP1-IV-HA was labeled with a monoclonal anti-HA antibody under non-permeabilizing conditions, and caveolin-1 was labeled with a polyclonal anti-caveolin-1 antibody after permeabilization. Images were captured and presented as described in A. Scale bar, 5 μm.

and b), which interfered with clear visualization of LRP1/caveolin-1 on the cell surface. To circumvent this problem, we infected the cells (both fibroblasts and adipocytes) with an LRP1 minireceptor (pAdCMV-LRP1-IV), namely LRP1-IV-HA, that contained an HA epitope tag at the N terminus. Thus, detection of cell surface-associated LRP1 minireceptor could be achieved by using an anti-HA antibody under non-permeabilizing conditions, which ensured that only cell surface but not intracellular LRP1 was labeled. In both infected fibroblasts and adipocytes, the tagged LRP1 minireceptor was presented on the cell surface (Fig. 6B, panels c, g, k, and o). A small fraction of the LRP1 minireceptor exhibited co-localization with caveolae in fibroblasts under basal conditions (Fig. 6B, panels a and b), with a slight increase in co-localization upon insulin treatment (panels e and f). In adipocytes where caveolin-1 became abundantly expressed on the plasma membrane (panels l and p) (32), a more pronounced increase in LRP1 minireceptor/caveolin co-localization was observed upon insulin treatment (compare panels i and j with panels m and n).

To ascertain that LRP1 indeed forms complexes with caveolin-1 in caveolae, we performed co-immunoprecipitation experiments with an anti-LRP1 β-chain antibody, caveolin-1 could be co-immunoprecipitated from caveolae in 3T3-L1 adipocytes. At concentrations of the antibody used to precipitate a similar amount of the LRP1 β-chain between cells treated with nothing (basal) and insulin (Fig. 7A, bottom panel on left), the amount of caveolin-1 that was co-immunoprecipitated with anti-LRP1 β-chain antibody was markedly elevated in cells treated with insulin (Fig. 7A, top panel on left). Because data shown in Fig. 4C have indicated that the amount of caveolin-1 present in caveolae was not increased in 3T3-L1 adipocytes treated with insulin, the elevated amount of caveolin-1 co-immunoprecipitated with LRP1 β-chain thus likely represented enhanced complex formation between LRP1 and caveolin-1. The amount of LRP1 β-chain precipitated and the amount of caveolin-1 co-immunoprecipitated with anti-LRP1 β-chain antibody in cells treated with PDGF-bb were below basal levels (Fig. 7A, left). The decreased amount of caveolin-1 co-immunoprecipitated with LRP1 β-chain was most likely attributable to dissociation of LRP1 from caveolae in response to PDGF-bb treatment but not due to disassembly of caveolin-1 from caveolae (Fig. 7A, top panel on right, also see Fig. 4, B and C). Attempts to co-immunoprecipitate LRP1 β-chain by using an anti-caveolin-1 antibody (BD Biosciences) from the caveolae fractions of 3T3-L1 adipocytes were unsuccessful (Fig. 7A, bottom panel on right), which could either be attributable to epitope shielding as a result of LRP1 β-chain-caveolin-1 complex formation or due to the fact that the amount of caveolin-1 in caveolae was in excess compared with that of LRP1.

**DISCUSSION**

To date, two major cell surface receptor proteins are known to localize within caveolae/lipid rafts: the receptor tyrosine kinases and the G protein-coupled receptors (57) that play important roles in signal transduction. Recent studies with apoER2 (20), LRP1 (21), and LDLR (22) have suggested that
members of the LDLR family may also have the capacity to associate with caveolae/lipid rafts, although the role of these LDLR family members in signal transduction has not yet been established. The data presented in this report demonstrate that insulin treatment not only stimulates LRP1 to the cell surface but also causes localization of LRP1 to caveolae in 3T3-L1 adipocytes. In undifferentiated 3T3-L1 fibroblasts, insulin treatment also resulted in LRP1 migration into caveolae, even though the translocation of LRP1 from intracellular membranes to the cell surface was absent. Thus in 3T3-L1 fibroblasts, the caveolae localization of LRP1 is achieved mainly through lateral transmigration along the plane of plasma membrane. Migration of LRP1 into caveolae shows hormone selectivity, in that it responds only to insulin but not to PDGF-bb. Moreover, insulin and PDGF-bb exert opposing effects on LRP1 association with caveolae; thus insulin enhances whereas PDGF-bb reduces LRP1 caveolae localization. The difference between insulin and PDGF-bb effects is indicative of a distinct mode of action for the two hormones, whereas insulin activates the PI 3-kinase pathway through phosphorylation of IRS-1, PDGF-bb selectively activates its cognate tyrosine kinase receptor. Results from co-immunoprecipitation experiments suggest a physical interaction (direct or indirect) between the LRP1 β-chain and caveolin-1. These studies provide strong evidence that LRP1, the largest member of the LDLR gene family, can become caveolae-associated in 3T3-L1 fibroblasts and adipocytes in an insulin-regulated manner.

Association of LRP1 with caveolae upon insulin stimulation demonstrates an exquisite compartmentalization of the receptor between three cell surface compartments, caveolae, bulk plasma membrane, and clathrin-coated pits (Fig. 8). The present study suggests that caveolae-associated LRP1 can originate from bulk plasma membrane and may also be derived from intracellular vesicular membrane fractions, such as the LDM (58). Both the transmigration of LRP1 from the bulk plasma membrane to caveolae and the translocation of LRP1 from LDM fraction to plasma membrane are dependent on PI 3-kinase activity. The mechanism by which LRP1 migrates into caveolae is currently unclear. In previous (34) and the present studies, we compared cell surface presentation of LRP1 in response to insulin with that of GLUT4. In 3T3-L1 adipocytes, the differentiation-dependent translocation of GLUT4 from LDM to the cell surface upon insulin treatment has been studied extensively, and multiple pathways have been postulated to explain this process. It has been shown that both PI 3-kinase-dependent and PI 3-kinase-independent pathways exist; the latter occurs within caveolae and involves an array of proteins including the kinase Fyn (that phosphorylates caveolin-1), the adaptor protein CAP, the insulin-receptor substrate Cb1, the G protein TC10, and the Rho-GTPase-activating protein (59–62). Phosphorylation of caveolin-1 in 3T3-L1 cells is also differentiation-dependent (63, 64) and is specific for insulin but not observed with PDGF-bb treatment (63). Moreover, evidence of microtubule-dependent (65, 66) and microtubule-independent mechanisms (66, 67) for GLUT4 translocation has been reported. It is thus likely that multiple mechanisms/pathways may also be present in 3T3-L1 adipocytes for insulin-stimulated LRP1 plasma membrane presentation and caveolar localization. Regardless of the mechanism, data from the current study demonstrate that the LRP1 migration into caveolae in response to insulin treatment shows features that are distinct from TIR, PDGFR, and GLUT4.

Compartmentalization of cell surface receptors in specialized membrane microdomains confers important function (68). The significance of LRP1 caveolae localization in response to insulin treatment has yet to be determined. Presentation of membrane proteins within different microdomains has been ob-

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**Fig. 7.** Immunoprecipitation of LRP1 and caveolin-1 protein complex. Caveolae fractions were isolated from 3T3-L1 adipocytes treated with none (basal), insulin, or PDGF-bb as in Fig. 4. After cell homogenization, detergent solubilization, and sucrose density ultracentrifugation, the caveolae were pooled from fractions 4–6 and lysed. The cleared lysates were subjected to immunoprecipitation (IP) with a polyclonal anti-LRP1 β-chain antibody (A) or a polyclonal anti-caveolin-1 antibody (B). The immunocomplexes were recovered with protein A-Sepharose beads, and proteins were resolved by SDS-PAGE and detected by immunoblot (IB) analysis using the indicated antibodies.

**Fig. 8.** A model of LRP1 compartmentalization in 3T3-L1 cells. Insulin stimulates translocation of LRP1 to the plasma membrane from an intracellular membrane compartment (i.e., LDM) through a PI 3-kinase-dependent pathway (34). Insulin also stimulates migration of LRP1 into caveolae, which requires the activity of PI 3-kinase as well. The caveolae localization is achieved through lateral transmigration of the receptor along the plane of plasma membrane in 3T3-L1 fibroblasts, and may be also achieved via translocation from an intracellular pool in 3T3-L1 adipocytes (indicated by the question mark).
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served for other receptor tyrosine kinases, transporters, and endocytic receptors. Studies with GLUT4 have suggested that internalization of this transporter can be achieved through caveolae (non-clathrin pathway) (67) and also through a pathway that requires both caveolae and caveolin-coated pits (69). Studies with transforming growth factor-β (TGFβ) have shown that the receptor resides in both non-raft and raft domains of the plasma membrane, and internalization of TGFβR can be achieved via the clathrin- or raft-dependent pathways with different consequences. Whereas internalization of TGFβR through clathrin-coated pits elicits signal transduction, internalization of TGFβR via the caveosomes results in accelerated degradation of the receptor (70). It has been suggested that protein localization to different microdomains may confer different biological functions. The function of caveola-localized LRP1 in 3T3-L1 adipocytes is being investigated in our laboratory.

We have confirmed the physical interaction between caveolin-1 and LRP1 in 3T3-L1 adipocytes by co-immunoprecipitation experiments. However, the amino acid sequences within LRP1 that are responsible for targeting LRP1 to caveolae have not yet been identified. Our data showing that the HA-tagged minireceptor LRP1-IV-HA responded to insulin and became internalized in transfected 3T3-L1 adipocytes suggest that the caveolae targeting information likely resides within the LRP1 β-chain. Proteins found within caveolae/raft lipid have a number of structural features, such as the glycosylphosphatidylinositol anchor (71, 72), the transmembrane domain (71, 73), fatty acylation (i.e. palmitoylation and myristoylation) (74), and cholesterol binding properties (75). Formation of a cholesterol and sphingolipid-rich lipid shell may attract and encape proteins within caveolae (68). Direct protein-protein interactions have also been observed in the localization of receptor tyrosine kinases within caveolae, which requires a “caveolin-binding motif” that recognizes and directly binds to caveolin-1 (ΔXΔXXXΔXXΦ, ΔXXXΔXXΦ, or ΔXXΔXXXΔXXΦ, where Φ = Phe, Tyr, or Trp, and Φ is any amino acid) (76). These motifs occur in the cytoplasmic tails of caveolin-1-binding proteins such as insulin receptor, PDGFR, endothelial nitric-oxide synthase, and EGFR (76). There are no caveolin-binding motif sequences within the cytoplasmic tail of LRP1. However, sequences resembling the caveolin-binding motif are found ~300 amino acid residues upstream of the transmembrane domain of the human LRP1. It remains to be determined if these extracellular caveolin-binding motif-like sequences are responsible for caveola targeting of LRP1. Recent mutagenesis studies (77) with the EGFR have suggested that the targeting information for lipid raft is located on the extracellular side of the EGFR very close to the membrane. The amino acid sequences within LRP1 that govern either the receptor retention within, or release from, caveolae is also currently being determined in our laboratory.

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