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The Low-Density Lipoprotein Receptor-Related Protein-1 Associates Transiently With Lipid Rafts

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Abstract The low-density lipoprotein receptor-related protein-1 (LRP-1) is a multifunctional receptor that undergoes constitutive endocytosis and recycling. To identify LRP-1 in lipid rafts, we biotin-labeled cells using a membrane-impermeable reagent and prepared Triton X-100 fractions. Raft-associated proteins were identified in streptavidin affinity-precipitates of the Triton X-100-insoluble fraction. PDGF β-receptor was identified exclusively in lipid rafts, whereas transferrin receptor was excluded. LRP-1 distributed partially into rafts in murine embryonic fibroblasts (MEFs) and HT 1080 cells, but not in smooth muscle cells and CHO cells. LRP-1 partitioning into rafts was not altered by ligands, including α_2-macroglobulin, platelet-derived growth factor-BB, and receptor-associated protein (RAP). To examine LRP-1 trafficking between membrane microdomains, we developed a novel method based on biotinylation and detergent fractionation. Association of LRP-1 with rafts was transient; by 15 min, nearly all of the LRP-1 that was initially raft-associated exited this compartment. LRP-1 in the Triton X-100-soluble fraction, which excludes lipid rafts, demonstrated complex kinetics, with phases reflecting import from rafts, endocytosis, and recycling. Potassium depletion blocked LRP-1 endocytosis but did not inhibit trafficking of LRP-1 from rafts into detergent-soluble microdomains. Our data support a model in which LRP-1 transiently associates with rafts but does not form a stable pool. Fluid movement of LRP-1 between microdomains may facilitate its function in promoting the endocytosis of other plasma membrane proteins, such as the urokinase receptor, which localizes in lipid rafts. J. Cell. Biochem. 96: 1021–1033, 2005.

Key words: low-density lipoprotein receptor-related protein-1; α_2-macroglobulin; platelet-derived growth factor; lipid rafts; clathrin

The low-density lipoprotein receptor-related protein (LRP-1) is a member of the LDL receptor family [Strickland et al., 2002]. LRP-1 is synthesized in the endoplasmic reticulum and cleaved by furin in the trans-Golgi to generate the mature form, which includes the 515-kDa heavy chain and an 85-kDa light chain [Willnow et al., 1996]. Two clusters of complement-like repeats in the LRP-1 heavy chain provide binding sites for most of the over 40 structurally and functionally diverse LRP-1 ligands [Neels et al., 1999; Strickland et al., 2002]. The LRP-1 heavy chain is anchored to the cell surface by noncovalent interactions with the light chain, which penetrates the plasma membrane and includes the intracytoplasmic tail [Strickland et al., 2002]. YxxL, NpxY, and dileucine motifs, in the light chain, function as clustering signals for receptor-mediated endocytosis in clathrin-coated pits [Li et al., 2000]. The more C-terminal of the two NPxY motifs also serves as a docking site for signaling-adaptor proteins, such as Dab-1, Fe65, Shc, and JIP-1/2 [Gotthardt et al., 2000].

Early hypotheses regarding the function of LRP-1 as an endocytic receptor were based on analogy to the LDL receptor, which localizes in clathrin-coated pits and undergoes endocytosis in the presence or absence of LDL [Goldstein et al., 1979]. The sequence, FxNPxY, is essential for LDL receptor clustering due to its direct interaction with clathrin [Chen et al., 1990; Kibbey et al., 1998]. Before LRP-1 was cloned, it was clear that LRP-1 ligands, such as activated α_2-macroglobulin (α_2M), undergo rapid endocytosis in vitro and in vivo [Pizzo and Gonias, 1984]. Immunoelectron microscopy studies of vascular smooth muscle cells (VSMCs) demonstrated LRP-1 located exclusively in

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clathrin-coated pits [Weaver et al., 1996], like the LDL receptor; however, we now understand that members of the LDL receptor gene family differ widely in properties. For example, in ApoER2, PxxP motifs make this receptor unable to cluster in clathrin-coated pits or undergo endocytosis [Sun and Soutar, 2003]. Instead, ApoER2 is found largely in lipid rafts [Riddell et al., 2001]. SorLA/LR11 and LRP-1 bind similar ligands; however, due to slow endocytosis, SorLA/LR11 may actually antagonize LRP-1 activity [Gliemann et al., 2004].

Boucher et al. [2002] identified LRP-1 in lipid rafts/caveolae in fibroblasts. LRP-1 was also identified in rafts in 3T3-L1 adipocytes treated with insulin [Zhang et al., 2004]. Although these results stand in apparent contradiction with our earlier immunoelectron microscopy studies of VSMCs [Weaver et al., 1996], localization of LRP-1 in lipid rafts helps explain tyrosine-phosphorylation of the light chain by platelet-derived growth factor-BB (PDGF-BB) [Boucher et al., 2002; Loukinova et al., 2002]. Furthermore, the presence of LRP-1 in rafts explains the formation of complexes in which LRP-1 is bridged by a common ligand to the urokinase receptor (uPAR), a glycosyl-phosphatidylinositol (GPI)-anchored protein known to localize in rafts [Conese et al., 1995; Weaver et al., 1997].

Lipid rafts are membrane microdomains enriched in cholesterol and sphingolipids in the outer leaflet and in cholesterol in the inner leaflet [Munro, 2003]. The cholesterol imposes a degree of rigidity on the phospholipid acyl chains so that the membrane assumes a structure referred to as liquid-ordered. Numerous proteins may cluster in lipid rafts, including GPI-anchored proteins, G protein-coupled receptors, and receptor tyrosine kinases, accounting for the potentially important role of rafts in cell-signaling [Simons and Toomre, 2000]. Lipid rafts may or may not be enriched in caveolin-1, the major structural protein found in the anatomic structure referred to as a caveolae. Raft-associated caveolin-1 may function in maintaining high cholesterol levels [Liu et al., 2002].

In this study, we compared LRP-1 localization in lipid rafts in a number of cells lines. Our method was based on the insolubility of raft-associated proteins in Triton X-100, but used surface-protein biotinylation and affinity-precipitation to analyze cell-surface proteins without interference from intracellular pools. PDGF-β receptor was identified entirely in the detergent-insoluble fraction, consistent with its known location in lipid rafts [Liu et al., 1996]. Transferrin receptor was identified entirely in the detergent-soluble fraction, consistent with its known location in clathrin-coated pits [Jing et al., 1990]. LRP-1 distribution into plasma membrane microdomains was cell-type specific. In murine embryonic fibroblasts (MEFs) and HT 1080 cells, LRP-1 distributed partially into lipid rafts; however, no LRP-1 was identified in rafts in CHO cells and VSMCs.

Because LRP-1 was identified in lipid rafts in MEFs, we applied our surface-protein biotinylation/detergent solubility method to test whether raft-associated LRP-1 forms a distinct and isolated pool of receptor. Our results demonstrate that LRP-1 associates with rafts transiently, due to rapid migration within the plasma membrane to detergent-soluble microdomains. LRP-1 that is exported from rafts probably transfers to clathrin-coated pits, where endocytosis occurs.

MATERIALS AND METHODS

Reagents

α_2M was purified from human plasma by the method of Imber and Pizzo [1981] and activated, to form the LRP-1-recognized form, by reaction with methylamine. Receptor-associated protein (RAP) was expressed as a glutathione-*S*-transferase (GST) fusion protein [Webb et al., 1995]. The activity of GST-RAP was confirmed by inhibition of activated α_2M-binding to LRP-1. Monoclonal antibody 11H4, which recognizes the LRP-1 light chain, was purified from hybridoma-conditioned medium. PDGF β-receptor-specific polyclonal antibody and clathrin heavy chain-specific polyclonal antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Human transferrin receptor-specific antibody was from ZYMED (South San Francisco, CA). Caveolin-1-specific antibody was from BD Biosciences (Franklin lakes, NJ). The biotinylation reagents, sulfo-NHS-LC-biotin and sulfo-NHS-SS-biotin, were from Pierce (Rockford, IL). Nystatin was from Calbiochem (La Jolla, CA). PDGF-BB was from R&D Systems (Minneapolis, MN).

Cell Culture

MEFs were obtained from the ATCC and cultured in DMEM with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. LRP-1

expression and activity in these cells, previously referred to as MEF-1 cells, has been described [Weaver et al., 1997]. To deplete potassium, MEFs were incubated in DMEM:water (1:1) for 5 min followed by potassium-depleted medium (20 mM HEPES, pH 7.5, 140 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5.5 mM glucose) for 1 h at 37°C. HT 1080 fibrosarcoma cells were obtained from the ATCC and cultured in MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. VSMCs were isolated from rat aortas as previously described [Geisterfer et al., 1988]. The VSMCs were cultured in DMEM/F12 supplemented with 10% FBS, 0.68 mM L-glutamine, 100-U/ml penicillin, and 100 μg/ml streptomycin. CHO-K1 cells and LRP-1-deficient CHO 13-5-1 cells [FitzGerald et al., 1995] were cultured in Ham's F-12 medium, supplemented with 5% FBS optimized for CHO cells (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Solubility-Based Fractionation of Plasma Membrane Proteins

Cell-surface proteins in confluent cultures were biotin-labeled with the membrane-impermeable reagent, sulfo-NHS-LC-biotin, at 4°C, as previously described [Wu et al., 2004]. The cells were detached by scraping and extracted in 1% Triton X-100 containing protease inhibitors for 30 min at 4°C. The extracts were subjected to centrifugation at 12,000*g* for 20 min at 4°C. Supernatants were collected as the Triton X-100-soluble fraction. The Triton X-100-insoluble pellets were re-extracted in RIPA buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors). The two detergent extracts were subjected to affinity precipitation with streptavidin–sepharose (Amersham Biosciences, Uppsala, Sweden). Precipitates and extracts were subjected to SDS–PAGE and immunoblot analysis. To sequester cholesterol, cultures were treated with Nystatin (100 μg/ml) for 1 h at 37°C.

Resolution of Membrane Fractions by Sucrose Gradient Ultracentrifugation

Lipid raft-rich and -depleted membrane fractions were resolved by sucrose density gradient ultracentrifugation, as described [Brown and Rose, 1992; Sargiacomo et al., 1993], with minor modifications. All steps were executed at 4°C. Briefly, cells were washed three times with 20 mM sodium phosphate, 150 mM NaCl, pH 7.4, collected by scraping into MBS buffer (25 mM 2-[*N*-morpholino] ethanesulfonic acid, 150 mM NaCl, pH 6.5 containing protease inhibitor cocktail), fragmented by passage ten times through a 25-G needle, combined with Triton X-100 to a final concentration of 1% (v/v), and incubated on ice for 30 min. This preparation (0.75 ml) was mixed with 90% (w/v) sucrose in MBS (0.75 ml), overlayed with 1.5 ml each of 35% and 5% (w/v) sucrose, and subjected to centrifugation at 40,000 rpm in a Beckman 50.1 rotor for 18 h at 4°C. After centrifugation, 11 fractions (0.4 ml each) were collected, beginning from the top of each tube. An equal aliquot of each fraction was subjected to immunoblot analysis.

LRP-1 Endocytosis From the Plasma Membrane

MEF LRP-1 endocytosis in the absence of ligand was studied using the reducible biotinylation reagent, sulfo-NHS-SS-biotin, as described [Weaver et al., 1996]. Cell-surface proteins were labeled with sulfo-NHS-SS-biotin (0.5 mg/ml) for 30 min at 4°C. The cells were transferred to 37°C for the indicated times, rapidly chilled back to 4°C, and then incubated in 10% FBS with or without glutathione (15.5 mg/ml) for 90 min at 4°C. Cultures were washed with iodoacetamide (5 mg/ml) to neutralize residual glutathione and then with PBS. Cell extracts were prepared in RIPA buffer and subjected to affinity-precipitation with streptavidin–sepharose. The affinity precipitates were subjected to immunoblot analysis.

For each time-point, two samples were analyzed, representing cultures that were incubated at 37°C and then treated with glutathione or vehicle. Glutathione functions as a reductant, dissociating the biotin marker from cellular proteins, but is not membrane permeable. Thus, in the glutathione-treated samples, affinity-precipitated (biotinylated) LRP-1 was interpreted as having entered an endosome or similar glutathione-resistant intracellular vesicle. Immunoblots were analyzed by densitometry. The intensity of the band from the culture that was not treated with glutathione is x, representing total biotin-labeled LRP-1. The intensity of the band from the culture that was treated with glutathione is y. The efficiency of the glutathione-reduction reaction (z) was

determined using cultures that were not warmed to 37°C as: $z = (x - y)/x$, and was typically 0.9–1.0. For samples that were warmed to 37°C, the fraction of biotinylated LRP-1 remaining on the cell surface (S) was determined as: $S = (x - y)/zx$.

LRP-1 Transfer Between Plasma Membrane Microdomains

MEFs were labeled with sulfo-NHS-SS-biotin and warmed to 37°C for various periods of time. After re-chilling to 4°C and treatment with glutathione or vehicle, cultures were extracted in sequence with cold Triton X-100 and RIPA buffer. The two detergent extracts from the glutathione-treated cultures and the two from the vehicle-treated cultures were separately affinity-precipitated with streptavidin–sepharose (four affinity precipitates per time point). The precipitates were subjected to immunoblot analysis for LRP-1. Thus, in all cases, this method detects biotinylated LRP-1. Band intensity was compared by densitometry. In these studies, changes in cell-surface LRP-1 in a specific detergent-extract may reflect endocytosis or transfer between plasma membrane microdomains.

For each incubation time at 37°C (t), we calculated S_t/S_0, which is the ratio of cell-surface LRP-1 in a specific detergent fraction at time = t against that present in the same detergent fraction at time = 0, according to the equation: $S_t/S_0 = (x_t - y_t)/zx_0$. x_t is the intensity of the band representing cells that were not treated with glutathione in the specified detergent fraction at time = t. y_t is the intensity of the band representing cells that were treated with glutathione and isolated in the same detergent fraction at the same time.

RESULTS

Localization of LRP-1 in Plasma Membrane Microdomains

Proteins in lipid rafts/caveolae typically demonstrate insolubility in cold Triton X-100 and low buoyant density in sucrose gradients [Sargiacomo et al., 1993; Smart et al., 1995]. By immunoelectron microscopy, we identified LRP-1 entirely in clathrin-coated pits in VSMCs [Weaver et al., 1996]; however, using a biochemical approach, Boucher et al. [2002] identified LRP-1 in caveolae in human fibroblasts. In 3T3-L1 adipocytes, LRP-1 was found primarily outside lipid rafts/caveolae; however, insulin treatment increased the proportion of LRP-1 in rafts [Zhang et al., 2004].

In this study, we labeled cell-surface proteins with a membrane-impermeable biotinylation reagent. Cells were extracted sequentially with Triton X-100 and RIPA buffer and the extracts were affinity-precipitated with streptavidin–sepharose. In MEFs, biotinylated transferrin receptor was present exclusively in the detergent-soluble fraction (Fig. 1A), consistent with its known localization in clathrin-coated pits [Jing et al., 1990]. Biotinylated PDGF β-receptor was present exclusively in the detergent-insoluble fraction, consistent with its reported localization in caveolae/lipid rafts [Liu et al.,

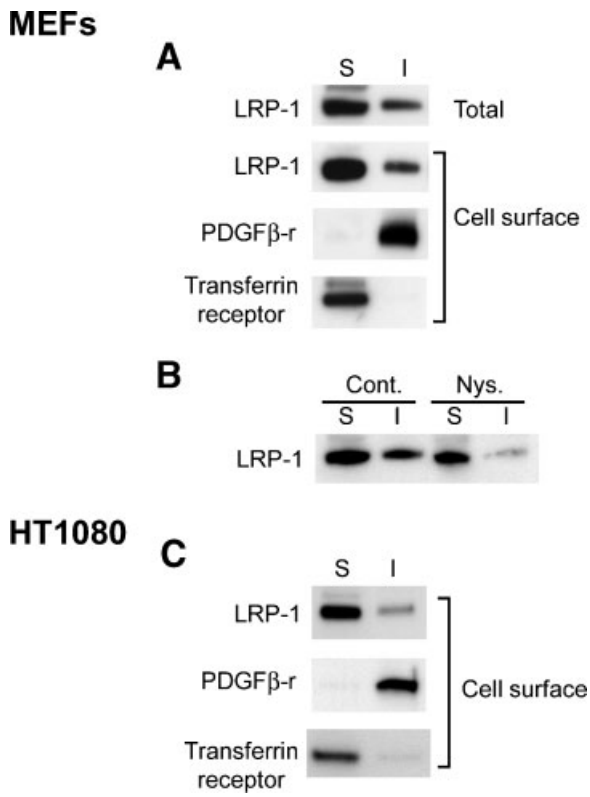

Fig. 1. Lipoprotein receptor-related protein-1 (LRP-1) distribution into lipid rafts in murine embryonic fibroblasts (MEFs) and HT1080 cells. **A**: MEFs were biotin-labeled. Triton X-100-soluble (S) and -insoluble (I) fractions were prepared. Extracts from the same number of cells were compared by immunoblot analysis with antibody 11H4 (total) or subjected to streptavidin-affinity precipitation and immunoblot analysis (cell surface). **B**: MEFs were treated with 100 μg/ml nystatin (Nys) or vehicle (Cont). Triton X-100-soluble and -insoluble fractions were prepared and compared by immunoblot analysis. **C**: HT 1080 cells were biotin-labeled. Triton X-100-soluble and -insoluble fractions were prepared. Streptavidin-affinity precipitates were subjected to immunoblot analysis.

1996]. These results support the validity of our method for identifying proteins in plasma membrane microdomains.

LRP-1 in MEFs was present in both the detergent-soluble and -insoluble fractions at steady state conditions (soluble: $68 \pm 2\%$; insoluble: $32 \pm 2\%$, $n = 4$) (Fig. 1A). The majority of the LRP-1 was present in the detergent-soluble fraction. When total cellular LRP-1 was examined, instead of biotinylated LRP-1, again protein was identified in both fractions; however, this method does not control for the contribution of LRP-1 in intracellular compartments. As a further control, we treated cells with Nystatin, to sequester cholesterol and disrupt lipid rafts. The fraction of LRP-1 in the detergent-insoluble fraction decreased, as anticipated (Fig. 1B).

In HT 1080 fibrosarcoma cells, biotinylated transferrin receptor partitioned entirely into the detergent-soluble fraction and PDGF β-receptor partitioned into the detergent-insoluble fraction (Fig. 1C). LRP-1 was present in both fractions, although the majority of the LRP-1 was in the soluble fraction. Thus, in MEFs and HT 1080 cells, LRP-1 is unique amongst the three proteins examined in that it is present in both detergent extracts, consistent with a model in which LRP-1 partitions into both lipid rafts and clathrin-coated pits.

Previously our laboratory identified VSMC LRP-1 entirely in clathrin-coated pits by immunoelectron microscopy [Weaver et al., 1996], we examined the distribution of VSMC LRP-1 into membrane microdomains using the detergent fractionation/streptavidin-affinity precipitation method (Fig. 2A). In whole cell extracts, LRP-1 was identified in both the detergent-soluble and -insoluble fractions. Clathrin heavy chain was present exclusively in the detergent-soluble fraction. By contrast, in streptavidin-affinity precipitates, biotinylated LRP-1 was present exclusively in the detergent-soluble fraction. Equivalent results were obtained with CHO-K1 cells. Again, biotinylated cell-surface LRP-1 was detected exclusively in the detergent-soluble fraction (Fig. 2B). These results support our prior immunoelectron microscopy studies [Weaver et al., 1996] and suggest that LRP-1 does not partition into lipid rafts in VSMCs and CHO cells in vitro. The difference in results obtained when VSMCs were analyzed without affinity precipitation underscores the importance of examining cell-surface proteins independently of intracellular pools.

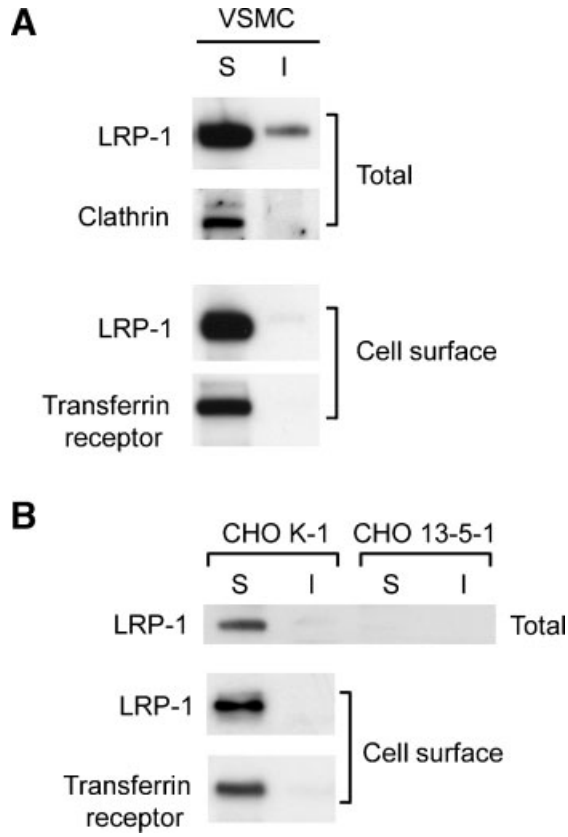

Fig. 2. LRP-1 distribution into lipid rafts in vascular smooth muscle cells (VSMCs) and CHO cells. VSMCs (**A**), CHO K1 cells, and CHO 13-5-1 cells (**B**) were biotin-labeled. Triton X-100-soluble (S) and -insoluble (I) fractions were prepared. Extracts from the same number of cells were compared by immunoblot analysis with 11H4 (total) or subjected to streptavidin-affinity precipitation followed by immunoblot analysis (cell surface).

Analysis of LRP-1 Localization in Membrane Microdomains by Buoyant Density Ultracentrifugation

Fragmented MEFs were suspended in Triton X-100 and subjected to sucrose density ultracentrifugation. We anticipated that cholesterol-rich membrane fragments would localize in low buoyant density fractions, whereas other membrane components would partition near the bottom of the centrifuge tube, together with incompletely disrupted cells and cell fragments. Figure 3A shows a representative experiment. Transferrin receptor was present near the bottom of the gradient, in high-density fractions. Caveolin partitioned mainly in low-density fractions, whereas PDGF β-receptor was recovered beginning in low-density fractions, but throughout the bottom of the gradient as well. Partial recovery of caveolin and PDGF

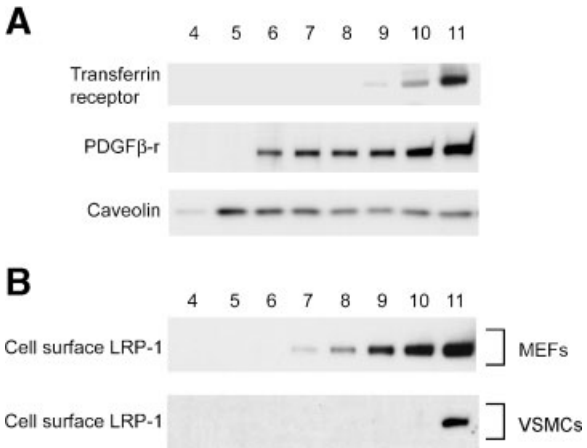

Fig. 3. Buoyant density analysis of LRP-1 in MEFs and VSMCs. **A**: Fragmented MEFs in Triton X-100 were subjected to sucrose density ultracentrifugation. Fractions were examined by immunoblot analysis. **B**: MEFs and VSMCs were biotin-labeled at 4°C. Cells were fragmented and subjected to sucrose density ultracentrifugation. Each fraction was streptavidin affinity-precipitated. The precipitates were analyzed by immunoblot analysis to detect LRP-1.

β-receptor in high-density fractions may represent incomplete cell fragmentation.

Next, we examined biotinylated LRP-1 in MEFs and VSMCs (Fig. 3B). Each fraction from the gradient was subjected to streptavidin-affinity precipitation, to isolate cell-surface protein, followed by immunoblot analysis. Figure 3B shows that MEF LRP-1 partitioned into fractions 7–11, whereas VSMC LRP-1 was recovered only in fraction 11, supporting the conclusion that LRP-1 may localize in lipid rafts in MEFs but not in VSMCs. The absence of biotinylated MEF LRP-1 in the lowest density fractions, where caveolin was observed, may reflect the fact that even in MEFs, most of the LRP-1 is present outside rafts. Alternatively, intermediate density fractions may reflect non-caveolar lipid rafts [Simons and Toomre, 2000; Mandal et al., 2005].

Regulation of LRP-1 Localization in Lipid Rafts

We tested whether ligands affect LRP-1 distribution into plasma membrane microdomains. No change was observed in MEFs that were treated with saturating concentrations of activated α_2M or RAP for 4 h at 37°C (Fig. 4A). We also tested PDGF-BB due to its effects on LRP-1 light chain phosphorylation [Boucher et al., 2002; Loukinova et al., 2002] and its reported ability to bind directly to LRP-1

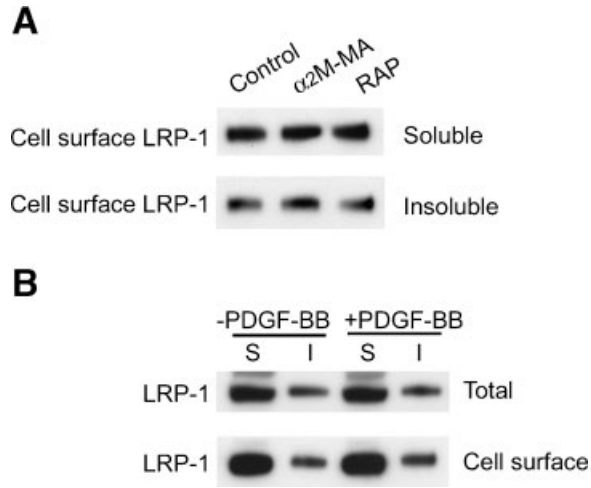

Fig. 4. Effects of ligands on LRP-1 localization in lipid rafts. **A**: MEFs were treated with methylamine-activated α_2M or GST-RAP for 4 h at 37°C. The cells were then biotin-labeled. Triton X-100-soluble and -insoluble fractions were prepared. Extracts were subjected to streptavidin-affinity precipitation followed by immunoblot analysis for LRP-1. **B**: MEFs were stimulated with PDGF-BB or vehicle for 30 min. Triton X-100-soluble (S) and -insoluble (I) fractions were prepared. Extracts were compared directly by immunoblot analysis with 11H4 (total) or affinity precipitates were prepared and subjected to immunoblot analysis (cell surface).

[Loukinova et al., 2002]. Treatment of MEFs with PDGF-BB for 30 min did not alter LRP-1 distribution in the plasma membrane (Fig. 4B). Thus, LRP-1 localization does not appear to be regulated by ligands.

LRP-1 Endocytosis in MEFs

Initially, we examined LRP-1 endocytosis in MEFs, without accounting for the distribution on LRP-1 into different membrane microdomains. Cell-surface proteins were labeled with a reducible biotinylation reagent. The cells were then warmed to 37°C. Intracellular LRP-1 was detected based on resistance of the biotin-marker to glutathione. A representative experiment is shown in Figure 5A. The total amount of biotinylated LRP-1 in cells that were not treated with glutathione remained unchanged as anticipated. Within 5 min at 37°C, substantial internalization of biotinylated LRP-1 was observed, as determined by an increase in glutathione-resistant receptor (Fig. 5A,B). The highest level of internalized LRP-1 was observed at 10 min; by that time, nearly 60% of the cell-surface LRP-1 had transferred to intracellular pools. Subsequently, the amount of internalized LRP-1 stabilized or decreased, suggesting LRP-1 recycling to the cell-surface.

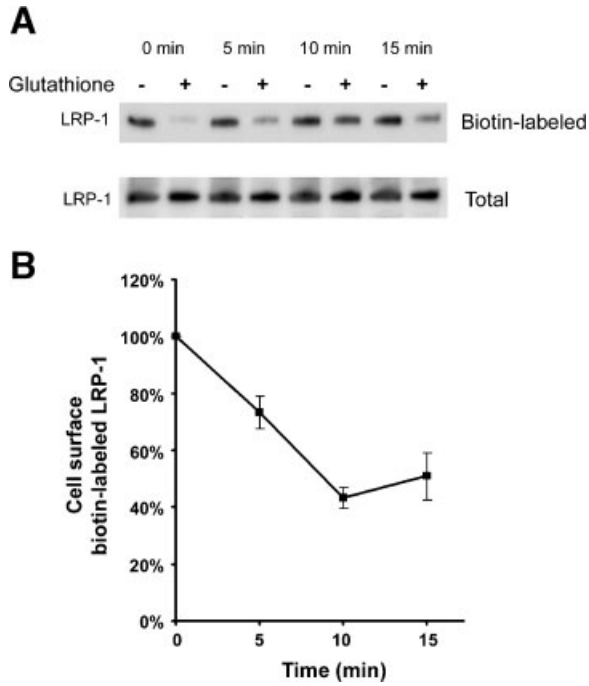

Fig. 5. LRP-1 endocytosis in MEFs. **A**: MEFs were labeled with sulfo-NHS-SS-biotin and incubated at 37°C for the indicated times. The cells were then rapidly chilled and treated with glutathione or vehicle. Extracts from the same number of cells were streptavidin-affinity precipitated and subjected to immunoblot analysis for LRP-1. As a control, immunoblot analysis of the original extracts prior to affinity precipitation (total) was performed. **B**: Cell-surface LRP-1 was determined as a function of time. The results of four separate experiments are averaged (mean ± SEM, n = 4).

Stability of LRP-1 in Lipid Rafts

Partial localization of LRP-1 in lipid rafts raised the question, "Does raft-associated LRP-1 represent a separate non-interacting pool of receptor?" To address this question, we developed a method for assessing LRP-1 translocation between membrane microdomains based on biotinylation and detergent fractionation. MEFs were labeled with the reductant-sensitive biotinylation reagent, sulfo-NHS-SS-biotin, incubated at 37°C for up to 15 min, chilled back to 4°C, treated with glutathione or vehicle, and extracted with Triton X-100 followed by RIPA buffer. The detergent extracts were subjected to affinity precipitation and immunoblot analysis for LRP-1. In these studies, cell-surface LRP-1 in a specific plasma membrane fraction (Triton X-100-soluble or -insoluble) may be affected by translocation between microdomains in the plasma membrane, endocytosis, or, late in the assay, recycling of biotinylated LRP-1 back to the cell surface. The amount of biotinylated LRP-1 in each cell-surface compartment was quantitated as described in Materials and Methods.

Figure 6A shows a representative experiment comparing total biotinylated LRP-1 and glutathione-resistant, biotinylated LRP-1 in the Triton X-100-soluble fraction, which is presumed to exclude lipid rafts. A progressive increase in the amount of glutathione-resistant, biotinylated LRP-1 was observed; however, in the first 5 min, the increase in the glutathione-resistant pool was offset by an increase in the total amount of biotinylated LRP-1, so that the level of cell-surface biotinylated LRP-1 remained unchanged ($P < 0.05$, $n = 5$) (Fig. 6B). By 10 min, there was a significant decrease in cell-surface biotinylated LRP-1, due to internalization. The level then stabilized or slightly recovered, probably due to recycling. As shown in Figure 6A, when total LRP-1 was examined by immunoblot analysis instead of biotinylated LRP-1 (no affinity precipitation step), no change was observed during the 15 min incubation. Thus, the graph shown in Figure 6B depicts a subpopulation of LRP-1 that was present at the cell surface when the cells were cultured at 4°C and biotinylated. Once the cells are transferred to 37°C, cell-surface, biotinylated LRP-1 may include protein that migrated out of the Triton X-100-insoluble pool.

Figure 6C shows a representative experiment tracking biotinylated LRP-1 in the detergent-insoluble fraction, which is presumed to include lipid rafts. The RIPA buffer extracts were prepared from the insoluble pellets of the cells shown in Figure 6A, however, the exposure time was extended in Figure 6C to allow visualization of the bands. Cell-surface, biotinylated LRP-1 was rapidly eliminated from the Triton X-100-insoluble fraction, primarily due to a decrease in the total amount of biotinylated LRP-1. By 15 min, there was also a reproducible increase in the glutathione-resistant, Triton X-100-insoluble fraction. Overall, nearly 50% of the biotinylated LRP-1 was eliminated from the Triton X-100-insoluble, cell-surface compartment by 5 min (Fig. 6D). By 15 min, biotinylated LRP-1 was entirely eliminated from this compartment.

Although we observed transfer of biotinylated LRP-1 from the Triton X-100-insoluble fraction to the -soluble fraction, the total amount of

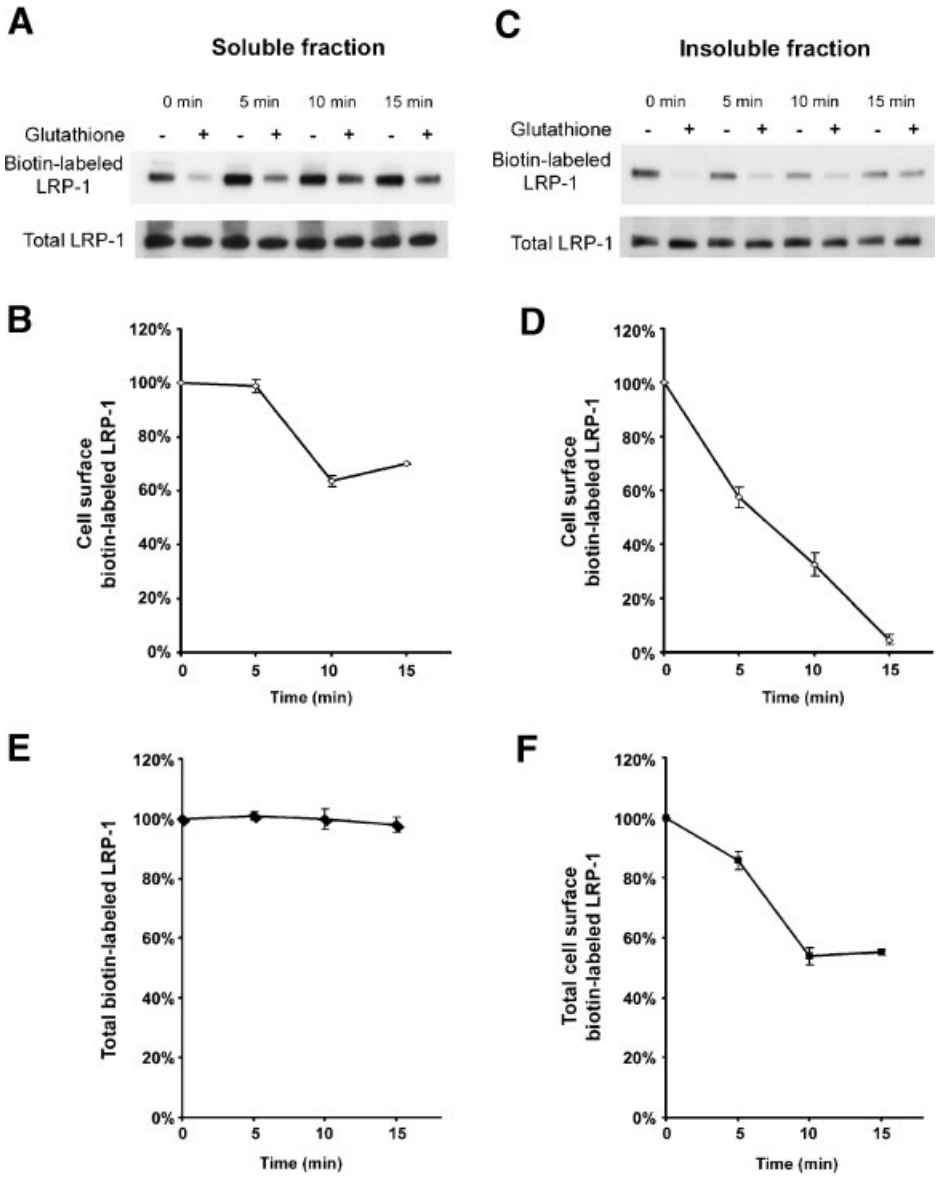

Fig. 6. LRP-1 stability in plasma membrane microdomains. MEFs were labeled with sulfo-NHS-SS-biotin and incubated at 37°C for the indicated time. The cells were then chilled to 4°C and treated with glutathione or vehicle. Triton X-100-soluble (**A**) and -insoluble (**C**) fractions were prepared and separately subjected to streptavidin-affinity precipitation and immunoblot analysis (biotin-labeled LRP-1). As a control, immunoblot analysis of the original extracts prior to affinity precipitation was performed (total LRP-1). Densitometry was performed. Biotinylated LRP-1 that remained at the cell surface (in the glutathione-sensitive fraction) is plotted against time for the Triton X-100-soluble fraction (**B**) and the Triton X-100-insoluble fraction (**D**). Each time point is plotted as a percent of that present in the same detergent fraction at time = 0. The results of five separate experiments were averaged (mean ± SEM, n = 5). **E**: The total amount of biotinylated LRP-1 in the Triton X-100-soluble and -insoluble fractions was added. Densitometry scans of equivalently exposed immunoblots were used for this purpose (note that the immunoblots shown in **panels A** and **C** were exposed differently). The results are expressed as a percentage of the level observed at time = 0 (mean ± SEM, n = 5). **F**: Cell-surface biotinylated LRP-1 in the Triton X-100-soluble and -insoluble fractions was added and plotted against that present at time = 0 (mean ± SEM, n = 5).

biotinylated LRP-1 recovered from the cells remained unchanged, as anticipated (Fig. 6E). Furthermore, when we calculated total cell-surface biotinylated LRP-1, recovered in both fractions collectively, the resulting graph reproduced that shown in Figure 5 (Fig. 6F). Taken together, our results suggest that LRP-1 associates transiently with cell-surface lipid rafts. Although the results in Figure 6 are most consistent with a model in which LRP-1

migrates from rafts to detergent-soluble membrane microdomains, based on these data alone, we could not rule out endocytosis from within lipid rafts [Nabi and Le, 2003].

LRP-1 Trafficking Between Membrane Microdomains

To further test the hypothesis that raft-associated LRP-1 rapidly exits this membrane compartment by migration to other microdomains, we subjected MEFs to hypotonic shock and potassium depletion, which selectively blocks clathrin-mediated endocytosis but has no effect on lipid raft/caveolae-mediated endocytosis [Larkin et al., 1983; Singh et al., 2003]. This procedure did not significantly alter the percentage of LRP-1 that partitioned into the two detergent fractions (results not shown). When the cells were warmed to 37°C, the level of glutathione-resistant, biotinylated LRP-1 in the detergent-soluble fraction did not increase with time, as was anticipated because endocytosis was blocked (Fig. 7A); however, by 5 min, the total amount of cell-surface biotinylated LRP-1 increased (Fig. 7B). Concurrently, we observed a time-dependent decrease in biotinylated LRP-1 in the detergent-insoluble fraction (Fig. 7C,D). The exposure time in Figure 7C was lengthened, compared with Figure 7A show the bands. Thus, the two panels cannot be directly compared.

As a control, we showed that the total amount of LRP-1 (no affinity precipitation) remained unchanged in the detergent-soluble and -insoluble fractions (Fig. 7A,C). We also demonstrated that the level of biotinylated LRP-1, recovered from both detergent fractions, remained unchanged despite the shift between fractions (Fig. 7E). The level of cell-surface biotinylated LRP-1, contained within both detergent fractions, also remained unchanged, suggesting that LRP-1 endocytosis occurs primarily in clathrin-coated pits (Fig. 7F). These results support our hypothesis that LRP-1 associates with lipid rafts transiently.

DISCUSSION

Plasma membrane microdomains may allow for co-localization of different proteins that function within biochemical systems. An excellent example is the clustering of proteins involved in cell signaling in lipid rafts [Simons and Toomre, 2000]. Identification of LRP-1 in rafts is intriguing given recent studies that implicate this receptor and related proteins in the LDL receptor family in cell-signaling. At least two mechanisms have been identified by which LRP-1 may signal. LRP-1 directly binds signaling adaptor proteins, such as Shc and Dab1 [Gotthardt et al., 2000]. In this capacity, LRP-1 may alter the subcellular localization of the adaptor protein or serve as a scaffold for presentation of the adaptor protein to kinases. In the second mechanism, bifunctional ligands bridge LRP-1 to other signaling receptors and alter the activity of the bridged signaling receptor [Webb et al., 2001]. In both cases, localization of LRP-1 in lipid rafts may facilitate interactions with co-receptors.

In this study, we determined that LRP-1 distributes partially into lipid rafts in MEFs and HT 1080 fibrosarcoma cells. We utilized a method that does not require pre-isolation of plasma membrane-enriched cellular fractions or ultracentrifugation. Instead, cell-surface proteins are isolated by affinity precipitation after detergent fractionation. The method is straightforward and allows for the analysis of multiple samples simultaneously. Complete resolution of transferrin receptor, PDGF β-receptor, and clathrin into the anticipated fractions supports the validity of the method. However, we are cautious because our method is open to alternative interpretations of membrane protein solubility in detergents [Munro, 2003]. Importantly, we did not detect LRP-1 in lipid rafts when we examined VSMCs. This result supports our previous immunoelectron microscopy study of VSMC LRP-1 [Weaver et al., 1996] and lends further support regarding the validity of this biochemical method. CHO cells also did not have raft-associated LRP-1.

If raft-associated LRP-1 represents a distinct and separately-functioning pool of receptor, we hypothesized that ligands may alter the distribution of LRP-1 into plasma membrane microdomains; however, this was not the case. We also did not observe a shift in LRP-1 distribution when cells were treated with PDGF-BB, despite the fact that PDGF-BB causes tyrosine phosphorylation of the LRP-1 light chain [Boucher et al., 2002; Loukinova et al., 2002]. These results support a model in which LRP-1 trafficking occurs constitutively and without regard to ligand. We did not study the effects of PDGF-BB on the rate of LRP-1 endocytosis, which may be altered without a change

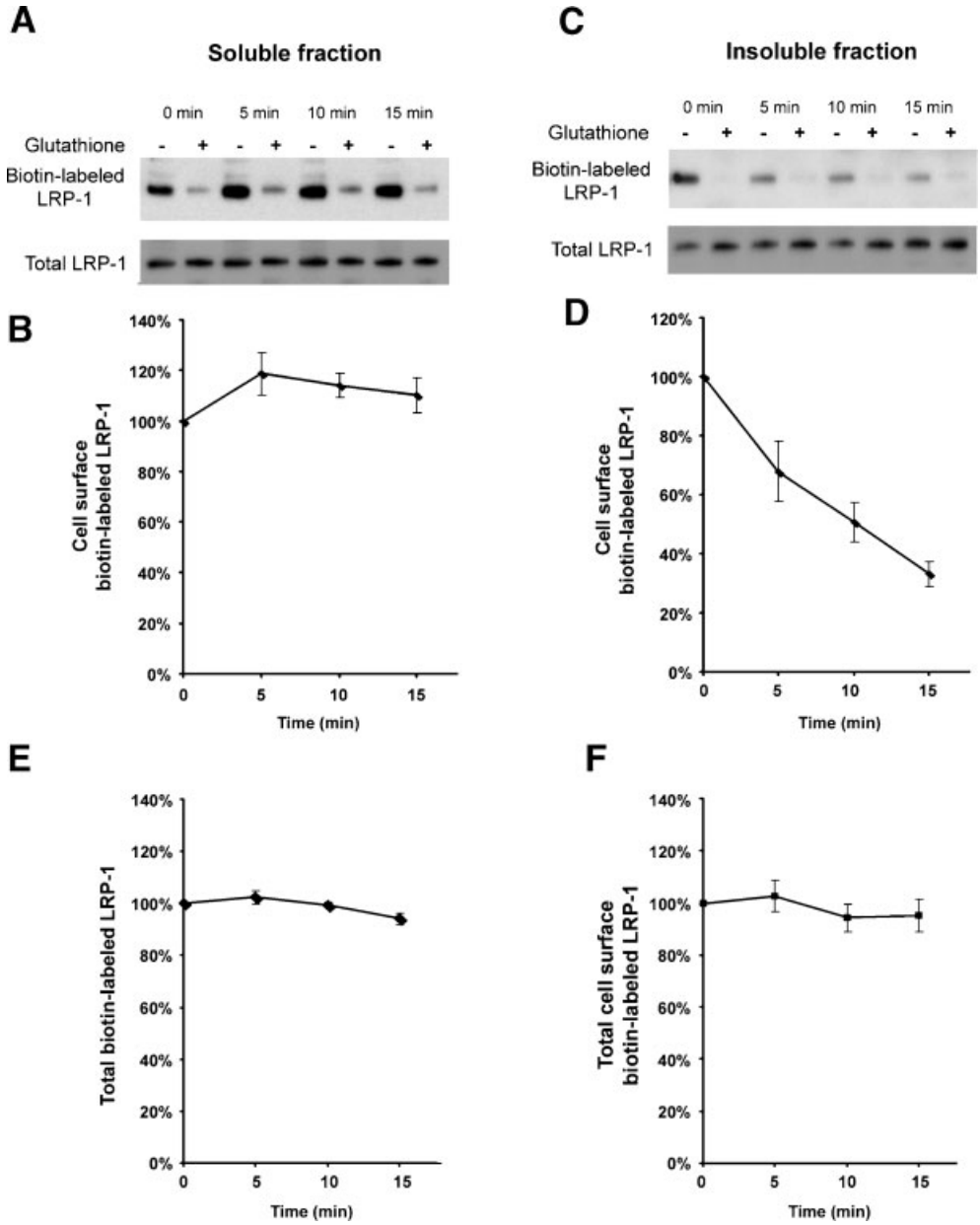

Fig. 7. LRP-1 trafficking between plasma membrane microdomains. MEFs were subjected to hypotonic shock/potassium depletion and then labeled with sulfo-NHS-SS-biotin. The cultures were warmed to 37°C for the indicated time. After re-chilling to 4°C, the cells were treated with glutathione or vehicle. Triton X-100-soluble (**A**) and -insoluble (**C**) fractions were prepared. The fractions were subjected to affinity precipitation and immunoblot analysis (Biotin-labeled LRP-1). As a control, immunoblot analysis of the original extracts prior to affinity precipitation was also performed (total LRP-1). Densitometry was performed. Biotinylated LRP-1 that remained at the cell surface (in the glutathione-sensitive fraction) is plotted against time for the Triton X-100-soluble fraction (**B**) and the Triton X-100-insoluble fraction (**D**). Each time point is plotted as a percent of that present in the same detergent fraction at time = 0. The results of five separate experiments were averaged (mean ± SEM, n = 5). **E**: The total amount of biotinylated LRP-1 in the Triton X-100-soluble and -insoluble fractions was added. Densitometry scans of equivalently exposed immunoblots were used for this purpose (note that the immunoblots shown in **panels A** and **C** were exposed differently). The results are expressed as a percentage of the level observed at time =(0 (mean ± SEM, n = 5). **F**: Cell-surface biotinylated LRP-1 in the Triton X-100-soluble and -insoluble fractions was added and plotted against that present at time = 0 (mean ± SEM, n = 5).

in plasma membrane distribution due to light chain phosphorylation.

A major goal of this study was to examine LRP-1 trafficking between plasma membrane microdomains. To assess endocytosis, use of a reducible biotinylation reagent may be preferable to assays in which ligand endocytosis is monitored because ligands may affect the endocytosis rate. Furthermore, if there is a pool of receptor that does not bind ligand or binds ligand with low affinity, this pool will not be monitored adequately by ligand-based methods. When the plasma membrane was considered as a single compartment, LRP-1 was internalized by MEFs with an apparent half-life of less than 10 min. Our assay did not include a sufficient number of early time points to precisely determine the endocytosis rate constant. For receptors that undergo rapid endocytosis like LRP-1, the degree of internalization observed in early time points may be influenced by the rate at which the cultures are warmed from 4 to 37°C. Using the equivalent method, we previously examined VSMCs and observed a slightly increased rate of endocytosis [Weaver et al., 1996]. In MEFs, recycling of biotinylated LRP-1 was apparent by 15 min, as suggested by a leveling off or slight rebound in the endocytosis graph.

The major finding of our LRP-1 trafficking studies, in which membranes were detergent-fractionated, was the transient nature of LRP-1 association with lipid rafts. Within 10 min, there was a significant decrease in the total amount of biotinylated LRP-1 recovered in the Triton X-100-insoluble fraction. This change was not artefactual because we confirmed that the total level of cellular biotinylated LRP-1 was unchanged. There are two explanations for the decrease in biotinylated LRP-1 in the Triton X-100-insoluble fraction. The most plausible is that LRP-1 in lipid rafts rapidly transfers to other membrane microdomains, such as clathrin-coated pits, when the cells are warmed. It is also possible that raft-associated LRP-1 is internalized by structures such as caveolae into vesicles that become detergent-soluble; however this explanation is not favored.

Strong results supporting our conclusion regarding the transient nature of LRP-1 association with lipid rafts were obtained when we subjected MEFs to hypotonic shock and potassium depletion. Under these conditions, LRP-1 internalization was entirely blocked (Fig. 7F); however, LRP-1 still rapidly exited the Triton X-100-insoluble fraction. Because endocytosis was inhibited, loss of LRP-1 from lipid rafts was paralleled by a significant increase in the level of biotinylated LRP-1 in the detergent-soluble fraction. The total level of cellular biotinylated LRP-1 (in both detergent fractions) and the level of cell-surface LRP-1 were unchanged over the 15 min incubation. The shift in biotinylated LRP-1 from the detergent-insoluble pool to the detergent-soluble pool in the absence of endocytosis argues strongly for the model in which LRP-1 translocates within the plasma membrane at the cell surface.

Our study demonstrated that LRP-1 distribution into membrane microdomains is cell type-specific; however, we did not address the mechanism by which LRP-1 associates with lipid rafts. This process may reflect continuous replenishing of LRP-1 at the cell surface by recycling endosomes. It is quite possible that LRP-1 localizes in lipid rafts in VSMCs and CHO cells, but to a level that is too low to measure by our technique. Cellular characteristics that may favor LRP-1 distribution into lipid rafts is an important topic for further study.

We and others have shown that LRP-1 promotes endocytosis of other plasma membrane proteins, including uPAR, amyloid precursor protein, and tissue factor [Gonias et al., 2004]. This process requires bifunctional ligands or intracellular adaptor proteins that bridge LRP-1 to the second plasma membrane protein. The possibility exists that LRP-1 drags entire membrane protein complexes into endosomes via this mechanism. Fluid trafficking of LRP-1 between plasma membrane microdomains may facilitate the internalization of receptors that localize in lipid rafts.

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