Low density lipoprotein receptor-related protein (LRP) is a member of the low density lipoprotein receptor family, which functions as an endocytic receptor for diverse ligands. In this study, we demonstrate that murine embryonic fibroblasts (MEF-2 cells) and 13-5-1 Chinese hamster ovary cells, which are LRP-deficient, accumulate greatly increased levels of cell-surface fibronectin (Fn), compared with LRP-expressing MEF-1 and CHO-K1 cells. Increased Fn was also detected in conditioned medium from LRP-deficient MEF-2 cells; however, biosynthesis of Fn by MEF-1 and MEF-2 cells was not significantly different. When LRP-deficient cells were dissociated from monolayer culture, increased levels of Fn remained with the cells, as determined by cell-surface protein biotinylation, suggesting an intimate relationship with cell surface-binding sites. The LRP antagonist, receptor-associated protein (RAP), promoted Fn accumulation in association with MEF-1 cells, whereas expression of full-length LRP in MEF-2 cells substantially decreased Fn accumulation, confirming the role of LRP in this process. Purified LRP bound directly to immobilized Fn, and this interaction was inhibited by RAP. Furthermore, MEF-1 cells degraded 125I-Fn at an increased rate, compared with MEF-2 cells. 125I-Fn degradation by MEF-1 cells was inhibited by RAP. These results demonstrate that LRP functions as a catabolic receptor for Fn. The function of LRP in Fn degradation and the ability of LRP to regulate levels of other plasma membrane proteins represent possible mechanisms whereby LRP prevents Fn accumulation on cell surfaces.

Fibronectin (Fn) is a multidomain glycoprotein found in the plasma as an ~450-kDa disulfide-linked dimer and in the extracellular matrix (ECM), in the form of larger multimers (1, 2). Structural variants of Fn arise from a single gene by alternative mRNA splicing and post-translational modification (3, 4). As one of the most ubiquitous and multifunctional ECM proteins, Fn plays a major role in such fundamental biological processes as cell adhesion, migration, growth, differentiation, cytoskeletal organization, hemostasis and thrombosis, and oncogenesis (5–7).

The structure of Fn includes well defined binding sites for multiple macromolecules, including cell-surface receptors, sulfated glycosaminoglycans, gelatin, and fibrin (8–10). Many of the cellular receptors that bind Fn are members of the integrin family, including the major Fn receptor, αvβ1, but also αvβ3, αvβ5, and αβ6 (11). Fn binding to integrins is particularly important because this interaction results in cell signaling involving multiple factors, including the Rho family of small GTP-binding proteins and phosphoinositide 3-OH kinase (12–14). Fn-αvβ1 interactions are also critical in supporting the formation of extracellular Fn fibrils (15), which may then function to suppress signaling pathways that lead to apoptosis (16–18). In addition to integrins, Fn fibril formation is controlled by macromolecules in the pericellular spaces and involves interaction of multiple Fn domains with other ECM components (19–21).

Processes that regulate Fn catabolism are not well understood. One hypothesis is that Fn levels are controlled by local proteolysis (22). Serine proteinases, and in particular urokinase-type plasminogen activator (uPA) and plasmin, may play an important role (23, 24); however, metalloproteinases may also be involved (25–27). In the liver, forms of Fn with terminal galactose residues may be cleared and catabolized by asialoglycoprotein receptors (28). Furthermore, certain integrins, including αvβ3, are known to undergo endocytosis and recycling (29–31). Thus, it is reasonable to assume that, under some circumstances, Fn may be internalized with αvβ3.

The low density lipoprotein receptor-related protein (LRP) is a member of the LDL receptor family, which is constitutively transported through a pathway that includes rapid endocytosis in clathrin-coated pits and efficient recycling (32–35). LRP binds many ligands, delivering these proteins to lysosomes, including activated αv-macroglobulin, apolipoprotein E, plasminogen activators, proteinase-inhibitor complexes, and thrombospondin (36). Other receptors in this family, such as the VLDL receptor and megalin/LRP-2, may be partially redundant with regard to ligand binding specificity; however, members of the LDL receptor family have different patterns of expression, at the cellular level, and generate different phenotypes when the genes are eliminated in knock-out mice (37–39).

In the present study, we demonstrate that Fn accumulates to...
greatly increased levels in the medium and ECM surrounding fibroblasts and CHO cells that are LRP-deficient. LRP binds directly to immobilized Fn, and this interaction is inhibited by receptor-associated protein (RAP), a 39-kDa protein that functions as a general antagonist of specific LRP interactions (40–42). Furthermore, we demonstrate that LRP mediates Fn catabolism by cells in culture. From these studies, we conclude that LRP may be a major regulator of Fn accumulation on cell surfaces.

EXPERIMENTAL PROCEDURES

Reagents and Proteins—Fn was purified from human plasma by the method of Ruošlahti et al. (43). LRP was purified from human placenta as described previously (44). GST-RAP was expressed in bacteria and purified as described previously (45) using a construct obtained from Dr. Joachim Herz (University of Texas Southwestern Medical Center, Dallas, TX). As a control, GST without fused RAP was also expressed and purified from bacteria transformed with the empty vector, pGEX-2T. Monoclonal antibody 8G1, which recognizes the 515-kDa LRP heavy chain, has been described previously (46). Fn-specific polyclonal antibody (F-3648) and globulin-free bovine serum albumin (BSA) were from Sigma. Peroxidase-conjugated donkey anti-rabbit IgG, sheep anti-mouse IgG, Na125I, and [35S]methionine were from Amersham Biosciences. The expression construct, which encodes full-length human LRP in pcDNA3.1, has been described previously (47). The biotinylation reagent, sulfo-NHS-LC-biotin, was from Pierce. Other chemicals were from Sigma, unless otherwise indicated.

Cell Culture—Murine embryonic fibroblasts (MEFs) that are genetically deficient in LRP (MEF-2 or PEA-13 cells), LRP(+/+), and normal MEFs (MEF-1 cells) from the same mouse strain were obtained from the ATCC (Manassas, VA) and cultured in DMEM with 10% fetal bovine serum, as described previously (48). B41 cells are MEF-2 cells that were transfected for stable expression of full-length human LRP and single-cell cloned. Transfection was performed using FuGENE 6 (Roche Molecular Biochemicals). Colonies were established by plating 100 μg/ml hygromycin B and analyzed for LRP expression and function. Wild-type Chinese hamster ovary (CHO-K1) cells and LRP-deficient, 13-5-1 CHO cells (49) were cultured in Ham’s F-12 medium, supplemented with 5% fetal bovine serum optimized for CHO cells (HyClone Laboratories, Logan, UT), 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Preparation of Cell Extracts, SDS-PAGE, and Immunoblotting—Cells were lysed in extraction buffer consisting of 50 mM HEPES, pH 7.5, 0.5 mM NaCl, 0.1% Triton X-100, 0.125% Tween 20, 0.5% deoxycholate, 10 μg/ml aprotinin, 10 μg/ml E64, and 10 μg/ml leupeptin. Equal amounts of cellular protein were subjected to SDS-PAGE under reducing conditions in 7.5% gels, transferred to nitrocellulose membranes, and probed with polyclonal anti-Fn antibody, followed by peroxidase-conjugated donkey anti-rabbit IgG. For LRP immunodetection, cell extracts were subjected to non-reducing 4–12% SDS-PAGE, and membranes were probed with monoclonal 8G1 antibody, followed by peroxidase-conjugated sheep anti-mouse IgG. Secondary antibodies were visualized by enhanced chemiluminescence (Renaissance-ECL, PerkinElmer Life Sciences).

Biotinylation and Recovery of Extracellular Fn—Monolayer cultures of MEFs and CHO cells were washed three times with ice-cold 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS), to remove contaminating fetal bovine serum and other soluble proteins and then treated with the membrane-impermeable biotinylation reagent, sulfo-NHS-LC-biotin (0.5 mg/ml), for 15 min at 22 °C. Alternatively, cells were treated with sulfo-NHS-LC-biotin after dissociation from monolayer culture using enzyme-free cell dissociation buffer (Invitrogen). In these experiments, the cells were pelleted, washed with ice-cold PBS, and resuspended at 4 × 106 cells/ml in Earle’s balanced salt solution (EBSS) with 25 mM HEPES, pH 7.5, containing NHS-LC-biotin and proteinase inhibitors. Biotinylation reactions were terminated by the addition of 50 mM Tris-HCl, 150 mM NaCl, and 100 mM glycine, pH 7.5, for 15 min at 22 °C. After washing with PBS, the cells were counted and lysed in extraction buffer. Biotinylated membrane proteins were precipitated with streptavidin-Sepharose (Amersham Biosciences). The affinity precipitates were recovered by centrifugation, washed, boiled in SDS sample buffer, and subjected to SDS-PAGE and immunoblot analysis to detect Fn.

Fn Accumulation as a Function of Time—MEF-1 and MEF-2 cells (5 × 104) were plated in 75-ml flasks in 10% fetal bovine serum-containing medium and allowed to adhere for 2 h. The medium was then replaced with serum-free DMEM containing Nutridoma NS8 sup-
increased by at least 20-fold, compared with that recovered from MEF-1 cells.

**Fn Recovered from LRP-deficient Cells Is Produced by the Cells**—The Fn detected by immunoblot analysis could have been derived from the cells in culture or the fetal bovine serum that was added to the medium. To distinguish between these possibilities, MEF-1 and MEF-2 cells were allowed to adhere for 2 h in serum-containing medium, which was then replaced by serum-free DMEM containing Nutridoma NS® supplement. Fn that was associated with the cells and in the medium was measured as a function of time. Under these conditions, the only source of Fn is cellular synthesis. As shown in Fig. 2A, cell-associated Fn was almost undetectable shortly after the cells adhered; however, Fn accumulated rapidly in the MEF-2 cell cultures. By 6 h, the level of Fn, which was recovered with MEF-2 cells, was significantly higher than that recovered with MEF-1 cells ($p < 0.01$). By 28 h, the difference in Fn recovery was further accentuated ($p < 0.001$).

As shown in Fig. 2B, Fn also accumulated to increased levels in conditioned medium from LRP-deficient MEF-2 cells, compared with conditioned medium from MEF-1 cells ($p < 0.01$). Thus, LRP deficiency is apparently associated with increased Fn accumulation in association with cells and in solution. To determine whether the differences in Fn accumulation were due to differential rates of Fn synthesis, L-[35S]methionine metabolic labeling experiments were performed. In five separate experiments, we did not observe a significant difference in the rate of Fn synthesis between MEF-1 and MEF-2 cells (results not shown).

**Fn Accumulates on the Cell Surface and in the Extracellular Spaces of LRP-deficient Cells**—To determine the location of Fn, which was recovered from cultures of LRP-deficient and expressing cells, we labeled cell-surface proteins, in MEF-1 and MEF-2 cells, with the membrane-impermeable biotinylation reagent, sulfo-NHS-LC-biotin. Biotinylated proteins were selectively recovered from cell extracts by streptavidin affinity precipitation, and Fn was detected in the affinity precipitates by immunoblot analysis. When the cells were labeled with sulfo-NHS-LC-biotin while in monolayer culture, biotinylated Fn was detected almost exclusively with the LRP-deficient MEF-2 cells (Fig. 3). Equivalent results were obtained when the MEFs were dissociated from monolayer culture, using enzyme-free cell dissociation buffer, and then labeled with sulfo-NHS-LC-biotin in suspension. These results indicate that Fn accumulates more significantly in the extracellular spaces surrounding LRP-deficient MEF-2 cells. At least a significant fraction of the Fn is intimately associated with the MEF-2 cell membrane because the Fn partitions with MEF-2 cells that are dissociated from monolayer culture.

Cell-surface biotinylation experiments were also performed using another model system, by comparing LRP-deficient 13-5-1 CHO cells and wild-type CHO-K1 cells (Fig. 4). Once again, the membrane-impermeable biotinylation reagent selectively labeled Fn in the LRP-deficient cell line. Equivalent results were obtained regardless of whether the sulfo-NHS-LC-biotin reacted with cells in monolayer culture or with cells in suspension.

**LRP Mediates the Degradation of Fn**—LRP undergoes rapid and constitutive endocytosis and recycling, delivering many ligands to lysosomes for degradation (36). We hypothesized that LRP functions as a catabolic receptor for Fn, which might explain the increase in cell-associated Fn in LRP-deficient cells. To test this hypothesis, $^{125}$I-Fn was incubated with MEF-1 and MEF-2 cells at 37 °C. Fn degradation was detected...
LRP Mediates the Catabolism of Fibronectin

by measuring trichloroacetic acid-soluble radioactivity in the medium.

Fig. 5 shows that LRP-expressing MEF-1 cells degraded $^{125}$I-Fn at a significantly increased rate compared with MEF-2 cells ($p < 0.01$). $^{125}$I-Fn degradation was specific because a 50-fold molar excess of unlabeled Fn blocked $^{125}$I-Fn degradation by greater than 90%. To confirm that 125I-Fn degradation by MEF-2 cells ($\Delta$) by adding $^{125}$I-Fn in the presence of 200 nM GST-RAP. All incubations were conducted in the presence or absence of a 50-fold molar excess of unlabeled Fn. At the indicated times, samples of medium were recovered, and trichloroacetic acid-soluble radioactivity in the medium was measured. Specific $^{125}$I-Fn degradation was determined as the difference between trichloroacetic acid-soluble radioactivity that accumulated in the presence and absence of unlabeled Fn. Specific $^{125}$I-Fn degradation (means ± S.E.), obtained from six independent experiments run in triplicate, is shown.

demonstrate that LRP mediates the catabolism of Fn and may alter cellular Fn accumulation by this mechanism.

Fn Binds Directly to LRP—Two possibilities were envisioned whereby LRP might promote Fn degradation. The first and most straightforward would involve direct binding of Fn to LRP so that the Fn is internalized by the cell and transferred to lysosomes. The second possibility is that LRP regulates the level of another plasma membrane protein, which in turn functions as a true catabolic receptor for Fn. To test whether Fn binds directly to LRP, we immobilized Fn in microtiter plates. Purified LRP demonstrated concentration-dependent binding to immobilized Fn and not to immobilized BSA in control wells (Fig. 6). GST-RAP blocked the interaction of purified LRP with immobilized Fn, providing evidence that the Fn-LRP interaction is specific. The inhibitory activity of GST-RAP was RAP concentration-dependent.

Blocking LRP Activity Promotes Fn Accumulation in MEF-1 Cells—From the results presented thus far, a model emerges in which LRP binds and internalizes Fn, promoting its degradation in lysosomes. LRP may also regulate levels of other plasma membrane proteins that function in Fn degradation or Fn fibril assembly. To confirm that the difference in Fn accumulation in MEF-1 and MEF-2 cultures was due to LRP, MEF-1 cells were treated with RAP, and MEF-2 cells were transfected to express LRP. If LRP is responsible for the observed differences in cell-associated Fn, then blocking the function of LRP with RAP should promote Fn accumulation. Conversely, stable expression of LRP in MEF-2 cells should inhibit Fn accumulation.

We confirmed that B41 cells, which are MEF-2 cells transfected with a cDNA construct encoding full-length human LRP, express LRP by immunoblot analysis (Fig. 7A). As shown in Fig. 7B, we also demonstrated that B41 cells internalize the LRP ligand, $\alpha_2$-macroglobulin, which was purified and converted into its receptor-recognized conformation, as described previously (46). The rate of $\alpha_2$-macroglobulin internalization was similar to that observed with heterozygous LRP-deficient

![Fig. 3. Cell-surface accumulation of Fn in MEFs. A, MEF-1 (LRP+) and MEF-2 cells (LRP−) were cultured in serum-containing medium until confluent. The cells were then treated with a membrane-impermeable biotinylation reagent while in monolayer culture. Cell extracts were prepared and probed directly for Fn by immunoblot analysis (cell extract) or subjected to streptavidin affinity precipitation. The precipitates were probed for Fn. B, MEF-1 and MEF-2 cells were dissociated from monolayer culture and surface-biotinylated in suspension. Streptavidin affinity precipitates were then probed for Fn by immunoblot analysis.](image)

![Fig. 4. Cell-surface accumulation of Fn in CHO cells. CHO-K1 (LRP+) and CHO 13-5-1 (LRP−) cells were treated with a membrane-impermeable biotinylation reagent while in suspension or in monolayer culture. Biotinylated proteins were recovered by streptavidin affinity precipitation. The affinity precipitates were subjected to immunoblot analysis for Fn.](image)

![Fig. 5. LRP mediates Fn catabolism. $^{125}$I-Fn was incubated with cultures of MEF-1 cells (●) or MEF-2 cells (△) at 37 °C. Equivalent incubations were carried out in parallel with MEF-1 cells (●) and MEF-2 cells (△) by adding $^{125}$I-Fn in the presence of 200 nM GST-RAP. All incubations were conducted in the presence or absence of a 50-fold molar excess of unlabeled Fn. At the indicated times, samples of medium were recovered, and trichloroacetic acid-soluble radioactivity in the medium was measured. Specific $^{125}$I-Fn degradation was determined as the difference between trichloroacetic acid-soluble radioactivity that accumulated in the presence and absence of unlabeled Fn. Specific $^{125}$I-Fn degradation (means ± S.E.), obtained from six independent experiments run in triplicate, is shown.](image)
were cultured under equivalent conditions. Cells were detached from subjected to immunoblot analysis for Fn.

Conditioned medium was isolated from the same cultures and also titrated in a [50x585]). The amount of Fn detected in association with RAP- (Fig. 7

Fn accumulation in conditioned medium was also observed associated Fn.

A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y
Z

LRP

MEFs (PEA-10 cells). These results demonstrate that LRP is functional in B41 cells.

When MEF-1 cells were cultured in the presence of 200 nm GST-RAP for 28 h, the level of cell-associated Fn was significantly increased, compared with cells that were cultured in the presence of 200 nm GST, as a control (Fig. 7C). An increase in Fn accumulation in conditioned medium was also observed (Fig. 7D). The amount of Fn detected in association with RAP-treated MEF-1 cells was comparable with that detected in LRP-deficient MEF-2 cells. These results support our model in which LRP functions to inhibit Fn accumulation on cell surfaces. B41 cells demonstrated substantially decreased Fn accumulation in the cell-associated fraction and in conditioned medium, compared with MEF-2 cells, further supporting the model.

**DISCUSSION**

The function of LRP and other proteins in the LDL receptor family, as endocytic receptors, is rather complicated at a number of levels. First, the breadth of ligands, which bind to LRP and are subsequently internalized, is extremely large (36). In some cases, LRP may bind complex ligands with potentially profound effects on cell physiology. For example, LRP may internalize growth factors including transforming growth factor-β and platelet-derived growth factor-BB that are bound to the primary LRP ligand, α2-macroglobulin (50, 51). Similarly, LRP mediates the endocytosis of matrix metalloproteinase 2 in association with another primary ligand, thrombospondin 2 (52).

There are a number of examples in which LRP functions as a catabolic receptor for a protein that binds first to another cell-surface site. Lipoprotein lipase and thrombin-protease nexin 1 are most frequently internalized by LRP after binding to cell surface heparan sulfates (53, 54), and collagenase 3 probably binds to a distinct cell-surface receptor prior to interacting with LRP (55). Perhaps most interestingly, LRP may regulate plasma membrane levels of other receptors. The most commonly described mechanism involves internalization of multimeric complexes, in which one ligand bridges LRP to another cell-surface receptor (56). An example of this process, cell-surface levels of uPAR are typically decreased in LRP-expressing cells (35, 57). Similarly, LRP mediates the endocytosis of matrix metalloproteinase 2 in association with another primary ligand, thrombospondin 2 (52).

Fn has the capacity to interact with the cell surface by binding to multiple macromolecules, including integrins and glycosaminoglycans (8–11); however, pathways that are responsible for Fn clearance from the extracellular spaces remain unclear. Because Fn is a major cell adhesion molecule that regulates cell signaling, pathways that alter cell-surface levels of Fn have the potential to impact on diverse aspects of cell physiology, including cell migration, proliferation, differentiation, and apoptosis. In this study, we identified LRP as a catabolic receptor for Fn. Direct binding of Fn to purified LRP
LRP Mediates the Catabolism of Fibronectin

In cells that are LRP-deficient, Fn accumulation to greatly increased levels. Our cell-surface biotinylation experiments studies suggest that the Fn is accumulating outside the cell, on the cell surface and in association with the ECM that forms surrounding LRP-deficient cells in culture. The ability of RAP to substantially increase Fn accumulation in MEF-1 cells supports the hypothesis that LRP-mediated Fn catabolism is directly linked to the differences in Fn accumulation observed in LRP-expressing and deficient cells. However, at the present time, other mechanisms cannot be excluded. For example, Aguirre-Ghiso et al. (61) demonstrated that uPAR may associate with α5β1, promoting the formation of cell-associated Fn fibrils. Because LRP decreases cell-surface levels of uPAR (35, 45, 57), LRP may indirectly regulate Fn accumulation at the cell surface by this mechanism.

LRP-deficient CHO 13-5-1 cells demonstrated increased Fn accumulation compared with LRP-expressing CHO-K1 cells, thus providing support for our hypothesis regarding the function of LRP in Fn catabolism in a second model system. The function of LRP as a regulator of Fn accumulation in both MEFs and CHO cells suggests that LRP may have an equivalent role in diverse cell types. In addition to LRP, CHO cells express the VLDL receptor, which is partially homologous to LRP in function (62). Ligands that bind to LRP and not to the VLDL receptor include α5-macroglobulin and Pseudomonas exotoxin A (46, 49). The increase in Fn accumulation in association with CHO 13-5-1 cells may suggest a selective role for LRP in Fn regulation; however, the activity of other LDL receptor homologues in this process should be taken into consideration and remains to be determined.

The relationship between cell-surface Fn and oncogenic transformation and cancer progression is intriguing but remains incompletely understood. Reduced cell-associated Fn levels may be linked to oncogenic transformation (64). However, cell-associated Fn fibrils may suppress the activity of p38MAPK leading to an increase in the activity of the MAP kinase, extracellular signal-regulated kinase, and increased cancer cell proliferation (61). Increased extracellular signal-regulated kinase activity may also inhibit cancer cell apoptosis (65).

We have demonstrated an association between LRP deficiency and increased cell migration on vitronectin- and fibronectin-coated surfaces. MEF-2 cells migrate more rapidly than MEF-1 cells on vitronectin (35). HT 1080 cells, which express an LRP antisense RNA expression construct and are thus LRP-efficient, migrate more rapidly than LRP-expressing HT 1080 cells (57). Furthermore, MCF-7 breast cancer cells, HT 1080 cells, and normal human fibroblasts that are cultured in RAP for 3–5 days, so that sustained neutralization of LRP or the VLDL receptor is achieved, also migrate more rapidly on vitronectin and fibronectin (45, 57). In all of these systems, LRP deficiency or neutralization of an LDL receptor homologue is associated with increased cell-surface uPAR levels and increased accumulation of endogenously produced uPA in conditioned medium. Furthermore, in RAP-treated MCF-7 cells and in antisense RNA-expressing HT 1080 cells, we have directly linked the increase in activity of the uPA/uPAR system to increased migration. However, in smooth muscle cells, LRP neutralization inhibits cell migration (66), suggesting that a uPAR-independent process may be operative. The effects of LRP on Fn accumulation represent a feasible mechanism whereby LRP may regulate cell migration. Similarly, LRP may regulate smooth muscle cell migration based on its ability to function as a receptor for apolipoprotein E (63). Understanding the full impact of Fn regulation by LRP, on cell phenotype, is an important goal for future studies.
The Low Density Lipoprotein Receptor-related Protein Mediates Fibronectin Catabolism and Inhibits Fibronectin Accumulation on Cell Surfaces
Ana M. Salicioni, Kellie S. Mizelle, Elena Loukinova, Irina Mikhailenko, Dudley K. Strickland and Steven L. Gonias

J. Biol. Chem. 2002, 277:16160-16166.
doi: 10.1074/jbc.M201401200 originally published online February 26, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M201401200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 64 references, 34 of which can be accessed free at http://www.jbc.org/content/277/18/16160.full.html#ref-list-1