Low Density Lipoprotein (LDL) Receptor-related Protein 1B Impairs Urokinase Receptor Regeneration on the Cell Surface and Inhibits Cell Migration*

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The low density lipoprotein (LDL) receptor-related protein 1B (LRP1B) is a newly identified member of the LDL receptor family and is closely related to LRP. It was discovered as a putative tumor suppressor and is frequently inactivated in lung cancer cells. In the present study, we used an LRP1B minireceptor (mLRP1B4), which mimics the function and trafficking of LRP1B, to explore the role of LRP1B on the plasminogen activation system. We found that mLRP1B4 and urokinase plasminogen activator receptor (uPAR) form immunoprecipitable complexes on the cell surface in the presence of complexes of uPA and its inhibitor, plasminogen activator inhibitor type-1 (PAI-1). However, compared with cells expressing the analogous LRP minireceptor (mLRP4), cells expressing mLRP1B4 display a substantially slower rate of uPA-PAI-1 complex internalization. Expression of mLRP1B4, or an mLRP4 mutant deficient in endocytosis, leads to an accumulation of uPAR at the cell surface and increased cell-associated uPA and PAI-1 when compared with cells expressing mLRP4. In addition, we found that expression of mLRP1B or the mLRP4 endocytosis mutant impairs the regeneration of unoccupied uPAR on the cell surface and that this correlates with a diminished rate of cell migration. Taken together, these results demonstrate that LRP1B can function as a negative regulator of uPAR regeneration and cell migration.

The plasminogen activation system consists of a cascade of enzymes and plays a central role in many physiological processes requiring the degradation of basement membrane and components of the extracellular matrix. When the regulation of this system is disrupted, as occurs in the pathogenesis of cancer, malignant cells are able to invade surrounding tissue and metastasize to distant body regions (1–3). Urokinase plasminogen activator (uPA) catalyzes the formation of plasmin from its inactive precursor, plasminogen. The activity of uPA is regulated by two proteins, the glycosylphosphatidylinositol-linked uPA receptor (uPAR) and plasminogen activator inhibitor type-1 (PAI-1). The binding of uPA to uPAR at the cell surface greatly increases its catalytic rate. In contrast, uPA is inactivated by binding to PAI-1. When active uPA is bound to uPAR, it is not internalized but remains at the cell surface. However, when receptor-bound uPA is complexed to its inhibitor, PAI-1, the complex is rapidly internalized and degraded. The mechanism by which this occurs was unknown until it was determined that low density lipoprotein receptor-related protein (LRP) is responsible for this process (4). Following the internalization, uPAR and LRP recycle back to the cell surface, while uPA and PAI-1 are degraded in lysosomes (4–7). The regeneration of unoccupied uPAR at the cell surface is thus critical for the maintenance of plasminogen activation and for regulation of cellular migration and invasion (8–10).

LRP1B is a recently discovered member of the LDLR family (11, 12). The LDLR family previously contained two large members, LRPs (LRP1), a dimer of 515- and 85-kDa subunits, and its closely related homolog, megalin (LRP2), a single species of ~600 kDa. LRP1B is more closely related to LRP (59% identity at the amino acid level) than to megalin (11). Protein domain structure comparison of LRP and LRP1B reveals that the overall organization of the two proteins is almost identical, except for the fragments encoded by the two extra exons in LRP1B (11, 12). Similar to LRP, LRP1B contains four putative ligand-binding domains (I, II, III, and IV from the N terminus) which consist of 2, 8, 10, and 12 cysteine-rich ligand-binding repeats, respectively. The one additional ligand-binding repeat in domain IV of LRP1B is encoded by one of its extra exons. Like LRP, LRP1B contains a furin endopeptidase processing site between its fourth ligand binding domain and transmembrane region. Once cleaved by furin in the Golgi, LRP1B exists as a noncovalently associated heterodimer, consisting of a large extracellular subunit and a smaller transmembrane subunit (12). Similar to that of LRP, the cytoplasmic tail of LRP1B contains five potential endocytosis motifs: two NPXY motifs, two dileucine motifs, and one YXXL motif. However, between the two NPXY motifs, LRP1B contains a unique insertion of a 33-amino acid sequence (encoded by the other extra exon), which is not present in LRP (11, 12). We recently demonstrated that the YXXL motif, but not the two NPXY sequences of LRP, serves as the dominant signal for receptor-mediated endocytosis (13).

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The abbreviations used are: uPA, urokinase plasminogen activator; uPAR, uPA receptor; ATF, uPA amino-terminal fragment; scuPA, single chain urokinase; LDLR, low density lipoprotein receptor; LRP, low density lipoprotein receptor-related protein; mLRP, miniLRP; PAI-1, plasminogen activator inhibitor type-1; CHO, Chinese hamster ovary; CHAPS, N,N,N-tris(3-cholamidopropyl)dimethylammonium)-1-propanesulfonic acid; HA, hemagglutinin; Mu, mutant.
In a previous study, we demonstrated that LRP1B can bind and internalize single chain urokinase (scuPA) and PAI-1, two components of the plasminogen activation system (12). In the present study, we further explore the role of LRP1B in modulation of the plasminogen activation system. By using an LRP1B minireceptor, which mimics the function and trafficking of LRP1B, we show that the expression of LRP1B impairs the regeneration of unoccupied uPAR on the cell surface and diminishes the rate of cell migration.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Recombinant human scuPA, uPA aminoterminal fragment (ATF; amino acids 1–135), and recombinant soluble urokinase receptor (suPAR; amino acids 1–281) were prepared and isolated as described (14–16). Human two-chain uPA was obtained by limited plasmin digestion of recombinant human scuPA (17). Recombinant human PAI-1, uPA-specific monoclonal antibody 3471, PAI-1-specific monoclonal antibody 379, and uPA-specific monoclonal antibody 3936 were from American Diagnostica. Rabbit polyclonal anti-HA antibody was from Upstate. Monoclonal anti-HA antibody has been described before (13). Human vitronectin and goat anti-mouse IgFITC were from BD Biosciences. Transwell cell culture chambers were from Costar. Carrier-free Na125I was purchased from PerkinElmer Life Sciences.

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Distribution of occupied uPAR to clathrin-coated pits. uPAR-LRP complexes are then endocytosed via clathrin-coated vesicles and traffic together to early endosomes. To examine whether uPAR and mLRP1B form a molecular complex after binding of uPA-PAI-1, we analyzed the potential interaction between uPAR and mLRP1B on the plasma membrane by carrying out cross-linking experiments in the presence of recombinant soluble (lacking the glycosylphosphatidylinositol-anchor) uPAR (amino acids 1–281). CHO cells stably transfected with mLRP1B4 were incubated with 125I-uPAR(1–281) (5 nM) for 90 min at 4 °C in the presence or absence of 5 nM uPA-PAI-1 and followed by cross-linking with DTSSP and immunoprecipitation with anti-HA. Immunoprecipitated proteins were processed for autoradiography.

Expression of mLRP1B4 or mLRP4-Mu Results in an Accumulation of Cell Surface uPAR—CHO cells express a relatively high level of cell surface uPAR. To examine the potential effects of LRP1B or LRP minireceptor expression on cell surface levels of uPAR, we analyzed cell surface uPAR in LRP-null CHO cells expressing various minireceptors via flow cytometric analyses. Fig. 3 shows that overexpression of mLRP1B4 or mLRP4-Mu results in a 3.5-fold increase in cell surface uPAR when compared with CHO cells stably transfected with pcDNA3 vector alone. In contrast, overexpression of wild-type mLRP4 results in no change in cell surface uPAR expression. These results suggest that in these CHO cells mLRP1B4 may function in a dominant-negative fashion for uPAR endocytosis. As a result, LRP1B4-bound uPAR is endocytosed at a very slow rate, which in turn results in cell surface accumulation of uPAR.

Expression of mLRP1B4 or mLRP4-Mu Results in an Accumulation of Cell Surface-associated uPA and PAI-1—Accumulation of uPAR at the cell surface may reflect a decreased rate of uPAR regeneration on the cell surface. To do this, we analyzed cells that had been saturated with unlabeled uPA-PAI-1 complexes. LRP-null CHO cells stably transfected with mLRP4, mLRP1B4, or mLRP4-Mu were incubated for 30 min at 37 °C with 10 nM unlabeled uPA-PAI-1 complexes to saturate cellular uPAR. Cells were then washed and incubated at 37 °C in fresh medium. At selected time points, cells were chilled to 4 °C, and the media containing 125I-ATF (a fragment of uPA that specifically binds to uPAR but not to LRP/LRP1B) was added. The amount of unoccupied cell surface uPAR was determined by measuring the amount of cell-bound 125I-ATF. As shown in Fig. 4, without additional incubation at 37 °C, CHO cells bearing mLRP4, mLRP1B4, and mLRP4-Mu bound equivalent small amounts of 125I-ATF, as cell surface uPAR was largely occupied by uPA-PAI-1 complexes. After incubation at 37 °C, progressively more 125I-ATF was bound to CHO cells expressing mLRP4 (increases of 4.0, 7.5, and 10.0-fold at 0.5, 1, and 2 h, respectively). In contrast, CHO cells expressing mLRP1B4 or mLRP4-Mu showed significantly less change in 125I-ATF binding. As shown in Fig. 3, steady-state uPAR levels at the cell surface were 3.5-fold greater in mLRP1B4- than mLRP4-expressing cells. However, when uPAR was initially saturated with uPA-PAI-1 complexes and allowed to regenerate to the cell surface for 2 h, the level of unoccupied uPAR on mLRP1B4-expressing cells was only one-third that of mLRP4-expressing cells. These results clearly indicate that LRP1B has a significantly reduced ability to regenerate unoccupied uPAR in CHO cells when compared with LRP.

Excess of mLRP1B4 or mLRP4-Mu Impairs Cell Migration—Having established that expression of mLRP1B4 or mLRP4-Mu impairs the regeneration of unoccupied uPAR on the cell surface, we next investigated the effects of mLRP1B4 and mLRP4-Mu on CHO cell migration. To study cellular migration, we utilized Transwell cell culture chambers in which the bottom pore membranes were precoated with vitronectin. Interestingly, stable expression of wild-type mLRP4 results in no change in CHO cell migration (Fig. 5), although these cells have lower levels of cell surface-bound uPA and PAI-1 (see Fig. 3). However, stable expression of mLRP1B4 or mLRP4-Mu results in an 80% decrease in CHO cell migration, compared with CHO cells transfected with empty pcDNA3 vector. These results demonstrate that expression of LRP1B results in a marked impairment in CHO cell migration.

Discussion

The structural similarity between LRP and LRP1B suggests that those two giant receptors may bind similar sets of ligands and display overlapping functions. In support of this, we found that at the cell surface, LRP1B, like LRP, forms immunoprecipitable complexes with uPAR in the presence of uPA-PAI-1 complexes and that LRP1B can also mediate the delivery of uPA-PAI-1 to the lysosome for degradation (data not shown). On the other hand, the unique sequences in LRP1B suggest that it may also have functions distinct from those of LRP. In this study, we demonstrate that expression of mLRP4 enhances unoccupied uPAR regeneration on the cell surface, whereas expression of mLRP1B4, as well as an mLRP4 endocytosis-defective mutant, causes the accumulation of cell surface uPAR and cell-associated uPA and PAI-1, impairs unoccupied uPAR regeneration on the cell surface, and diminishes cell migration.
mLRP1B, like the mLRP4 endocytosis mutant, displays a substantially slower rate of uPA/PAI-1 internalization when compared with mLRP4. The cytoplasmic tails of LRP1B and LRP contain the same five potential endocytosis motifs, including two NPXY motifs, two dileucine motifs, and one YXXL motif. However, the tail of LRP1B has a unique insertion of 33 amino acid residues that is not present in the tail of LRP (11, 12). This insert may be responsible for the slower internalization rate of LRP1B by binding cytosolic adapter proteins and masking the neighboring endocytosis signal(s). This slower endocytosis mediated by LRP1B may allow for more sustained signal transduction upon ligand binding. Consistent with this hypothesis, recent studies have revealed new roles for several members of the LDLR family as obligate components of signal transduction pathways (23). The 33-amino acid insert in the
tail of LRP1B may itself, or together with a common motif in the LRP tail, also interact with some cytosolic proteins that participate in signal transduction. Future studies similar to those that have dissected the signals within the cytoplasmic tails of LRP and apolipoprotein E receptor-2 (24, 25) will be conducted to address this issue.

Continuous high level plasminogen activation at the cell surface depends upon the removal of uPA/PAI-1 complexes. LRP-mediated internalization of uPA/PAI-1-uPAR complexes results in the lysosomal degradation of uPA and PAI-1 and recycling of uPAR to the cell surface (4–7). This process allows for the regeneration of unoccupied uPAR, immobilization of fresh uPA in the form of its zymogen, pro-uPA, and the eventual re-expression of active uPA at the cell surface (8, 10). Failure to remove uPA/PAI-1-complexes from the cell surface may diminish the cellular capacity for plasminogen activation and may impair cell migration/invasion mediated by uPAR (8–10, 26). CHO cells bearing mLRP4B display approximately a 90% reduction in ligand endocytosis when compared with CHO cells expressing mLRP4. The physiological significance of this may be that LRP1B acts in a dominant-negative fashion to limit the effectiveness of LRP- or caveola-mediated uPA/PAI-1-uPAR clearance. If cells co-express LRP and LRP1B, the net result of uPAR regeneration likely depends on the relative expression of the two receptors. For example, if the expression level of LRP1B in a given cell type exceeds that of LRP, LRP1B may function as a dominant-negative regulator for the regeneration of unoccupied uPAR at the cell surface and therefore inhibit uPAR-mediated cell migration. A model depicting this hypothesis is shown in Fig. 6. Consistent with this hypothesis, we have demonstrated that expression of mLRP1B in CHO cells impairs the regeneration of unoccupied uPAR on the cell surface and reduces cell migration. Although our previous studies with LRP minireceptors have clearly demonstrated that these receptors traffic and function similarly to their endogenous full-length receptor (13, 20), it is important to realize that the use of minireceptors may abolish or diminish the functional differences between full-length LRP and LRP1B. Future studies with endogenous LRP1B should help us to fully understand the biological functions of this receptor.

In addition to regulating plasminogen activation at the cell surface, recent studies indicate that uPAR possesses other cellular activities, including an ability to generate intracellular signal transduction, to regulate integrin function, and to directly bind vitronectin (27–29). Thus, it is possible that LRP1B expression also mediates uPAR interaction with integrin sub-units and uPAR/integrin-mediated signaling.

Two lines of evidence strongly support an important and apparently causal role for the plasminogen activation system in cancer metastasis: the results from experimental model systems with animal tumor metastasis and the finding that high levels of uPA, PAI-1, and uPAR in many tumor types predict poor patient prognoses (1). The discovery that LRP mediates the internalization of uPA/PAI-1-uPAR complexes has generated interest in the role of LRP in malignant transformed cells. Although the role of LRP in tumor progression remains unclear at present, several studies show a correlation between LRP expression and human cancer cell invasion and metastasis (9, 30–36). LRP1B was originally discovered as a putative tumor suppressor gene and is frequently inactivated in non-small cell lung cancer cell lines (11) and in high grade of urothelial cancer (37). Therefore, our present results warrant further studies on the potential role of LRP1B as a tumor suppressor and in inhibition of tumor metastasis.

In summary, using an LRP1B minireceptor, we have demonstrated that expression of LRP1B impairs unoccupied uPAR regeneration on the cell surface and diminishes cell migration. These results suggest that LRP1B mimics an LRP endocytosis mutant and negatively regulates uPAR regeneration and function.

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