**γ-Secretase-mediated Release of the Low Density Lipoprotein Receptor-related Protein 1B Intracellular Domain Suppresses Anchorage-independent Growth of Neuroglioma Cells**

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The low density lipoprotein receptor related protein 1B (LRP1B) is a large endocytic receptor that was first identified as a candidate tumor suppressor gene. In the current investigation we demonstrate that LRP1B undergoes regulated intramembrane proteolysis in a γ-secretase-dependent process. The released intracellular domain (ICD) then translocates to the nucleus via a nuclear localization signal that is present within this domain. ICD release first requires shedding of the LRP1B ectodomain, which appears to be catalyzed by a member of the metalloproteinase family. Employing site-directed mutagenesis studies, we identified lysine residues 4432 and 4435 and arginine 4442 as key amino acids important for ectodomain shedding of LRP1B. We also demonstrate that an LRP1B minireceptor as well as the ICD domain alone suppresses anchorage-independent growth of LRP1B-deficient neuroglioma cells (H4 cells). Interestingly, abrogating ectodomain shedding resulted in a loss of the ability of LRP1B minireceptors to suppress anchorage-independent growth. Together, these studies reveal that LRP1B has tumor suppression function that is mediated by proteolytic processing of the receptor resulting in ICD release.

The low density lipoprotein (LDL) receptor-related protein 1B (LRP1B) is a member of the LDL receptor gene family and was initially identified as a candidate tumor suppressor gene by positional cloning (1, 2). The LRP1B gene is frequently inactivated by homozygous genomic deletions or by the expression of aberrant mRNA transcripts (1). In human esophagecancer cell lines and primary tumors the LRP1B gene was shown to be inactivated by transcriptional silencing through hypermethylation of its promoter CpG island (3). Homozygous deletions of LRP1B gene have also been reported in human liver cancers (4) and urothelial cancers (5). By conventional and array-based comparative genomic hybridization analysis, loss of the LRP1B allele was noted in endocervical adenocarcinomas of uterus (6). Thus, collective data indicate that this gene is inactivated during cellular transformation, suggesting that LRP1B is a candidate tumor suppressor gene.

Structurally, LRP1B is closely related to LRP1, a receptor that recognizes numerous ligands and is essential for normal development (7). Like LRP1, LRP1B contains a furin cleavage site (REKR) within a β-propeller domain that is cleaved by furin during receptor trafficking (8–10). The major structural differences between LRP1 and LRP1B occur within their intracellular domains (ICD). First, the LRP1B ICD contains an alternatively spliced exon (exon 90) that is not present in LRP1 (1). Second, the LRP1B ICD contains a cluster of basic residues (KRKRRTK) that is similar to basic clusters in B RCA2 tumor suppressor which have been shown to function as a nuclear localization sequence (NLS) (11).

Currently, the physiological function of LRP1B remains unknown. In mice, LRP1B expression is primarily restricted to the brain, adrenal glands, salivary gland, and testis (12). However, in humans, LRP1B is widely expressed (13). Like LRP1, LRP1B is expressed in vascular smooth muscle cells where it is thought to attenuate the migration of these cells by regulating activity and function of the PDGFR-β (9). Mice in which the LRP1B gene has been deleted develop normally (12), possibly due to its restricted expression pattern and functional compensation by other members of the LDL receptor superfamily. Studies using LRP1B minireceptors have revealed that this receptor recognizes ligands such as the receptor-associated protein, urokinase and tissue-type plasminogen activator (8), and Pseudomonas exotoxin A (15) that are also recognized by LRP1, revealing that LRP1B and LRPI exhibit overlapping ligand specificity.

A number of transmembrane receptors have been found to undergo regulated intramembrane proteolysis (RIP). The common features of RIP involve ectodomain shedding of the transmembrane protein, generating a membrane-bound product with a short ectodomain, which is then cleaved within the transmembrane domain releasing the ICD. The first transmembrane proteins discovered to undergo RIP were Notch (16, 17) and the amyloid precursor protein (APP) (18). More recently, several other cell surface proteins that undergo RIP have been identified and include Notch ligands Jagged and Delta (19, 20), ErbB4 (21), N-cadherin (22), E-cadherin (23),
CD44 (24), the colony-stimulating factor 1 receptor (25), p75 neurotrophin receptor (26), syndecan 3 (27), Nectin-1α (28), γ-proteocadherins (29), and tumor suppressor DCC (30). For Notch (31) and ErbB4 (21), it has been established that the initial proteolytic events are triggered upon ligand binding to the receptors, leading to shedding of the ectodomain. Ectodomain shedding is usually mediated by ADAM family members such as ADAM17 (TACE) (32) or ADAM10 (kuzbanian) (33) or by matrix metalloproteinases (34). This intramembrane cleavage is mediated by an γ-secretase complex in most cases. The released ICD from Notch enters the nucleus as a transcription co-activator (31, 35), whereas the ICD derived from APP appears to function as a transcription co-repressor (36). In the case of the N-cadherin, its released ICD promotes the degradation of transcription factor CBP in the cytosol (37), whereas in the case of E-cadherin, its ICD domain modulates the disassembling of cell-cell interaction complexes (23).

In addition to LRP1 (38), several other members of the LDL receptor family have recently been demonstrated to undergo RIP. These include LRP2/megalin (39) and LRP8/apoER2 (40, 46). To begin to investigate the biological function of LRP1B, we initiated studies to determine whether LRP1B also undergoes RIP. Our results revealed that LRP1B undergoes proteolytic processing via a γ-secretase-mediated release of the ICD. Furthermore, we demonstrate that LRP1B suppresses anchor-independent growth of neuroglioma cells (H4 cells) and that this function requires LRP1B proteolysis.

**EXPERIMENTAL PROCEDURES**

**LRP1B Expression Vector Constructs**—All of the transmembrane domain-containing truncated constructs of mLRP1B4 (8) were generated by PCR using the Advantage cDNA amplification kit (Clontech) with the mLRP1B4 as a template. The PCR fragments were cloned into the BamH1 site and Xhol site of the pcDNA3 vector by using the following primer sets: LRPIB 425, 5′-AAATATGGATCCCCATGGATTAGCATGGGAAATTCTTT-3′ and 5′-TTCTCATTCGAGTTAGTGCCACTGTCTCTTATACCAGTTATT-3′; LRPIB 172, 5′-TTCTATGGATCCGAGGAGCCGCAGCCGAGGAGCAGC-3′ and 5′-TTCTACCTCGAGATGTTAGCCACGTGCTCTTTATACCATTTATCCTATT-3′. Constructs contained the signal peptide of LRP and an HA epitope fused to the N terminus of the expressed protein. In the case of LRPIB ICD, the PCR fragment was subcloned into the EcoRI and NotI sites of pcDNA3 cloning vector (Invitrogen) with a neomycin-resistant gene. For the EGFP fusion constructs, the expression vector pEGFP-C1 (Clontech) was used; for C-terminal Gal4-VP16 (GV) fusion constructs, the GV coding sequence from pEGFP-C1 (Clontech) was used; for C-terminal Gal4-VP16 expression vector (Clontech). All of the constructs were validated by DNA sequencing.

**Cell Culture and Transfection**—HEK 293, COS1, H4, and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Chinese hamster ovary (CHO) cells and furin-deficient CHO cell lines (a kind gift from Dr. Toni Antalis) were cultured in Ham’s F-12 medium supplemented with 10% fetal bovine serum. All of the transient transfections of these cells with the indicated vector plasmids were performed with transfection reagent FuGENE 6 (Roche Applied Science) following the manufacturer’s procedure.

**Reporter Gene Assay**—Cells grown overnight in 6-well plates were co-transfected with GV-fused protein expression constructs (0.5 μg), luciferase reporter plasmid pFR-Luc (0.5 μg) (Stratagene), and pCMV-LacZ expression vector (0.05 μg). pFR-Luc contains 5′ upstream activation sequence Gal4 binding sites immediately upstream of the TATA box promoter, and the firefly luciferase open reading frame is driven by this promoter. pCMV-LacZ served as an internal control to normalize for transfection efficiency. Luciferase activity assay was performed on the cell lysates 24 h after transfection using the luciferase assay kit from Clontech. β-Galactosidase activity of the same lysates was assayed using Galacto-Light Plus chemiluminescent reporter assay purchased from Clontech. Normalized luciferase activity was obtained by dividing the relative light unit value of luciferase assay by the β-galactosidase value from the same lysate. To study the effect of γ-secretase inhibitor L485,658 on cleavage processing, the inhibitor was applied to the cells 16 h after transfection, and the reporter gene assays were performed 24 h after the treatment. All transfections for reporter gene assays were done in triplicate and repeated in at least three independent experiments.

**Antibodies, Cell Extract Preparation, and Immunoblotting**—Whole cell lysates were prepared using modified radioimmune precipitation cell lysate buffer (50 mm Tris, pH 7.4, 150 mm NaCl, 1% Nonidet P-40 with protease inhibitors). Precast 10–20% Tricine or 4–20% Tris-glycine gels (Invitrogen) were used for Western blotting analysis. Mouse monoclonal and rabbit polyclonal antibodies against HA epitope were purchased from Sigma and Santa Cruz biotechnology. Rabbit anti-VP16 IgG was purchased from Clontech. Rabbit 489 polyclonal antibodies against C terminus LRP1B (intra cellular domain) was raised using a synthetic oligopeptide representing the C-terminal sequence of LRP1B (ELLPKKIEIGIRETV). To analyze the soluble mLRP1B4 in the condition medium, cells transfected with wild type and mutant LRP1B constructs were cultured in advance Dulbecco’s modified Eagle’s medium (Invitrogen) with 0.5% fetal calf serum for 24 h. Collected condition medium was concentrated using Centrprep 30 (Millipore). To the concentrated samples, an equal volume of 2× Nonidet P-40 cell lysis buffer was added and immunoprecipitated with 4 μg/ml rabbit polyclonal anti-HA antibodies (Santa Cruz). Immunoprecipitates were immunoblotted with monoclonal anti HA antibodies (Santa Cruz).

**Florescence Microscopy Analysis**—H4 cells grown on cover-slips in 6-well plates were transiently transfected with the EGFP fusion constructs. 36 h after transfection the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline, pH 7.0. The fluorescence analysis was performed using a Nikon ECLIPSE E800 microscope.
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A

B

FIGURE 1. Expression constructs of mLRP1B4 and truncated receptors with GV fused at their C termini. A, scheme showing proteolysis fragments and the relative position of the furin cleavage site, which occurs between residues 3954 and 3957, and the shedding site (a), which occurs between residues 4432 and 4435. The PS1 cleavage site that occurs within the transmembrane domain is shown as b. 8, GV is a chimeric artificial transcription factor composed of the DNA binding domain of yeast Gal4 transcription factor and herpesvirus VP16 transcription factor activation domain. LRP1B 172-GV contains the LRP1B transmembrane domain and ICD, whereas LRP1B 172Δ-GV represents alternatively spliced exon 90 is deleted. All the receptor constructs contain a HA epitope at their N termini. The R489 epitope is located at the C terminus of mLRP1B and is shown.

Anchorage-independent Growth by Soft Agar Assay—The cell transformation detection assay kit from Chemicon International was used following the recommended procedure. Neuroglioma H4 cells were grown overnight in 150-cm culture dishes and transfected with (10 μg/plate) WT and mutant LRP1B constructs using FuGENE 6 (Roche Applied Science). 72 h after transfection cells were selected in the presence of 600 μg/ml G418 for 2 weeks. Cell surface expression of LRP1B containing the transmembrane domain in these cells was analyzed by flow cytometry using a monoclonal anti-HA IgG (Sigma). After confirming the cell surface expression, 2.5 × 10⁵ cells per well were mixed with the 0.4% top agar in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum and grown for 18 days at 37 °C. After incubation, colonies were counted by size for transfectants of each indicated construct.

RESULTS

LRP1B Is Cleaved by an γ-Secretase-like Activity—To systematically examine if LRP1B undergoes RIP we developed a set of plasmids capable of expressing different forms of LRP1B (Fig. 1). One expression plasmid encodes for a minireceptor, termed mLRP1B4, which contains the light chain of LRP1B along with the fourth cluster of ligand binding repeats that has been previously demonstrated to bind and endocytose ligands (8). Additional expression plasmids were generated that contain deletion mutants of mLRP1B4 and include LRP1B 425, a 425-amino acid-containing protein that includes 6 EGF repeats of the LRP1B light chain, the transmembrane domain, and the entire ICD. LRP1B 172 represents a receptor in which most of the ectodomain is deleted and is similar to the mNotch mutant described (41). As a control for our experiments, we also expressed the LRP1B ICD. LRP1B differs from LRP1 in the inclusion of an alternatively spliced exon 90 insertion within its ICD, and thus, in addition to these constructs, we also prepared LRP1B 172Δ in which the inserted sequence encoded by the alternatively spliced exon 90 was deleted from LRP1B 172. To assess cleavage analysis, a sensitive reporter gene assay was employed in which the yeast Gal4 DNA binding domain and a VP16 transactivation domain from herpes simplex virus was fused to the C-terminal end of the cytoplasmic tail as described previously (38).

HEK 293 cells were transfected with the LRP1B plasmids, and LRP1B ICD cleavage was assessed by measuring luciferase activity, which is only expressed when the Gal4-VP16-fused LRP1B ICD is released from membrane tethering by proteolysis. The results (Fig. 2A) demonstrate that expression of LRP1B 425-GV, LRP1B 172-GV, and LRP1B 172Δ-GV all resulted in the generation of luciferase activity and the latter two at a level very close to that of the positive controls (LRP1B ICD-GV and GV). The data also showed that the alternative spliced form of LRP1B in which the sequence encoded by exon 90 was deleted (LRP1B 172Δ) was also cleaved as efficiently as wild type LRP1B 172-GV, revealing that both forms of the receptor undergo similar proteolytic processing, and that the cleavage site is not located within the sequence encoded by exon 90.

To determine whether the cleavage was mediated by γ-secretase-like activity, the specific γ-secretase inhibitor L-685,458 was used. HEK 293 cells were transfected with LRP1B 172-GV plasmid and then treated with increasing concentrations of the γ-secretase inhibitor L-685,458. After treatment, the luciferase activity was measured, and the results (Fig. 2B) show that the reporter gene activity generated from cleavage of LRP1B 172-GV was inhibited by L-685,458 in a dose-dependent manner, similar to the results obtained for the APP fused to the GV domains which served as a positive control in this experiment. These results suggest that the release of the GV-fused intracellular domain is mediated by γ-secretase cleavage. Similar results were obtained using H4 human neuroglioma cell lines (data not shown).

Interestingly, the reporter gene activity from cells transfected with LRP1B 425-GV was consistently much lower than that of LRP1B 172-GV. These results were not due to higher protein expression by the shorter forms, as immunoblotting of cell extracts using anti-HA epitope located at the N-terminal portion of the expressed protein (Fig. 2C) or an antibody that recognizes the VP16-domain located at the C terminus (Fig. 2D) revealed that LRP1B 425-GV was expressed at equal or even higher levels than LRP1B 172-GV in the transfected cells. This difference in reporter gene activity between these two minireceptors suggests that LRP1B 172-GV is a direct substrate of γ-secretase, whereas LRP1B 425-GV may require an extra step of ectodomain shedding to generate the γ-secretase substrate. Finally, although the LRP1B ICD gave a signal in the luciferase assay, the ICD was not easily detected by immunoblot analysis, suggesting that it is rapidly degraded.

The Putative γ-Secretase Substrate is a 21-kDa C-terminal Fragment Generated from LRP1B Ectodomain Shedding—To determine whether we could detect cleavage products of LRP1B, we next investigated the processing of mLRP1B4, LRP1B 425, and LRP1B 172 in the presence of γ-secretase
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Together with the reporter gene assay results, the data suggest that the LRP1B ICD is proteolytically released by a γ-secretase like activity.

We also noted that the band density of unprocessed LRP1B-172 was dramatically increased in the presence of γ-secretase inhibitor (Fig. 3B, lanes 3 and 4), suggesting that LRP1B 172 itself may be a direct substrate of γ-secretase, similar to the findings for truncated forms of Notch (41, 43).

The Ectodomain of LRP1B Is Shed by a Metalloproteinase—To identify the class of proteinase required for proteolytic processing of LRP1B, we examined the effect of various proteinase inhibitors on the generation of the 21-kDa γ-secretase substrate. When the cells were preincubated with TAPI-2, an inhibitor of ADAM 17 (TACE), we noticed a decrease in the levels of the 21-kDa fragment (Fig. 4) in the presence of the γ-secretase inhibitor L-685,458. These results suggest that ectodomain shedding by ADAM 17 or a related proteinase is necessary to generate the 21-kDa γ-secretase substrate. Consistent with these results, we found that the general metalloproteinase inhibitor GM6001 also reduced the generation of the 21-kDa fragment.

We also examined if inhibitors of other classes of proteinases could inhibit the generation of the 21-kDa C-terminal fragment. We found that the general serine proteinase inhibitor AEBSF reduced the amount of the 21-kDa fragment, suggesting inhibitors by immunoblot analysis using antibodies against the HA tag located at the N-terminal region of the receptor (Fig. 3B) as well as antibodies prepared against the C-terminal region of LRP1B (Fig. 3C). Cells transfected with mLRP1B4 constructs express the ~160-kDa band of the unprocessed mLRP1B4 protein (Fig. 3B, lanes 1 and 2). A 96-kDa furin processed LRP1B light chain is detected when the extracts are immunoblotted with a C-terminal antibody (Fig. 3C, lanes 1 and 2). In the case of LRP1B 172, the expected 26-kDa polypeptide representing the mature form of this truncated receptor was detected (Fig. 3B, lane 3), whereas in the case of LRP1B 425, a 74-kDa polypeptide representing the intact protein was detected (Fig. 3B, lane 5). When the same immunoblots were probed with a C-terminal antibody, a 21-kDa band was observed to accumulate in the presence of L-685,458 (Fig. 3C, arrow, lanes 2, 4, and 6). Because this band accumulates only when γ-secretase inhibitor is present, we hypothesize that this 21-kDa band represents a substrate for γ-secretase that arises from shedding of the LRP1B ectodomain.

FIGURE 3. A 21-kDa C-terminal fragment from mLRP1B4 accumulates in the presence of γ-secretase inhibitors. A, constructs used in this experiment. Note that these constructs lack the Gal4-VP16 domain and, thus, differ in size from the constructs used in Fig. 2. B, immunoblot analysis of cleaved LRP1B using anti-HA recognizing the N-terminal region (a) or C terminus antibody R489 (b). 293 cells were transfected with the indicated constructs in the presence or absence of 0.5 μM γ-secretase inhibitor L658,458. 16 h after the addition of L658,458, cell extracts were prepared and analyzed by immunoblot analysis. The migration position of mLRP1B4, LRP1B 425, and LRP1B 172 are shown. The arrow denotes the migration position of the 21-kDa fragment that accumulates in the presence of γ-secretase inhibitors. a and b depict the shedding site and PS-1 cleavage site, respectively.

FIGURE 2. The LRP1B ICD is released in a γ-secretase-dependent manner. A, reporter-based cleavage assay in which HEK 293 cells were transfected with vector or plasmid expressing LRP1B 425-GV, LRP1B 172-GV, LRP1B 172Δ-GV, LRP1B ICD-GV, or GV as a control. The cells were co-transfected with reporter plasmid and pCMV promoter-driven β-galactosidase expression plasmid, which was used to normalize for transfection efficiency. Twenty-four hours after transfection, luciferase and β-galactosidase activity of the cell lysates was measured. Values are the mean of triplicate determinations ± S.E., and each experiment was performed in triplicate. B, luciferase activity of LRP1B 172-GV and APP-GV-transfected HEK 293 cells is inhibited by γ-secretase-specific inhibitor L658,458 in a dose-dependent manner. C, immunoblotting analysis of cell extracts from HEK 293 cells transfected with indicated plasmids using anti-HA antibodies, which detects the tag on the N-terminal portion of each construct. D, immunoblotting analysis of cell extracts from HEK 293 cells transfected with the indicated plasmids using anti-VP16 antibody, which detects the C-terminal portion of each protein. NS denotes a nonspecific band detected by the anti-VP16 IgG.
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an effect of serine proteinases on LRP1B proteolytic processing. Because AEBSF is known to inhibit furin, which activates ADAM17 by processing of the pro-domain, this is the most likely explanation for its effect on LRP1B shedding.

Mutational Analysis Identifies Candidate Ectodomain Shedding Sites on LRP1B—Because LRP1B 172 contains a relatively small ectodomain and is processed to generate the 21-kDa membrane-bound fragment (see Fig. 3C, lane 4), the proteolytic cleavage site resulting in shedding of the ectodomain must be relatively close to the transmembrane domain. In LRP1B a stretch of amino acids linking the transmembrane domain with the first EGF repeat contains a number of basic residues (Fig. 5A) that are potential targets sites for proteolysis. We selected Lys-4432, Lys-4435, and Arg-4442 as targets for site-directed mutation analysis, and two different mutants of mLRP1B4 were generated, one in which Lys-4432 and Lys-4435 were both replaced by Gly (mutant 1) and a second mutant in which Arg-4442 was replaced by Gly (mutant 2). To determine whether the ectodomain of these mutant receptors was shed from the cell surface, H4 cells were transfected with WT and mutant receptors, and flow cytometry analysis was performed to confirm their surface expression. These experiments (data not shown) confirm that each mutant receptor was appropriately expressed on the cell surface. We next examined cell extracts for the presence of the 21-kDa C-terminal membrane-bound fragment. Transfected cells were cultured in the presence of the γ-secretase inhibitor, and accumulation of this fragment was detected by immunoblotting. The results (Fig. 5B) reveal that cells transfected with WT receptor accumulate the 21-kDa fragment and that cells transfected with either mutant receptor did not accumulate this fragment. The results also showed that the processing of the mutant receptors by furin during trafficking through the Golgi was not impaired when compared with WT receptor (Fig. 5B). To assess ectodomain shedding of WT and mutant receptors, conditioned media from transfected cells were examined for expression of soluble LRP1B by immunoblotting. The results (Fig. 5C) reveal that an ~116-kDa soluble form of LRP1B was detected in the conditioned media of cells transfected with WT receptor but not in cells expressing either of the K4432G/K4435G or R4442G mutant receptors. This was not related to expression levels, as WT and mutant receptors were readily detected at similar levels in cell extracts upon immunoblotting (Fig. 5D). Together, these results reveal that the ectodomain of LRP1B is shed and can be readily detected in conditioned media and that mutation of Lys-4432/Lys-4435 or Arg-4442 to glycine generates mutant receptors whose ectodomains are not released from the cell surface, which in turn seems to prevent subsequent proteolytic processing.

Like Notch, LRP1B also contains a furin cleavage site at residues 3927–3930 (REKR), and thus, we also examined the effect of mutating the LRP1B furin cleavage site on its proteolytic processing. By converting Arg-3927 and Arg-3930 to glycine, we completely abolished the furin cleavage of mLRP1B. However, the 21-Da polypeptide fragment was still detected in the presence of γ-secretase inhibitor (data

FIGURE 4. Proteolytic processing resulting in generation of the 21-kDa LRP1B fragment is inhibited by an ADAM 17 inhibitor. Top panel, H4 cells were transfected with mLBP4, and the generation the 21-kDa LRP1B C-terminal fragment was measured in the presence of 0.5 μM γ-secretase inhibitor L658,458 by immunoblotting with antibody R489 that recognizes the C-terminal fragment. Middle panel, as a control, the amount of mLBP4 in the cell extracts is shown. Bottom panel, blots were probed for actin as a loading control.

FIGURE 5. Mutation analysis identifies candidate proteolysis sites on the LRP1B ectodomain. A, alignment of several species of LRP1B demonstrate conserved lysine and arginine residues in a region close to the transmembrane domain. B, H4 cells were transfected with mLBP4 WT and mutant receptors. After transfection the cells were cultured in the presence of 0.5 μM γ-secretase inhibitor L658,458 for 16 h. After this period cell extracts were prepared and analyzed for C-terminal LRP1B products by immunoblot analysis using R489, which recognizes an epitope at the C terminus of LRP1B. C, conditioned media from transfected H4 cells were analyzed for shed ectodomain by immunoblotting with anti-HA IgG. D, cell extracts were assessed for mLBP4 WT and mutant expression by immunoblotting with anti-HA IgG.
not shown), revealing that furin cleavage at this site is not necessary for ectodomain shedding. In addition, we transfected furin-deficient Chinese hamster ovary cells with mLRP1B4 and found that the 21-Da fragment was generated in the presence of the γ-secretase inhibitor, further demonstrating that furin activity is not required for the ectodomain shedding.

The Released LRP1B-ICD Can Be Detected in the Presence of Proteasome Inhibitors—Because immunoblotting experiments failed to detect the LRP1B ICD, we suspected that this fragment is rapidly degraded in cells. Thus, we performed an experiment in the presence of the proteasome inhibitor lactacystin to determine whether we could detect the LRP1B ICD after inhibition of this degradation pathway. When cells were transfected with mLRP1B4 and cultured in the presence of lactacystin, we detected an ~18-kDa polypeptide that is the appropriate size for the LRP1B ICD (Fig. 6, lane 2). This fragment was not present when the cells were cultured with the γ-secretase inhibitor L-685,458 (Fig. 6, lane 3), revealing that the 18-kDa fragment arises after γ-secretase cleavage. Furthermore, neither fragment was detected when the two shedding mutant forms of LRP1B were used for these experiments (Fig. 6, lanes 5 and 8).

The Released LRP1B ICD Translocates to the Nucleus—The LRP1B ICD contains a cluster of basic residues (KKRRKRTK) that is similar to basic clusters in BRCA2 tumor suppressor, which have been shown to function as a NLS sequence. This raised the possibility that the KKRRKRTK motif within LRP1B ICD may function as NLS and mediate the transport of the cleaved ICD to the nucleus (see Fig. 1 for location of NLS with the LRP1B ICD). To determine if this is the case, the LRP1B ICD was fused to EGFP, and cells transfected with this fusion protein were examined by fluorescence microscopy (Fig. 7). As controls, cells were also transfected with EGFP and the ICD derived from LRP-1. The results showed that although EGFP and LRP1-ICD-EGFP are diffusely spread across the cell (Fig. 7, A and B), the LRP1B ICD fusion protein predominantly existed in the nucleus, with little fluorescence detected in the cytosol (Fig. 7C). Deletion of the putative NLS sequence in the LRP1B ICD resulted in a cellular distribution similar to that of EGFP alone (Fig. 7D). Finally, mutation of the two NPXY motifs in the LRP1B-ICD to APXXA or deletion of the insertion sequence present in LRP1B did not affect the nuclear localization of the fusion proteins (data not shown). These results reveal that the LRP1B ICD localizes to the nucleus as a result of a basic cluster that functions as a nuclear localization signal sequence. Interestingly, our results also indicate that the LRP1 ICD, which does not contain the basic cluster found in LRP1B, does not localize to the nucleus. This result is in contrast with a previous study (44) but in agreement with the studies of Yoon et al. (45).

mLRP1B4 Suppresses Tumor Cell Anchorage-independent Growth—LRP1B is known to be frequently inactivated by genetic or epigenetic alterations in tumor cells (3–6). We employed PCR to examined LRP1B transcripts in neuroglioma H4 cells and detected aberrant transcripts with deletions in exon 2 to exon 7 leading to a loss of protein (data not shown), revealing that these LRP1B-deficient cells represent a good model system to explore the potential of LRP1B to alter cellular function. Thus, H4 cells were transfected with wild type mini-receptor mLRP1B4 pcDNA3 expression vector, and pooled stable transfectants were isolated by culturing the transfected cells with G418 for 2 weeks. The transfected cells were then used to measure anchorage-independent growth in soft agar. We used pooled stable transfectants rather than isolated clones derived from individual colonies to avoid artifacts due to the high heterogeneity of tumor cell populations in the assays (42). Expression of mLRP1B4 and the mutant versions of this receptor was confirmed by flow cytometry analysis (Fig. 8, A–C). Colony formation in cells transfected with the WT minireceptor was suppressed (Fig. 8E), whereas the H4 cells transfected with empty vector developed colonies identical to the untransfected parental H4 cells (Fig. 8, panel D). These results suggest the potential of mLRP1B4 to suppress tumorigenesis as assessed by this assay. Next, we determined if RIP processing of LRP1B contrib-
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FIGURE 8. mLRP1B4 inhibits anchorage-independent growth of H4 neuroglioma cells. H4 cells were transfected with vector (A and D), mLRP1B4 WT (B and E), mLR1B4 (K4432G/K4435G mutant) (C and F), and LRP1B ICD (G). Surface expression of mLRP1B4 WT (B) and K4432G/K4435G mutant (C) was assessed by flow cytometry analysis using anti-HA IgG. The cells were grown for 2 weeks under the selection of the antibiotic G418, and then the cells (~2.5 × 10^4 cells/well) were grown in 0.4% soft agar with 0.8% agar as base layer using 6-well agar plates for 2.5 weeks. Representative fields are shown in panels D–G; H, four representative fields for each well were examined under a 10× objective, and all of the colonies in each of the 4 fields for each well were counted and classified based on colony size: 30–40, 40–50, and >50 μm.

mLRP1B4 WT and the LRP1B4 K4432G/K4435G mutant were assessed by immunoblot analysis with antibodies to HA. The cells were grown for 48 h under the selection of G418, and then the cells (~2.5 × 10^4 cells/well) were grown in 0.4% soft agar with 0.8% agar as base layer using 6-well agar plates for 2.5 weeks. Representative fields are shown in panels D–G; H, four representative fields for each well were examined under a 10× objective, and all of the colonies in each of the 4 fields for each well were counted and classified based on colony size: 30–40, 40–50, and >50 μm.

In the current study we provide compelling evidence that the LRP1B ICD is released by RIP. First, using a reporter gene assay approach, we demonstrate that the LRP1B ICD is released in a γ-secretase-dependent fashion. Immunoblots of cell extracts identified a 21-kDa fragment derived from the LRP1B C-terminal domain that accumulated only when γ-secretase activity was blocked with inhibitors. An 18-kDa fragment was found to accumulate when cells were treated with the proteasome inhibitor lactacystin. The generation of this fragment was prevented by γ-secretase inhibitors, implying that it is a product of γ-secretase-mediated proteolysis. Together, these data suggest that the 18-kDa fragment represents the LRP1B ICD. Once released, the LRP1B ICD localizes within the nucleus. This was demonstrated by transfecting cells with a plasmid expressing the LRP1B ICD fused to EGFP. Nuclear localization required the putative NLS within the LRP1B ICD, as depletion of this short cluster of basic residues resulted in cytosolic distribution of the LRP1B ICD. In contrast to LRP1B, the LRP1 ICD was not detected in the nucleus. This is in contrast to a previous study using a slightly different construct (44). The reason for this discrepancy is not readily apparent.

In regulated intramembrane proteolysis of type I transmembrane proteins that is mediated by γ-secretase, ectodomain shedding is first required. This proteolysis then allows the truncated protein to undergo additional cleavage by γ-secretase, resulting in a release of the ICD. Our evidence suggests that ectodomain shedding of LRP1B is also required before γ-secretase-mediated cleavage occurs. This was demonstrated by generating mutant receptors that were not shed and were unable to be further processed by γ-secretase.

In the search for the candidate proteinases responsible for the ectodomain shedding, we found that an ADAM 17 inhibitor and a wide spectrum inhibitor of matrix metalloproteinases could inhibit ectodomain shedding of LRP1B minireceptor, suggesting that a member(s) of the metalloclopeptase superfamily is involved in the shedding process.

For some proteins, such as Notch and Erb4, RIP processing is initiated upon ligand binding, which in turn triggers ectodomain shedding. At this time we do not know if ligand binding to LRP1B is required for ectodomain shedding and ICD release. It is interesting to note that both ligand binding cluster deletion constructs, LRP1B 425 and LRP1B 172, are efficiently processed. LRP1B 425 contains only EGF repeats in the extracellular domain, and no ligands have thus far been identified that bind to this portion of the receptor. This raises the possibility that ectodomain shedding of LRP1B may be a constitutive process that can be modulated by ligand binding, as has been demonstrated for APP (14), but this will need to be determined in future studies.

DISCUSSION

Members of the LDL receptor superfamily recognize a variety of lipoproteins from the extracellular environment and efficiently deliver them to sites of processing and degradation within cells. These ligands include lipoproteins, proteinases and proteinase-inhibitor complexes, viruses, bacterial toxins, matrix components, and growth factors. Although transport of these ligands is an important function for LDL receptor family members, evidence is now accumulating to suggest a prominent role for certain members in signal transduction pathways and in regulating cell physiology. This may occur by regulated proteolysis of the receptor resulting in release of ICD.
As a candidate tumor suppressor LRP1B has not been tested until now for its tumor suppression function in vitro or in vivo. Using anchorage-independent growth assay, we found that a LRP1B minireceptor (mLRP1B4) significantly suppresses the anchorage-independent growth of LRP1B-deficient tumor cells in soft agar. This activity of LRP1B minireceptor appears related to its proteolysis and ICD release, as mutant receptors in which the shedding process is inhibited significantly decrease the suppression effects of LRP1B on tumor cell growth on soft agar. In light of the nuclear localization of the released intracellular domain, the events that lead to tumor suppression most probably occur in the nucleus by modulating expression of genes related to cell transformation.

In conclusion, this study provides new data supporting a role for LRP1B as a tumor suppressor and demonstrates that this function requires proteolytic processing of LRP1B. Deciphering the mechanism by which LRP1B functions as a tumor suppressor and identifying the modulated gene(s) is likely to provide new reagents for the therapy of human cancers.

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