Differential Functions of Members of the Low Density Lipoprotein Receptor Family Suggested by Their Distinct Endocytosis Rates*

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The low density lipoprotein receptor (LDLR) family is composed of a class of cell surface endocytic receptors that recognize extracellular ligands and internalize them for degradation by lysosomes. In addition to LDLR, mammalian members of this family include the LDLR-related protein (LRP), the very low density lipoprotein receptor (VLDLR), the apolipoprotein E receptor-2 (apoER2), and megalin. Herein we have analyzed the endocytic functions of the cytoplasmic tails of these receptors using LRP minireceptors, its chimeric receptor constructs, and full-length VLDLR and apoER2 stably expressed in LRP-null Chinese hamster ovary cells. We find that the initial endocytosis rates mediated by different cytoplasmic tails are significantly different, with half-times of ligand internalization ranging from less than 30 s to more than 8 min. The tail of LRP mediates the highest rate of endocytosis, whereas those of the VLDLR and apoER2 exhibit least endocytosis function. Compared with the tail of LRP, the tails of the LDLR and megalin display significantly lower levels of endocytosis rates. Ligand degradation analyses strongly support differential endocytosis rates initiated by these receptors. Interestingly apoER2, which has recently been shown to mediate intracellular signal transduction, exhibited the lowest level of ligand degradation efficiency. These results thus suggest that the endocytic functions of members of the LDLR family are distinct and that certain receptors in this family may play their main roles in areas other than receptor-mediated endocytosis.

The low density lipoprotein receptor (LDLR) family includes five members in mammals: the LDLR itself, the apolipoprotein E receptor 2 (apoER2), the very low density lipoprotein receptor (VLDLR), the LDLR-related protein (LRP), and megalin. These modules include 1) ligand-binding repeats of 40 amino acids that include six cysteine residues forming three disulfide bonds; 2) epidermal growth factor precursor repeats, which also contain six cysteines residues each; and 3) modules of ~50 amino acids with a consensus tetrapeptide, Tyr-Trp-Thr-Asp (YWTD). In addition to these extracellular modules, each of these receptors also contains a single transmembrane domain and a relatively short cytoplasmic tail with potential endocytosis signals (1–5). A cluster of several complement-type ligand binding repeats constitutes a ligand-binding domain, and differential clustering of these repeats within a domain may impart specificity with respect to ligand recognition (3). The epidermal growth factor precursor homology domains and YWTD repeats are necessary for the dissociation of ligands from the receptor in endosomes (6, 7). Ligand interactions with all members of the LDLR family can be antagonized by a receptor-associated protein (RAP), a unique ligand frequently used as a tool in the study of ligand-receptor interaction (8). RAP also functions intracellularly as a molecular chaperone to facilitate receptor folding and trafficking within the early secretory pathway (8).

Traditionally, all members of the LDLR family have been regarded as cell surface endocytosis receptors that function in delivering their ligands to lysosomes for degradation (1–3). However, recent studies have revealed new roles for these receptors in signal transduction (9). A set of cytoplasmic adaptor and scaffold proteins containing protein interaction domain or PSD-95/DLG/ZO-1 domains have been shown to bind to the cytoplasmic tails of members of the LDLR family (10–15). These new findings suggest that members of the LDLR family may participate in several signal transduction pathways including the regulation of mitogen-activated protein kinases, cell adhesion, vesicle trafficking, neurotransmission, and neuronal migration (9). Cellular signaling through this class of receptors may be regulated by receptor endocytosis (13). For example, binding of the adaptor protein Dab1 (Disabled-1) to the cytoplasmic domain of the LDLR impedes its interaction with the endocytic machinery (13).

The relatively short cytoplasmic tails of LDLR, VLDLR, apoER2, LRP, and megalin contain 50, 54, 115, 100, and 209 amino acid residues, respectively (16–20). A common characteristic of the LDLR family members is that at least one copy of the NPXY sequence is found within their cytoplasmic tails. For LDLR, this NPXY motif serves as a signal for receptor endocytosis through coated pits (4). However, we recently reported that the YXXL motif, but not the two NPXY sequences, within the cytoplasmic tail of LRP serves as the dominant signal for...
receptor-mediated endocytosis (5). We also demonstrated that the distal dileucine motif and a serine phosphorylation within the LRP tail contribute to receptor endocytosis (5, 21). These results suggest that each member of the LDLR family may utilize different potential signal(s) within their cytoplasmic tails for receptor-mediated endocytosis.

In the present study, we have directly compared the endocytic functions of the members of the LDLR family using LRP minireceptors, its chimeric receptor constructs, and full-length VLDLR and apoER2 stably expressed in LRP-null Chinese hamster ovary (CHO) cells. We find that the initial endocytosis rates mediated by the cytoplasmic tails of the LDLR family members differ significantly, suggesting that members of the LDLR family may play their main roles in either receptor-mediated endocytosis or signal transduction.

EXPERIMENTAL PROCEDURES

Materials—Plasmid pDLR-2 was obtained from American Type Culture Collection. Plasmid pCDL-SR containing human VLDLR cDNA, and plasmid pCDL-SR containing human apoER2 cDNA were kindly provided by Dr. Tokuo Yamamoto (Tohoku University Gene Research Center, Sendai, Japan). A human kidney cDNA was obtained from CLONTECH for polymerase chain reaction (PCR) cloning of the megalin tail. Human recombinant RAP was expressed in a glutathione S-transferase expression vector and isolated as described previously (22). Human α2-macroglobulin (α2M) was purified from plasma and activated with methanolamine (to yield receptor-binding form, α2M*) as described before (23). Polyclonal rabbit anti-human apoER2 antibodies were kindly provided by Dr. James S. Owen (University College London, London, London, UK; see Ref. 24) and Dr. Johannes Nimptsch (University of Vienna, Vienna, Austria). Monoclonal anti-HA antibody has been described before (25). Peroxidase labeled anti-mouse antibody and ECL system were from Amersham Pharmacia Biotech. All tissue culture media, serum, and plasticware were from Life Technologies, Inc. Immobilon-P transfer membrane was obtained from Millipore, Bedford, MA. The construction of full-length human VLDLR with an HA epitope near the amino terminus in pcDNA3 vector has been described previously (28). To generate the chimeras, we cloned a PCR fragment encoding the cytoplasmic tail of the LDLR, or megalin cytoplasmic tail. Human recombinant RAP was expressed in a glutathione S-transferase expression vector and isolated as described previously (22). Plasmid pcDL-SR containing human apoER2 cDNA was digested with XhoI and EcoRI, and the full-length apoER2 cDNA was subcloned into pcDNA3.1(−) vector. The construction of the membrane-containing minireceptor of LRP (mLRP4) (see Fig. 1) with an HA epitope near the amino terminus in pcDNA3 vector has been described previously (26). To generate the chimeras, we cloned a PCR fragment encoding the cytoplasmic tail of the LDLR, or megalin cytoplasmic tail into a “tail-less” mLRP4 construct. To create the tail-less LRP4 construct, a unique restriction site XhoI was introduced after the transmembrane domain. Plasmid pDLR-2 was used as the PCR template for LDLR tail, whereas a human kidney cDNA library was used as template for the megalin tail. Each of the 5′ PR primers included XhoI sites. These PCR fragments were digested with XhoI and XbaI and ligated into the tail-less mLRP4 construct, digested with the same enzymes. Thus, compared with mLRP4T100, these chimeric receptors include two extra amino acids (leucine and glutamic acid) immediately after the transmembrane domain. All oligonucleotides were synthesized at Washington University School of Medicine Protein Chemistry Laboratory. All DNA sequences generated by PCR were verified by DNA sequencing.

Cell Culture and Transfection—LRP-null CHO cell line and CHO-K1 (kindly provided by Dr. David FitzGerald, National Institutes of Health; see Ref. 29) were cultured in Ham’s F-12 medium containing 10% fetal bovine serum. Stable transfection into LRP-null CHO cells was achieved by transfection of 30 μg of plasmid DNA in 10-cm dishes. Stable transfectants were selected using 700 μg/ml G418 and maintained with 500 μg/ml G418.

Metabolic Pulse-Chase Labeling and Immunoprecipitation—Metabolic labeling with [35S]Cysteine was performed essentially as described before (22, 30). For pulse-chase experiments, cells were generally pulse-labeled for 30 min with 200 μCi/ml [35S]Cysteine in cysteine-free medium and chased with serum-containing medium for 0 or 120 min. Cells were lysed with 0.5 ml of lysis buffer (phosphate-buffered saline containing 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride) at 4 °C for 30 min. Immunoprecipitation was carried out essentially as described before (28).

Western Blot Analysis—Stably transfected CHO cells were lysed with 0.5 ml of lysis buffer at 4 °C for 30 min. Equal quantities of proteins were subjected to SDS-PAGE (6%) under reducing conditions. Following transfer to polyvinylidene difluoride membrane, successive incubations with proper primary antibody and horseradish peroxidase-conjugated secondary antibody were carried out for 60 min at room temperature. The immunoreactive proteins were then detected using the ECL system.

Analytic Analysis of Endocytosis—CHO cells were plated in 12-well plates at a density of 2 × 105 cells/well and used after overnight culture (5). Cells were rinsed twice in ice-cold ligand binding buffer (minimal Eagle’s medium containing 0.6% bovine serum albumin), and [125I]RAP was added to 5 nM final concentration in cold ligand binding buffer (0.5 mM/well). The binding of [125I]RAP was carried out at 4 °C for 30 min with gentle rocking. Binding of [125I]RAP was specific, i.e. the addition of 100-fold excess unlabeled RAP inhibited binding by 90–95%. Unbound ligand was removed by washing cell monolayers three times with cold binding buffer. Ice-cold stop/strip solution (0.2 mM acetic acid, pH 2.6, 0.1 M NaCl) was added to one set of plates without warming up and kept on ice. The remaining plates were then placed in a 37 °C water bath, and 0.5 ml of ligand binding buffer prewarmed to 37 °C was quickly added to cell monolayers to initiate internalization. After each time point, the plates were quickly placed on ice, and the ligand binding buffer was replaced with cold stop/strip solution. Ligand that remained on the cell surface was stripped by incubation of cell monolayers with cold stop/strip solution for a total of 20 min (0.75 ml for 10 min, twice) and counted. Cell monolayers were then solubilized with low SDS lysis buffer and counted. The sum of ligand that was internalized plus that remained on the cell surface after each assay was used as the maximum potential internalization. The fraction of internalized ligand after each time point was calculated and plotted. α2M* internalization was carried out in the same way of RAP internalization, except that the ligand binding buffer contained 5 mM CaCl2.

Analyses of [125I]RAP Binding and Degradation—Cells (2 × 105) were seeded in 6-well plates precoated with a 3% agarose gel. RAP assay (minimal Eagle’s medium containing 0.6% bovine serum albumin with 5 nM radioligand, 0.6 mM/well) was added to cell monolayers, in the absence or the presence of 500 nM unlabeled RAP, followed with incubation for 1 h at 4 °C. Thereafter, overlying buffer containing unbound ligand was removed, and cell monolayers were washed and lysed in low SDS lysis buffer and counted.

[125I]RAP degradation was measured using the methods described (4, 5, 31). Briefly, 2 × 105 cells were seeded into 12-well dishes 1 day prior to assays. Prewarmed assay buffer with 5 mM [125I]RAP was added to cell monolayers in the absence or the presence of unlabeled 500 nM RAP followed with incubation for 4 h at 37 °C. Thereafter, the plates were quickly placed on ice. The medium overlying the cell monolayers was collected, and proteins were precipitated by addition of bovine serum albumin to 10 mg/ml and trichloroacetic acid to 20%. Degradation of radioligand was defined as the appearance of radioactive fragments in the overlying medium that were soluble in 20% trichloroacetic acid. The cell monolayers were washed, and the ligand that remained on the cell surface in the steady state was stripped by incubation of cell monolayers with cold stop/strip solution and counted. The protein concentration of each cell lysate was measured in parallel dishes that did not contain RAP ligand. The degradation efficiency was the value of the ratio of the degraded [125I]RAP divided by the cell surface bound [125I]RAP in the steady state and calculated relative to mLRP4T100.

RESULTS

Endocytosis Rates of Endogenous LRP and Its Minireceptor mLRP4T100—LRP is synthesized as a 600-kDa single chain precursor that undergoes post-translational proteolytic processing within the trans-Golgi compartment, by the endoprotease furin (32, 33). This posttranslational processing results in the formation of mature LRP as a noncovalently associated heterodimer, consisting of the extracellular 515-kDa protein and the transmembrane 85-kDa chain (32). We generated an LRP minireceptor that mimics the function and trafficking of LRP. This LRP minireceptor composed of residues 3274–4525 of the full-length LRP (28), which includes the fourth cluster of ligand binding repeats and the entire carboxyl terminus of the receptor (designated mLRP4T100), with “m” representing mem-
brane-containing, “4” representing the fourth cluster of ligand binding repeats, “T” representing cytoplasmic tail, and “100” representing the 100 amino acid residues within the LRP tail; see Fig. 1A and Ref. 28). To facilitate immunodetection, an HA epitope was included near the amino terminus of mLRP4T100.

Our previous studies have shown that the fourth ligand-binding domain of LRP binds RAP with high affinity (25) and that mLRP4T100 internalizes RAP with high efficiency (5). To examine whether there is a difference between the endogenous LRP and mLRP4T100 for ligand internalization, we compared the endocytosis rates of these two receptors. Because LRP is the only receptor that binds RAP with high affinity in CHO-K1 cells (34), we utilized 125I-RAP for our endocytosis assays. Fig. 2A shows the endocytosis rates of endogenous LRP in CHO-K1 cells and mLRP4T100 stably expressed in LRP-null CHO cells. The endocytosis rates of LRP and mLRP4T100 were extremely fast with internalization half-times of less than 30 s and were virtually indistinguishable from one another.

To confirm this finding with a physiological ligand, we measured the endocytosis rate of LRP in CHO-K1 cells using LRP unique ligand α-M. Similar to that seen with 125I-RAP internalization, LRP exhibited fast internalization of 125I-α-M with a half-time of less than 30 s (Fig. 2B).

VLDLR and apoER2 Exhibit Low Endocytosis Rates—Using LRP-null CHO cells, we generated stably transfected cell lines expressing human VLDLR and human apoER2. To examine whether these two receptors are expressed in the correct forms, we performed metabolic pulse-chase labeling and immunoprecipitation. Thus, stably transfected CHO cells were metabolically pulse-labeled with [35S]cysteine for 30 min and chased for 0 or 120 min with complete medium, followed by immunoprecipitation with anti-HA antibody for VLDLR or polyclonal anti-apoER2 antibody for apoER2 (Fig. 3A). For both VLDLR and apoER2, only one band around 130 kDa is detected prior to the chase. This band represents the full-length ER precursor form of VLDLR or apoER2 that lacks complex sugar modification (Fig. 3A, indicated by an arrow). After 120 min of chase, one additional band representing receptor mature form (indicated by an arrowhead) is seen. It is also noted that apoER2 has higher level of mature form than VLDLR does following the chase (Fig. 3A). At the steady state, the ER form and the mature form of VLDLR are expressed at similar levels and are migrated closely to each other as analyzed by Western blotting (Fig. 3B). For apoER2, the level of apoER2 ER form is very low, whereas the level of apoER2 mature form is relatively high. As expected, the apoER2 mature form migrated significantly more slowly than its ER form (Fig. 3B). Taken together, these results indicate that VLDLR and apoER2 are expressed in correct forms in LRP-null CHO cells.

We next compared the endocytosis rates of mLRP4T100 with VLDLR and apoER2 stably expressed in LRP-null CHO cells. Surprisingly, the endocytosis rates of VLDLR and apoER2 were extremely low (Fig. 3C), with half-times of RAP internalization over 8 min (Table I). At 1 min, VLDLR and apoER2 internalized 9.2 and 10%, respectively, of the total cell-associated 125I-RAP, corresponding to 15–18% of mLRP4T100 endocytosis (Figs. 3C and 5B). These results indicate that VLDLR and apoER2 exhibit extremely low endocytosis rates compared with LRP.
Endocytosis Rates of Members of the LDLR Family

**FIG. 2. Endocytosis rates of endogenous LRP and its minireceptor mLRP4T100.** A, endocytosis of 125I-RAP by endogenous LRP and mLRP4T100. CHO-K1 and LRP-null CHO cells stably transfected with mLRP4T100 were incubated with 5 nm 125I-RAP at 4 °C for 30 min and then shifted to 37 °C for the indicated times. The amounts of ligand internalized as the fraction of the total cell-associated ligand (the sum of the internalized ligand plus the ligand remaining on the cell surface at the end of the assay; see “Experimental Procedures” for further explanation) are plotted against time. Values are the averages of triple determinations with the S.E. indicated by error bars. B, endocytosis of 125I-αM* by endogenous LRP. CHO-K1 cells were incubated with 0.5 nm 125I-αM* at 4 °C for 2 h, and 125I-αM* internalization was carried out for indicated time points as described for 125I-RAP internalization. These experiments are representatives of several such experiments performed with similar data.

**TABLE I**

| Receptor        | 125I-RAP internalization (t1/2) |
|-----------------|--------------------------------|
| mLRP4T100       | 0.5                            |
| mLRP4-LDLR      | 4.8 ± 0.5                      |
| mLRP4-megalin   | 3.1 ± 0.1                      |
| VLDLR           | 8.2 ± 0.1                      |
| apoER2          | 8.1 ± 0.6                      |

Data are calculated from the experiments described as in the legends to Figs. 2–5. Values are the averages of at least three independent experiments with the S.E.

**FIG. 3. VLDLR and apoER2 exhibit extremely low endocytosis rates.** A, metabolic pulse-chase labeling and immunoprecipitation for VLDLR and apoER2. LRP-null CHO cells stably transfected with full-length VLDLR and apoER2 were pulse-labeled with [35S]cysteine for 30 min and chased for 0 or 120 min. After each chase, cells were lysed, immunoprecipitated with anti-HA antibody for VLDLR and with a polyclonal rabbit antibody for apoER2, and analyzed via 6% SDS gels under reducing conditions. The positions of the ER forms and mature forms are indicated by arrows and open arrowheads, respectively. B, Western blotting analysis of VLDLR and apoER2 stably transfected in LRP-null CHO cells. The positions of the ER forms and mature forms are indicated by arrows and open arrowheads, respectively. C, endocytosis of 125I-RAP by mLRP4T100, VLDLR, and apoER2. 125I-RAP internalization in LRP-null CHO cells stably transfected with mLRP4T100, VLDLR, and apoER2 was carried out for indicated time points as described in the legend to Fig. 2A. Values are the averages of triple determinations with the S.E. indicated by error bars. These experiments are representatives of several such experiments performed with similar data.

**LDLR Tail and Megalin Tail Display Reduced Endocytosis Rates Compared with LRP Tail**—At present, the ligand binding regions of extracellular domain of megalin are yet to be defined. The very large size of megalin (~600 kDa) and the lack of full-length CDNA also limits its molecular manipulation and expression via transfection. For LDLR, RAP binding affinity to LDLR is significantly lower than that of other LDLR family members. Thus, we made two chimeric receptors that contain LRP fourth ligand-binding domain, and LDLR or megalin cytoplasmic tail. To generate the chimeras, we replaced the LRP tail of mLRP4T100 with cytoplasmic tails derived from LDLR and megalin (Fig. 1B). Using LRP-null CHO cells, we generated stably transfected cell lines expressing mLRP4-LDLR and mLRP4-megalin.

To examine whether the chimeric receptors were expressed in the correct forms when they were stably transfected in LRP-null CHO cells, we performed metabolic pulse-chase labeling and immunoprecipitation with anti-HA antibody. For mLRP4T100, only one band around 200 kDa is detected prior to the chase. This band represents the full-length ER precursor form that lacks complex sugar modification (Fig. 4A, indicated by arrow). After 120 min of chase, two additional bands are seen. The 85- and 120-kDa bands represent the furin-processed minireceptor forms that correspond to the LRP-85 (Fig. 4B, indicating open arrowhead) and LRP-ligand-binding domain 4 (Fig. 4A, indicated by closed arrowhead), respectively (28). mLRP4-LDLR and mLRP4-megalin exhibited a similar banding pattern on SDS-PAGE. The 120-kDa furin-processed forms are identical to that of mLRP4T100, whereas the ER forms and the other processed forms migrate faster or slower than that of mLRP4T100 depending upon the length of the cytoplasmic tail. The two processed forms of mLRP4-megalin migrate at similar positions on 6% SDS-PAGE. At the steady state levels, these three receptors exhibit a similar expression pattern (Fig. 4B). For mLRP4T100, two distinct bands are seen on 6% SDS-PAGE gel under reducing conditions. Because the HA epitope is near the amino terminus of the minireceptor, Western blot analyses with anti-HA antibody do not detect the LRP-85 band. Taken together, these results indicate that mLRP4-LDLR and mLRP4-megalin are properly expressed and processed in LRP-null CHO cells.

We next compared the endocytosis rate of mLRP4T100 with that of the chimeric receptors stably expressed in LRP-null CHO cells. As shown in Fig. 4C, the endocytosis rates of...
mLRP4-LDLR and mLRP4-megalin were similar. However, compared with mLRP4T100, the endocytosis rates mediated by LRP tail and megalin tail were significantly low, with half-times of RAP internalization of 4.8 and 3.1 min, respectively (Fig. 4C and Table I). At 1 min, mLRP4-LDLR and mLRP4-megalin internalized 14 and 18%, respectively, of the total cell-associated 125I-RAP, corresponding to 22–30% of mLRP4T100 endocytosis (Figs. 4C and 5A). These results indicate that the LRP tail and the megalin tail display reduced endocytosis compared with the LRP tail.

**Comparison of Endocytosis Rate of mLRP4T100 Endocytosis Mutant with That of mLRP4-LDLR, mLRP4-megalin, VLDLR, and apoER2**—To further characterize the endocytosis rates of mLRP4-LDLR, mLRP4-megalin, VLDLR, and apoER2, we compared these with that of mLRP4T100(Y63A), which is a LRP minireceptor endocytosis mutant. This mutant contains a substitution of an alanine for the tyrosine residue, which is within the LRP dominant endocytosis motif YXXL (Fig. 1B). Our previous studies have demonstrated that the endocytosis rate of mLRP4T100(Y63A) was significantly lower than that of mLRP4T100 (5). Fig. 5 shows that the endocytosis rate of mLRP4T100(Y63A) is slightly lower than that of mLRP4-megalin, similar to that of mLRP4-LDLR, but is significantly higher than that of VLDLR and apoER2. These results confirm that the LDLR tail and the megalin tail display reduced endocytosis compared with the LRP tail, whereas VLDLR and apoER2 exhibit extremely low endocytosis rates.

**Low Efficiency in Ligand Degradation by mLRP4-LDLR, mLRP4-megalin, VLDLR, and apoER2**—Having established that there are significant differences in the endocytic function among the tails of the LDLR family members, we then investigated ligand degradation efficiency for these receptors. To assess relative amounts of the receptors on the cell surface, we analyzed the ligand binding activity of the receptors stably expressed in CHO cells at 4 °C. As shown in Fig. 6, CHO cells expressing mLRP4T100 exhibited a moderate level of cell surface 125I-RAP binding, whereas CHO cells transfected with the pcDNA3 vector alone exhibited only ~10% of RAP binding seen with mLRP4T100. This minimal amount of RAP binding to pcDNA3-transfected cells is likely mediated by cell surface heparan sulfate proteoglycan (35). However, CHO cells expressing mLRP4-LDLR, mLRP4-megalin, VLDLR, and apoER2 exhibited higher levels of cell surface RAP binding activity, which correspond to 225, 299, 289, and 220% of mLRP4T100 125I-RAP binding activity, respectively (Fig. 6). Because RAP has similar binding affinities to mLRP4T100, LDLR, and apoER2 (21, 36, 37), we concluded that the cell surface level of mLRP4T100 is significantly lower than that of mLRP4-LDLR, mLRP4-megalin, VLDLR, and apoER2.

To quantify the efficiency of ligand degradation, we incubated stably transfected cell lines with 5 nM 125I-RAP for 4 h at 37 °C, and the amount of 125I-RAP bound at the surface in the steady state was determined by its susceptibility to release with acid buffer (pH 2.6). We also measured the amount of 125I-RAP that had been degraded and released into the me-
null CHO cells. Binding of 5 nM 125I-RAP to LRP-null CHO cells stably transfected with pcDNA3 or various receptors was carried out for 1 h at 4 °C in the absence or presence of 500 nM unlabeled RAP. Values are the average specific binding of triplicate determinations with the S.E. indicated by error bars. This experiment is a representative of two such experiments performed with similar data.

**DISCUSSION**

All members of LDLR family are recognized as cell surface endocytosis receptors. However, the endocytosis rates mediated by these receptors are unclear. In the present study, we provide direct evidence that there are distinct differences in endocytic functions among the tails of the LDLR family members. We found that the tail of LRP supports the most efficient endocytosis, whereas LDLR tail and megalin tail display reduced endocytosis rates compared with LRP tail, while VLDLR and apoER2 exhibit minimal endocytosis function.

It is not surprising that among all members of the LDLR family examined, the LRP tail supports receptor endocytosis to the greatest degree. LRP belongs to the class of receptors that undergo constitutive endocytosis in the presence or absence of ligand. This feature may be determined by the constant exposure of its endocytosis signals and is highlighted by its cell surface distribution concentrated within clathrin-coated pits (5, 38). The tail of LRP consists of 100 amino acid residues and contains multiple potential endocytosis signals including two NPXY, one YXXL, and two dileucine motifs. Our recent studies indicate that the YXXL motif within the cytoplasmic tail of LRP serves as the dominant signal for LRP endocytosis. Furthermore, the distal dileucine motif and a serine phosphorylation in its cytoplasmic tail also contributes to the endocytosis of LRP (21). At present, it has been reported that LRP can bind and internalize over 20 structurally and functionally distinct ligands. In this report, we have demonstrated that LRP possesses extremely fast endocytosis rate, which is consistent with its major function as a clearance receptor.

In the present study, we found that the endocytosis rates mediated by mLRP4-LDLR, mLRP4-megalin, VLDLR, and apoER2 are significantly lower than that of mLRP4T100. One common characteristic of the LDLR family members is that each contains at least one copy of the NPXY sequence within the cytoplasmic tail. The tails of LDLR, VLDLR, and apoER2 contain one copy of the NPXY sequence, whereas the megalin tail contains two copies of the NPXY sequence. The NPXY motif in the LDLR has been shown to serve as a signal for receptor endocytosis through coated pits (4). Thus, it is possible that the NPXY motifs within the tails of VLDLR, apoER2, and megalin also function as their dominant endocytosis signals.

In contrast to other family members, we found that the tails of VLDLR and apoER2 support limited endocytosis. Recently, it has been demonstrated that VLDLR and apoER2 serve as obligate components in Reelin/Dab1-mediated neuronal migration (10, 11, 39, 40). Mice that lack the genes for both VLDLR and apoER2 demonstrate a neurological and neuroanatomical...
endothelial phenotype that is indistinguishable from that seen in animals deficient in either Reelin or Dab1 (11). Thus, signal transduction is likely the main function of VLDLR and apoER2.

It is worthy of note that compared with other members of the LDLR family, apoER2 exhibits extremely low level of ligand degradation efficiency. This observation is in agreement with previous report by Sun and Soutar (37), who found that β-VLDL was poorly degraded by apoER2 or its spliced variants compared with LDLR. The mechanistic basis thereof is not presently known. apoER2 shows high homology to both the LDLR family (10–15). Thus, the present studies extend our understanding of the differences in the biological activities of members of the LDLR family. Rapid receptor internalization mediated by receptors such as LRP may result in rapid desensitization of signals initiated by receptor ligation, whereas slow endocytosis mediated by the VLDLR and apoER2 may allow for more sustained signal transduction upon ligand binding.

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