Rapid Endocytosis of the Low Density Lipoprotein Receptor-related Protein Modulates Cell Surface Distribution and Processing of the β-Amyloid Precursor Protein

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The low density lipoprotein receptor-related protein (LRP) is a ~600-kDa multifunctional endocytic receptor that is highly expressed in the brain. LRP and its ligands apoE, α2-macroglobulin, and β-amyloid precursor protein (APP) are genetically linked to Alzheimer disease and are found in characteristic plaque deposits in brains of patients with Alzheimer disease. To identify which extracellular domains of LRP interact with APP, we used minireceptors of each of the individual LRP ligand binding domains and assessed their ability to bind and degrade a soluble APP fragment. LRP minireceptors containing ligand binding domains II and IV, but not I or III, interacted with APP. To test whether APP trafficking is directly related to the rapid endocytosis of LRP, we generated stable Chinese hamster ovary cell lines expressing either a wild-type LRP minireceptor or its endocytosis mutants. Chinese hamster ovary cells stably expressing wild-type LRP minireceptor had less cell surface APP than pcDNA3 vector-transfected cells, whereas those stably expressing endocytosis-defective LRP minireceptors accumulated APP at the cell surface. We also found that the steady-state levels of the amyloid β-peptides (Aβ) are dictated by the relative expression levels of APP and LRP, probably reflecting the dual roles of LRP in both Aβ production and clearance. Together, these data establish a relationship between LRP rapid endocytosis and APP trafficking and proteolytic processing to generate Aβ.

Alzheimer disease (AD) is one of the leading causes of dementia in elderly persons. Although the cause of AD dementia is unknown, it is probably related to the deposition of characteristic extracellular amyloid plaques and intracellular neurofibrillary tangles found in the brains of patients with AD. Amyloid plaques are composed of fibrillar aggregates of amyloid-β peptides (Aβ), which are derived from the processing of a ~120-kDa transmembrane protein known as β-amyloid precursor protein (APP). APP, which exists in three main isoforms (APP695, APP715, and APP770), can follow two alternate post-translational processing pathways. In the amyloidogenic pathway, APP is cleaved first at a β-secretase site by the enzyme β-site APP-cleaving enzyme and subsequently by a γ-secretase within its intramembrane region to release the Aβ peptide.

Several studies have shown that APP internalization through the endocytic pathway can lead to Aβ generation (4–7). The APP tail has a YENPTY motif similar to tyrosine-based NPXY and YXXØ endocytic motifs (where X can be any amino acid and Ø is an amino acid with a bulky hydrophobic group) found in other endocytic receptors (8). A decrease in APP endocytosis caused either by mutations within its endocytosis motif or by potassium depletion, which inhibits the formation of clathrin-coated pits, results in an accumulation of APP at the cell surface and favors the non-amyloidogenic processing of APP.

The low density lipoprotein receptor-related protein (LRP) interacts with APP, and its expression facilitates APP processing via the amyloidogenic pathway. Cells overexpressing APP that are transiently transfected with LRP produce higher levels of Aβ and lower levels of sAPPα than mock-transfected cells (9). LRP may influence the processing of APP through both extracellular and intracellular interactions (10–12). Expression of the C-terminal fragment of LRP increases Aβ production and decreases the release of sAPPα, similar to the expression of full-length receptor (13). Blocking the extracellular interaction between APP and LRP with receptor-associated protein (RAP) results in a decrease in Aβ levels and an increase in cell surface APP and sAPPα levels (9). Consistent with these in vitro studies, overexpression of a functional LRP minireceptor in the PDAPP amyloid mouse model increases soluble brain Aβ in aged mice (14).

In this study, we found that minireceptors containing domains II and IV of LRP can bind and degrade a soluble APP fragment. Using endocytosis-deficient LRP minireceptors containing domain IV, we also found that the endocytosis rate of LRP influences the cell surface level of APP and Aβ secretion in...
LRP Endocytosis Alters APP Trafficking and Aβ Production

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MATERIALS AND METHODS

Antibodies and Reagents—The C-terminal APP antibody BC1 (recognizes amino acids 704–730) was the kind gift of Barbara Cordell (Scrio). The monoclonal APP antibody 6E10 raised against residues 1–17 of Aβ was purchased from Signet. A previously described monoclonal anti-HA antibody was used for FACS analysis of LRP minireceptors (15). GE10, 22C11 (RDI, Inc.), and 8E5 (gift from Kelly Bales, Lilly Research Laboratories) were used for FACS analysis of APP. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG was obtained from BD Biosciences. Soluble APP770α (sAPP770α) was purified from the media of human embryonic kidney cells stably overexpressing APP770 as described previously (18). Soluble APP695α (sAPP695α) and endogenous APP were precipitated with Protein-A agarose beads for 45 min, washed, and then incubated overnight with antibody at 4 °C. Immunocomplexes were eluted with 0.1% saponin. Successive incubations with anti-APP antibody 6E10 (Signet) or 22C11 (RDI, Inc.)/50 μg/ml and goat anti-mouse IgG-fluorescein isothiocyanate were carried out at 4°C for 1 h. Aβ was specifically degraded by CHO-mLRP2 cells. Aβ in the conditioned media was captured with antibody 2G3 or 21F12 for Aβ40 and Aβ42, respectively, and subsequently detected with biotinylated 266 or 3D6 antibody. Endogenous Aβ values were normalized to the amount of total protein in cell lysates. Human Aβ values were normalized to the amount of APP expression determined by Western blot analysis.

Human Aβ Clearence—Conditioned media from primary neurons derived from PDAPP mice were used as source of human Aβ. PDAPP mice overexpress mutated human APPV717F under the platelet-derived growth factor promoter (21). Diluted media were added to CHO-pDNA3 and CHO-mLRP4 cells (~500 pg of total Aβ), or blank wells without cells. After 24 h, media were collected with the addition of proteinase inhibitors and then spun at 20,200 × g for 5 min to remove cellular debris. Aβ remaining in the conditioned media was analyzed by ELISA using capturing antibody 2G3 for Aβ40 or 21F12 for Aβ42, and subsequent detection with biotinylated 3D6 antibody, specific for human Aβ. Remaining Aβ in the conditioned media was normalized to the amount of total protein in cell lysates. Values were normalized to that of CHO-pDNA3 cells.

RESULTS

To determine which extracellular domains of LRP are important for an interaction with APP, we assessed the degradation of a soluble purified APP770 fragment (sAPP770α) by cells expressing minireceptors of LRP containing each individual ligand-binding domain (CHO-mLRP1, mLRP2, mLRP3, or mLRP4) (22). To determine receptor-specific degradation, 125I-sAPP770α degradation was assessed in the absence or presence of excess amounts of the LRP antagonist RAP. RAP binds to domains II, III, and IV of LRP with high affinity (23). We found that 125I-sAPP770α was specifically degraded by CHO-mLRP2 and CHO-mLRP4 cells but not CHO-mLRP1 or CHO-mLRP3 cells (Fig. 1A). We detected similar levels of the 85-kDa subunit of the mature minireceptors among these cell lines (Fig. 1B) suggesting that the differences in the ability to degrade 125I-sAPP770α were not caused by differences in the expression levels of these minireceptors. In addition, mLRP2 and mLRP4, two LRP domain minireceptors that degraded 125I-sAPP770α, exhibited similar rates of endocytosis (Fig. 1C). None of the cell lines tested significantly degraded 125I-sAPP695α, which lacks a KPI domain (data not shown). These findings are in agree-
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Because APP and LRP interact through extracellular interactions mediated by the KPI domain of APP and also intracellular interactions via the adaptor protein FE65 (10–13,16), we tested whether endogenous APP within CHO LRP-null cells could interact with overexpressed LRP domain IV minireceptors. In a previous study from our laboratory, mLRP4 minireceptors containing site-directed mutations within putative cytoplasmic endocytosis motifs were generated and stably transfected into CHO LRP-null cells (15). Minireceptors bearing mutations within the YXXL motif (Y63A and L66A) and distal di-leucine repeat (L68A/L78A) (Fig. 3A) have a reduced rate of endocytosis compared with wild-type minireceptor (15). To determine whether an extracellular interaction could occur between the minireceptors and endogenous APP, we first examined whether CHO cell APP is KPI-containing APP similar to APP751 or non KPI-containing APP similar to APP695. Lysates from cells transiently transfected with hAPP751 or hAPP695 were compared with CHO LRP-null lysates. Immunoblot of lysates for APP revealed that CHO LRP-null cells contained an APP immunoreactive band that migrated at the same molecular size as APP751 and was larger than the molecular size of APP695, suggesting that endogenous CHO cell APP was primarily KPI-containing APP (Fig. 3B).

Next, we determined whether endogenous APP could interact with mLRP4 and its endocytosis mutants by co-immunoprecipitation. Immunoblot of APP immunoprecipitates with anti-HA antibody revealed immunoreactive bands that migrate at the same molecular sizes as the furin-cleaved processed form (~120 kDa) and the unprocessed form (~210 kDa) of LRP minireceptors (Fig. 3C). No anti-APP or anti-HA signals were detected in lysates immunoprecipitated with control IgG antibody (data not shown). We also detected an interaction between chimeric receptor compared with the wild-type LRP minireceptor (mLRP4), we found mLRP4-APPtail had a much slower rate of endocytosis (~10%/min) than mLRP4 (~70%/min) (Fig. 2B). These results support our hypothesis that LRP endocytosis could facilitate APP internalization and processing to the amyloidogenic pathway.

Fig. 2. The APP cytoplasmic domain confers a slower endocytosis rate to mLRP4 than the LRP cytoplasmic domain. A, schematic of mLRP4 and mLRP4-APPtail receptors. mLRP4 contains the fourth ligand binding domain of LRP, transmembrane region, and 100 amino acid tail of LRP. mLRP4-APPtail contains the extracellular and transmembrane regions of mLRP4 and the cytoplasmic domain of APP. mLRP4; □, APP, TM, transmembrane domain. B, CHO LRP-null cells stably transfected with mLRP4 (●) or mLRP4-APPtail (▲) were incubated with 5 nM 125I-RAP for 1 h at 4 °C and then shifted to 37 °C for the indicated times. The percentage of ligand internalized at each time point is equal to the amount of ligand internalized divided by the total cell associated ligand (see “Experimental Procedures”). Values are the mean average of triplicate determinations with the S.E. indicated by error bars.

Fig. 1. Soluble APP770 is degraded by CHO-mLRP2 and CHO-mLRP4 cells. A, CHO LRP-null cells stably transfected with mLRLP1, mLRLP2, mLRLP3, and mLRLP4 were incubated with 1 nM 125I-sAPP770a at 37 °C for 6 h in the presence or absence of excess unlabeled RAP (1 μM). Specific degradation was evaluated as described under “Experimental Procedures.” Values are the mean average of triplicate determinations; error bars indicate S.E. B, CHO LRP-null cells stably transfected with mLRLP1, mLRLP2, mLRLP3, and mLRLP4 express similar cell surface levels of the minireceptor. Equal amounts of cell protein were separated by SDS-PAGE and blotted with a polyclonal antibody raised to the 85-kDa furin-cleaved C-terminal fragment of LRP. After detection with ECL Plus, fluorescence intensity in the 85-kDa band (indicated by arrowhead in the blot on the right) was quantitated for each cell line using a variable mode imager. Values are the mean average of triplicate determinations with the S.E. indicated by error bars. No significant differences were observed between the cell lines by analysis of variance. C, CHO LRP-null cells stably transfected with mLRLP2 or mLRLP4 were incubated with 2 nM 125I-labeled anti-HA IgG for 1 h at 4 °C and then shifted to 37 °C for the indicated times. The percent of ligand internalized at each time point is equal to the amount of ligand internalized divided by the total cell-associated ligand (see “Experimental Procedures”). Values are the mean average of triplicate determinations with the S.E. indicated by error bars.
APP and mLRP4Tailess. Because the major form of APP in CHO cells contained the KPI domain that can bridge an extracellular interaction with LRP, it is probably that the association between LRP minireceptors and APP are predominantly extracellular and independent of cytoplasmic interactions.

After confirming an interaction between endogenous APP and the LRP minireceptors, we tested whether the rate of LRP endocytosis could influence the trafficking of APP by comparing cell surface APP between cells expressing mLRP4 and mLRP4 endocytosis mutants by FACS analysis. We detected significantly less cell surface APP in CHO-mLRP4 cells compared with CHO-pcDNA3 cells (Fig. 4A). In support of our hypothesis, cells expressing endocytosis mutants of mLRP4, mLRP4(Y63A), mLRP4(L66A), mLRP4(Y63A, L66A/L87A), and mLRP4Tailess exhibited ≥3-fold more cell surface APP compared with CHO-mLRP4 wild-type cells (Fig. 4B). To confirm that the effect of LRP overexpression on APP distribution was specific to APP, we assessed for changes in the amount of another cell surface receptor, the transferrin receptor, within another cell surface receptor, the transferrin receptor, within cell lines and equal amounts of protein were subjected to SDS-PAGE. Blots were probed with anti-APP antibody and detected with fluorescein isothiocyanate-conjugated goat anti-mouse IgG by FACS analysis. We found that expression of mLRP4 or its endocytosis mutants did not alter total cellular APP. By both FACS analysis (Fig. 4C, top) and immunoblot (Fig. 4C, bottom), total APP levels were similar in pcDNA3, mLRP4, and endocytosis mutants of mLRP4. Taken together, these results suggest that changes in cell surface APP were caused solely by altered trafficking of APP mediated by the expression of LRP minireceptors.

Mutations in the LRP tail that do not influence the endocytosis rate of LRP may also change APP trafficking because of potential structural alterations that may interfere with their ability to interact with APP. Therefore, we measured levels of cell surface APP in CHO cells stably transfected with LRP minireceptors containing mutations that had no change in endocytosis rate compared with wild-type mLRP4. CHO cells expressing LRP minireceptors with mutations within the proximal NPXY (N26A and Y29A), proximal di-leucine motifs (L43A/L44A), or the distal NPXY motif (N60A) had no significant effects on cell-surface distribution of APP compared with cells expressing wild-type LRP minireceptor (Fig. 5). These data indicate that the fast endocytosis rate of LRP is the major contributor to alterations in the cellular distribution of APP.

The above results suggest that LRP endocytosis decreases cell surface APP levels by increasing its endocytosis rate. Be-
cause Aβ has been shown to be produced within endocytic compartments, we assessed the effect of LRP minireceptor expression on APP processing to Aβ by measuring endogenous levels of Aβ40 and Aβ42 in conditioned media of cells expressing pcDNA3 or mLRP4 by ELISA. We found a trend toward less Aβ40 and significantly less Aβ42 in 48-h conditioned media from CHO-mLRP4 cells compared with CHO-pcDNA3 cells (Fig. 6A).

Based on our hypothesis and previous published studies (9, 13), we expected to find increased secreted Aβ levels in CHO-mLRP4 cells. Because CHO-mLRP4 cell media had less Aβ than that of the vector control cells, we considered the possibility that the Aβ clearance pathway mediated by the LRP minireceptor was dominant over the production pathway. To test whether Aβ could be cleared by CHO-mLRP4 cells, we incubated CHO-pcDNA3 and CHO-mLRP4 cells with human Aβ obtained from conditioned media of PDAPP mouse primary cultured neurons. To measure the disappearance of human Aβ without the interference of endogenously produced CHO cell hamster Aβ, we used an ELISA that specifically detects human Aβ but not the endogenously produced hamster Aβ. We found that cells expressing mLRP4 cleared significantly more human Aβ40 and Aβ42 than CHO-pcDNA3 cell lines (Fig. 6B). These findings suggest that the lower levels of endogenous Aβ detected in CHO-mLRP4 cells could be caused by increased clearance mediated by the overexpressed LRP minireceptor.

Because previous studies reporting increased Aβ levels with LRP expression used cells overexpressing human APP (9, 13), we generated CHO LRP-null stable cell lines that overexpressed human APP770 together with pcDNA3 or mLRP4. After selecting stable clones with equal expression of APP as detected by Western blot analysis, we measured steady state levels of human Aβ40 and Aβ42 in conditioned media of CHO-pcDNA3 cells compared with CHO-mLRP4 cells. Opposite to our findings with endogenous Aβ, but in agreement with previously published reports, we found ~3-fold more Aβ40 and Aβ42 levels in CHO-mLRP4 cells compared with CHO-pcDNA3 with overexpression of APP770 (Fig. 6C).

**DISCUSSION**

Several lines of evidence suggest that LRP has a role in AD pathogenesis either by interacting with APP and influencing its processing (9, 13, 16), or as a clearance receptor for Aβ (25–27). The objective of the current study was to investigate how LRP expression and rate of endocytosis influence APP cellular distribution and processing to Aβ. Using LRP-deficient CHO cells overexpressing functional LRP minireceptors, we found that the extracellular domains II and IV of LRP can both individually bind and degrade a soluble form of the KPI-containing APP (sAPP770α), but not a soluble KPI-lacking APP (sAPP695α). In addition, the cellular degradation of soluble APP770 by CHO-mLRP2 and CHO-mLRP4 was blocked by excess RAP, an LRP antagonist. These results are consistent with previous findings that a KPI domain is necessary for the interaction between APP and LRP (18). Similar to our findings, it has been shown that another KPI-containing ligand of LRP, tissue factor pathway inhibitor, specifically interacts with ligand-binding domains II and IV of LRP but not domains I or III (28). Most LRP ligands have been shown to bind domains II and IV of LRP, and no ligands have been reported to bind to domain I (29). Although the predominant APP isoform in neurons does not contain a KPI domain (APP695), the proportion of KPI-containing forms of APP is significantly elevated in AD brains (30). Therefore, it is possible that increased extracellular interactions between LRP and KPI-containing isoforms of APP could contribute to the Aβ accumulation seen in AD.

We also established that the LRP minireceptor containing the ligand binding domain IV of LRP (mLRP4) interacts with endogenous CHO cell APP and that this interaction leads to decreased cell surface APP levels compared with pcDNA3 vector-transfected cells. In addition, cells expressing endocytosis-deficient mLRP4, which were able to interact with endogenous CHO cell APP, showed an accumulation of APP at the cell surface. These results are consistent with our previous findings showing that overexpression of a minireceptor of LRP1B, a member of the LDLR family that has high homology to LRP but significantly slower rate of endocytosis, interacts with APP and leads to its cell surface accumulation (31). More importantly, mLRP1B4-expressing CHO cells also produce less Aβ and secrete more soluble APP (31).

Previous studies have shown that retention of APP at the cell surface decreases Aβ production and increases sAPPα (5–7, 32). Overexpression of an LRP C-terminal fragment in MEF LRP−/− cells bearing a point mutation within its distal NPXY motif increased sAPPα levels compared with a wild-type LRP fragment (13). This mutation is equivalent to the mLRP4(Y63A) endocytosis mutant from which we detected an increase in cell surface APP. Our findings that a chimeric mLRP4/APPtail receptor had markedly decreased rate of endocytosis compared with wild-type mLRP4 further suggest that, upon interaction of LRP and APP at the cell surface, the fast rate of endocytosis of LRP could indeed facilitate the trafficking of APP within the endocytic compartments.

It has been postulated that APP endocytosis facilitates Aβ generation because it brings both APP and the β-secretase β-site APP-cleaving enzyme, which is concentrated in lipid rafts, into close proximity (33, 34). Furthermore, β-site APP-cleaving enzyme optimum activity would be favored at the slightly acidic pH of endosomes (19). If LRP association with APP at the cell surface increases APP trafficking within the endocytic compartments where Aβ can be generated, we expected Aβ secretion in the media to be higher in CHO-mLRP4 cells than in CHO-pcDNA3 control cells. Past reports in the literature have indeed shown that LRP expression is associated with increased Aβ levels in the media (9, 13). Ulery et al. (9)
reported a 3-fold increase in Aβ levels in CHO cells stably transfected with APP751 and transiently transfected with full-length LRP. Pietzrik et al. (13) found that Aβ levels were increased in LRP+/− mouse embryonic fibroblasts stably transfected with APP751 compared with LRP−/− mouse embryonic fibroblasts. However, contrary to our expectations and to these previous studies, we found decreased endogenous Aβ in the media of CHO-mLRP4 cells compared with CHO-pcDNA3 cells and no alterations in Aβ levels with mLRP4 endocytosis mutant receptor expression. Although we were initially surprised with these findings, a detailed comparison between these studies and ours revealed a major difference between them. Whereas we analyzed the effect of LRP overexpression on endogenous Aβ secretion, the other studies used cells overexpressing human APP to evaluate the effect of LRP on Aβ secretion. In view of these differences, we considered the possibility that when cells did not overexpress APP, the Aβ clearance pathway mediated by the LRP minireceptor could be dominant over its effect on increasing Aβ production, leading to the decreased steady-state levels of Aβ in the media.

Supporting this hypothesis, we found that the disappearance of exogenously added human Aβ was significantly greater in CHO-mLRP4 than that of CHO-pcDNA3 control cells. These results were consistent with the findings that LRP can bind and clear Aβ either directly (25) or indirectly via LRP ligands (e.g. apolipoprotein E and α2-macroglobulin) (26, 27). The ability of CHO-mLRP4 cells to clear Aβ more effectively could therefore explain why lower levels of Aβ were detected in CHO-mLRP4 than in CHO-pcDNA3 control cells. This hypothesis was further confirmed by the generation of CHO-mLRP4 stably transfected with human APP770, which showed increased Aβ levels over pcDNA3 control cells, similarly to previous reports (9, 13).

Taken together, our data support a link between LRP endocytosis, APP trafficking, and APP proteolytic processing. Previous in vivo studies from our laboratory have shown that overexpression of mutated human APPV717F and LRP minireceptor increases Aβ levels. Subsequent CHO LRP-null cells stably transfected with APP770 together with pcDNA3 vector or mLRP4 were incubated in low-serum media. After 24 h, media were analyzed for Aβ40 and Aβ42 levels by ELISA. Aβ levels were normalized to the amount of Aβ in the corresponding cell extracts. Values shown are normalized to pcDNA3 control and are the mean average of triplicate determinations from two experiments with Student’s two-tailed t test. * , p < 0.001 compared with control by Student’s two-tailed t test.

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