Sequences from the Low Density Lipoprotein Receptor-related Protein (LRP) Cytoplasmic Domain Enhance Amyloid β Protein Production via the β-Secretase Pathway without Altering Amyloid Precursor Protein/LRP Nuclear Signaling*

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Increasing evidence suggests that the low density lipoprotein receptor-related protein (LRP) affects the processing of amyloid precursor protein (APP) and amyloid β (Aβ) protein production as well as mediates the clearance of Aβ from the brain. Recent studies indicate that the cytoplasmic domain of LRP is critical for this modulation of APP processing requiring perhaps a complex between APP, the adaptor protein FE65, and LRP. In this study, we expressed a small LRP domain consisting of the C-terminal 97 amino acids of the cytoplasmic domain, or LRP-soluble tail (LRP-ST), in CHO cells to test the hypothesis that the APP-LRP complex can be disrupted. We anticipated that LRP-ST would inhibit the normal interaction between LRP and APP and therefore perturb APP processing to resemble a LRP-deficient state. Surprisingly, CHO cells expressing LRP-ST demonstrated an increase in both sAPP secretion and Aβ production compared with control CHO cells in a manner reminiscent of the cellular effects of the APP “Swedish mutation.” The increase in sAPP secretion consisted mainly of sAPPβ, consistent with the increase in Aβ release. Further, this effect is LRP-independent, as the same alterations remained when LRP-ST was expressed in LRP-deficient cells but not when the construct was membrane-anchored. Finally, deletion experiments suggested that the last 50 amino acid residues of LRP-ST contain the important domain for altering APP processing and Aβ production. These observations indicate that there are cellular pathways that may suppress Aβ generation but that can be altered to facilitate Aβ production.

The low density lipoprotein receptor-related protein (LRP) is a large type I transmembrane protein that functions as a multifunctional endocytosis receptor for a diverse array of extracellular ligands (1, 2). LRP is synthesized as a 600-kDa precursor protein that is subsequently cleaved in the trans-Golgi compartment by furin to generate a large 515-kDa α-chain and a smaller 85-kDa membrane-associated β-chain that remain non-covalently linked (3, 4). The cytoplasmic tail contains two NPXY endocytosis motifs and binds a number of cytoplasmic adaptor and scaffold proteins, such as FE65, Mint2, Disabled-1 (Dab1), Shc, and JIP-1 and -2, probably through the second NPXY motif (5–7). In addition, LRP itself undergoes a presenilin-dependent γ-secretase intramembrane proteolysis, releasing a potentially transcriptionally active fragment in a manner similar to APP and Notch (8, 9).

Recent studies have implicated a role for the LRP pathway in Alzheimer disease pathogenesis. LRP and three of its key ligands, apoE, α2-macroglobulin, and APP, are genetically associated with Alzheimer disease and are found in senile plaques in the brains of Alzheimer disease patients (10–13). LRP also mediates the binding and clearance of amyloid β peptide (Aβ) complexes bound to apoE or α2-macroglobulin in cultured cells and in the brain (14–16). In addition, LRP may play a crucial role in brain efflux of Aβ isoforms at the blood-brain barrier (17, 18). These findings therefore support a model in which LRP plays an important role in Aβ uptake and removal.

In a dual but opposing effect, it has been proposed that LRP can also promote Aβ production by altering the trafficking and processing of APP, possibly by APP/LRP interactions via the Kunitz protease inhibitor domain as well as through cytoplasmic adaptor proteins (7, 19–23). Absence of LRP or treatment of receptor-associated protein, an antagonist of all known LRP ligands, substantially reduced Aβ release, a phenotype that was reversed when full-length or truncated LRP was transfected in LRP-deficient cells (20, 21). The domain in LRP that is responsible for regulating APP processing and Aβ production was mapped to the cytosolic tail, specifically, a seven-amino acid motif that included the distal NPVY site (20). These results suggested that the cytoplasmic domain of LRP plays a major role in APP processing.

We hypothesized that formation of a LRP-APP complex is important for normal APP processing and have shown that indeed, APP and LRP appear to be functionally linked by the adaptor protein FE65 (24). In this study, we tested the idea that blocking the interaction between LRP and APP could reduce Aβ production in a manner similar to that observed in LRP-deficient cells. We hypothesized that expression of a LRP construct, consisting of only the last 97 amino acid residues of the cytosolic tail of LRP, will block LRP/APP interaction and, in so doing, alter APP processing and Aβ generation. Surprisingly, expression of the soluble LRP cytosolic fragment did not impair Aβ production as expected. Instead this fragment al-
its N terminus using PCR. Myc-LRP-ST constructs contain the C-terminal 97 amino acid residues of LRP fused to a Myc tag (abbreviated as LRP-ST for soluble tail). The Myc-LRP-STΔNP"Y and Myc-LRP-STΔNP"Y are engineered from the Myc-LRP-ST construct, in which the first and second NPXY motif of LRP-ST was deleted, respectively. cDNA constructs used in this study. Diagram represents cDNA constructs used in this study. Myc-LRP-ST constructs were cloned into the pcDNA3 vector (Clontech, Palo Alto, CA) for retrovirus production. Plasmid pRL-TK encoding firefly luciferase (pG5E1B-luc) have been described (26)

**FIG. 1.** cDNA constructs used in this study. Diagram represents cDNA constructs used in this study. Myc-LRP-ST constructs contain the C-terminal 97 amino acid residues of LRP fused to a Myc tag (abbreviated as LRP-ST for soluble tail). The Myc-LRP-STΔNP"Y and Myc-LRP-STΔNP"Y are engineered from the Myc-LRP-ST construct, in which the first and second NPXY motif of LRP-ST was deleted, respectively. (LRP-ST1–47) contains the first 47 amino acid residues of LRP, (LRP-ST48–97) contains the last 50 amino acid residues of LRP-ST and has a FLAG tag at its N terminus. The FA-LRP-ST construct, in which the fatty acylation (i.e. myristoylation and palmitoylation) signal sequence of Fyn tyrosine kinase at its N terminus using PCR. Myc-LRP-ST constructs contain the C-terminal 97 amino acid residues of LRP fused to a Myc tag (abbreviated as LRP-ST for soluble tail). The Myc-LRP-STΔNP"Y and Myc-LRP-STΔNP"Y are engineered from the Myc-LRP-ST construct, in which the first and second NPXY motif of LRP-ST was deleted, respectively. (LRP-ST1–47) contains the first 47 amino acid residues of LRP, (LRP-ST48–97) contains the last 50 amino acid residues of LRP-ST and has a FLAG tag at its N terminus. The FA-LRP-ST construct, in which the fatty acylation (i.e. myristoylation and palmitoylation) signal sequence of Fyn tyrosine kinase at its N terminus using PCR.
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FIG. 2. Stable expression of Myc-LRP-ST (MycST) in CHO cells overexpressing APP751 (APP-WT CHO). Full-length (FL-LRP) and the β-chain of LRP are recovered in the membrane fraction (A, upper panel) and detected using LRP polyclonal antibody 1704. The Myc-LRP-ST fragment in the cytosolic fraction is detected using LRP (A) or Myc (B) antibodies. Asterisk indicates the ~25-kDa C-terminal fragment of LRP representing the substrate for γ-secretase intramembrane proteolysis (9).

lysates were separated into cytosolic and membrane fractions, full-length LRP (~600 kDa) and LRP β-chain (~85 kDa) were detected in membrane fractions from both parental control and Myc-LRP-ST-transfected cells (Fig. 2A, upper panel). As anticipated, the Myc-LRP-ST lacking the transmembrane domain was detected in the cytosolic fraction of MycST cells only. The recently detected ~25-kDa C-terminal fragment of LRP representing the substrate for γ-secretase intramembrane proteolysis (9) was also detected in membrane fractions of both cell lines (Fig. 2A, lower panel, asterisk). Expression of the Myc-LRP-ST fragment was also detected by immunoblotting with an antibody that recognized the Myc epitope tag (Fig. 2B). Expression of Myc-LRP-ST did not alter the expression of APP (Fig. 3, top panel). However, there was a significant ~2-fold increase in the level of APP CTF in MycST cells as compared with control APP-WT CHO cells (Fig. 3, middle panel). Previously, we reported that in LRP-deficient cells, the levels of APP CTFs were substantially reduced (20).

We next assayed for sAPP secretion, because CTF generation and sAPP secretion are frequently correlated with each other. As predicted, there was an ~2-fold increase in total sAPP secretion in conditioned medium from MycST cells as compared with controls (Fig. 4A). To determine whether the α- or β-secretase-derived sAPP was preferentially affected by LRP-ST, the increased sAPP, CTFs, and Aβ levels in MycST cells were because of the increased stability of APP, we next examined the turnover of full-length APP by pulse-chase analysis in APP-WT CHO and MycST cells. However, there was no change in the turnover rate of APP in MycST cells as compared with APP-WT CHO cells (Fig. 4A and B). Similarly, the increase in CTF levels in MycST cells might also be due to its enhanced stability. As with full-length APP, there was no significant difference in the half-life of APP CTFs (~2 h) in MycST cells as compared with controls (Fig. 5C), suggesting that the increased CTF level in MycST cells is secondary to enhanced production.

In CHO cells, a major pathway for the generation and secretion of Aβ appears to be derived from endocytic processing of APP from the cell surface (30, 31). Accordingly, we next measured the internalization of APP using an iodinated APP antibody (1G7). The internalization of APP was expressed as a ratio between the ~125I-1G7 antibody bound to intracellular and cell-surface APP. Although APP internalization was essentially unchanged (7% reduction, Fig. 5D), a 30% reduction in steady-state levels of cell-surface APP was observed in MycST cells as compared with control APP-WT CHO cells (Fig. 5E). These results suggested that LRP-ST altered the processing of APP in compartments prior to trafficking to the cell surface. Such a phenotype resembles that of the APP “Swedish mutation” where decreased cell-surface APP and increased β-secretase cleavage products were documented (31, 32). However, we failed to document any increase in either the protein or in the mRNA level of β-secretase (data not shown).

The alterations in the level of cell-surface APP level as well as the changes in APP/Aβ processing in MycST cells indicated that there may be perturbed trafficking of APP to different subcellular organelles. Therefore, we examined the subcellular localization of APP by immunolocalization and sucrose density gradient fractionation after sodium carbonate extraction or Triton X-100 solubilization. However, we could not detect any significant changes in APP subcellular localization in MycST cells with either of these two approaches (data not shown).

Effect of MycST Deletion Mutants on APP Processing—A recent study using the yeast two-hybrid assay reported that the cytoplasmic tail of LRP could interact with several functionally distinct proteins, such as JIP1 and -2, PSD-95, and Mint2 (6). In addition, it has been shown that the NPXY motifs of both LRP and APP cytoplasmic tails bind to adaptor proteins FE65, mDab, and Shc (5–7, 24, 26, 33). Because FE65, Shc, and Mint proteins have been shown to differentially modulate Aβ secretion (33–37), it is possible that binding of the cytoplasmic adaptor proteins to the NPXY motifs of the LRP cytoplasmic tail region could account for the increased Aβ secretion observed in MycST cells. Thus, we first expressed Myc-LRP-ST constructs lacking the first (Myc-LRP-ST ∆NPVTY) or second (Myc-LRP-ST ∆NPVY) NPXY motifs in parental APP-WT CHO cells.
Surprisingly, expression of both Myc-LRP-ST deletion constructs still increased the levels of Aβ as compared with control APP-WT CHO cells (Fig. 6A), indicating that neither NPYY core motif alone in LRP-ST is functional with respect to the increase in Aβ production. Based on these results, we therefore hypothesized that regions flanking either one or both NPYY motifs must account for the increased Aβ secretion by LRP-ST. To test this possibility, additional mutant LRP-ST constructs were engineered. Although expression of the last 50 amino acid residues of LRP-ST (LRP-ST48–97) still increased Aβ secretion, expression of the first 47 amino acid residues of LRP-ST (LRP-ST1–47) had no effect on Aβ secretion in APP-WT CHO cells (Fig. 6B). These results suggest that amino acid residues in between positions 48 and 97 but not the core NPYY motif of LRP-ST (residues 57NFTNPVYATL66) are critical for the increased production of Aβ following forced expression of the LRP cytosolic tail.

**Effect of Translocation of the LRP-ST to the Membrane Fraction on APP Processing**—Because LRP-ST is soluble, we infer that it modulates APP processing in the cytoplasm. This notion predicts that translocation of LRP-ST to the membrane should attenuate or even abolish its effect on APP processing. To test this hypothesis, we engineered a mutant LRP-ST construct (FA-LRP-ST), which contains the fatty acylation (i.e. myristoy-
FIG. 5. Turnover of full-length APP and APP CTF, APP internalization and surface APP level in MycST cells. There is no change in either APP maturation and turnover or APP CTF turnover in MycST cells as compared with APP-WT CHO cells. Representative autoradiograph from pulse-chase experiments after metabolic labeling with [35S]methionine/cysteine is shown for APP (A) and quantitation of full-length APP and APP CTF turnover from three separate experiments expressed as mean ± S.E. (B and C, respectively). Steady-state internalization of APP from the cell surface (D) and the cell-surface APP levels (E) were measured with iodinated 1G7 antibody at 37 °C and at 4 °C, respectively, as described under “Experimental Procedures.” The ratios of internalized to cell-surface APP and the surface APP levels in MycST cells are normalized to the APP-WT CHO cells. The APP internalization was comparable between the two cell lines. However, there was ~30% reduction in the cell-surface APP level in MycST cells. *, p < 0.05; **, p < 0.0001.
Reduced Aβ level in conditioned media from APP-WT CHO cells stably expressing N-terminal 47 amino acids of LRP-soluble tail (LRP-ST1–47). Deletion of the first (Myc-LRP-ST-NPTY) or second (Myc-LRP-ST-NPVY) NPXY domain of LRP-ST had no effect on the elevation of Aβ levels induced by LRP-ST construct as compared with APP-WT CHO cell level (A). When APP-WT CHO cells were stably transfected with the construct in which the second half of LRP-ST was deleted (LRP-ST1–47), but not the proximal half (LRP-ST48–97), Aβ levels in conditioned media were restored to control levels (B). When the fatty acylation signal peptide of Fyn tyrosine kinase was appended to the N terminus of LRP-ST (FA-LRP-ST), most of the expressed FA-LRP-ST was found in the membrane fraction (E, middle panel). Prolonged exposure of the immunoblot revealed low levels of FA-LRP-ST fragment in the cytosolic fraction (E, bottom panel). The translocation of the LRP-ST fragment from cytosolic to membrane fraction caused a reduction in both secreted sAPP (C) and Aβ levels (D). *, p < 0.001; †, p < 0.05 compared with control APP-WT cells.
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**Fig. 7.** Effect of Myc-LRP-ST is independent of endogenous LRP expression. Transfection of Myc-LRP-ST in LRP-deficient 13-5-1 CHO cells increased sAPP and Aβ secretion similar to that seen in APP-WT CHO cells. Comparable levels of full-length APP (FL-APP) are seen in the two 13-5-1 cell lines. Immunoblots for FL-APP, sAPP, and Aβ were performed using antibodies CT15, 1G7/5A3, and 26D6, respectively.

**Fig. 8.** Myc-LRP-ST does not inhibit AICD-mediated transcription in a heterologous reporter assay system. Co-expression of an APP-Gal4 fusion protein induced only minimal transactivation of the gene (luciferase). Co-expression of FE65 and APP-Gal4 fusion protein strongly stimulated transcription of the Gal4-dependent reporter that was not attenuated by Myc-LRP-ST. No induction of reporter gene activity was seen by co-expression of FE65 with a mutant APP-Gal4 construct, where the NPTY motif was deleted (APPΔ-Gal4). The results are expressed as fold induction in transcription over control cells expressing the Gal4 DNA-binding domain alone.

**Discussion**

Increasing evidence suggests that LRP has opposing effects on APP processing and Aβ metabolism. On one hand, LRP mediates the clearance of Aβ either by itself or following complex formation of Aβ with various LRP ligands (14–18). On the other, LRP also influences multiple steps in APP processing to modulate Aβ formation (20–22). The latter appears to involve an interaction between LRP and APP that is mediated by FE65 as an adaptor molecule linking the respective cytosolic domains to form a functional tripartite complex (24). To further investigate the mechanism by which LRP modulates Aβ generation, we hypothesized that expression of the LRP cytosolic domain, LRP-ST, should influence multiple steps in APP processing to modulate Aβ generation. To our surprise, expression of LRP-ST in CHO cells not only did not impair Aβ generation, we found that Aβ generation was paradoxically increased. These results indicate that the subcellular localization of LRP-ST is independent for its role in APP processing.

**Effects of LRP-ST on APP Processing Do Not Require Endogenous LRP Expression**—Although we initially predicted that LRP-ST would block the APP/LRP interaction to lower Aβ production, this outcome was not seen in the studies. This indicated that LRP-ST may not be blocking the APP/LRP complex. Accordingly, to test whether the perturbations in APP processing we observed are independent of LRP, the LRP-ST construct was expressed in the CHO 13-5-1 cell line that is deficient in LRP. Surprisingly, both sAPP and Aβ levels were indeed increased in 13-5-1 cells expressing Myc-LRP-ST, much like that seen in LRP-expressing APP-WT CHO cells (Fig. 7). Thus, these results are consistent with the hypothesis that the LRP-ST effect on APP processing is independent of endogenous LRP and therefore did not inhibit APP/LRP interaction as predicted.

**Myc-LRP-ST Expression Does Not Impair AICD/FE65-mediated Transcription**—Recently, it was reported that the expression of the cytoplasmic domain of LRP abolished the transcriptional activation mediated by the APP intracellular domain released after β-secretase cleavage, AICD, and FE65 (8). Therefore, we examined whether abnormal APP processing by Myc-LRP-ST expression is associated with changes in AICD/FE65-mediated transcription. As expected, co-transfection of FE65 together with APP-Gal4 substantially increased the transactivation of the reporter, whereas FE65 was unable to augment the transactivation of the mutant APP lacking the NPXY domain (APPΔ-Gal4) (Fig. 8). However, co-transfection of Myc-LRP-ST had no effect on the transcriptional activation mediated by AICD/FE65 (Fig. 8). Thus, these data indicate that transcriptional consequences associated with AICD/FE65 do not underlie the changes in abnormal APP processing seen by LRP-ST expression.
JNK-interacting proteins (JIP1b and JIP2) (41) and mDab (7, 42, 43). These studies suggested that interactions between the GY-ENPTY motif of APP and the phosphotyrosine-binding/protein interaction (PTB/PI) domains of some of these adaptor proteins can modulate Aβ secretion (34, 36, 41). Because LRP also binds to JIP-1, mDab, and FE65 probably through its distal NPXY motif (6, 7, 24), it is possible that LRP-ST might effectively compete with APP for binding to adaptor proteins to influence Aβ secretion. In our study, expression of the distal half of LRP-ST (LRP-STΔ48–97 fragment) containing the distal NPXY domain, but not the proximal half (LRP-ST1–47 fragment), increased Aβ secretion (Fig. 6). This suggests that the sequestration of adaptor proteins (e.g. JIP-1b, mDab, and FE65) by LRP-ST might account for this phenotype. However, removal of the second NPVY motif alone (Myc-LRP-STΔNPVY) was not sufficient to abrogate the effects of LRP-ST on Aβ secretion, indicating that LRP-ST was interfering with other unknown target molecules that interact with additional sequences beyond the NPVY motif. These findings led us to postulate that LRP-ST may either facilitate the trafficking of APP to compartments containing BACE or enhance BACE-mediated cleavage of APP. The latter scenario is consistent with the recent observations that phosphorylation of APP on BACE-mediated cleavage of APP. The latter scenario is consistent with the recent observations that phosphorylation of APP on

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J. Biol. Chem. 2005, 280:20140-20147.
doi: 10.1074/jbc.M413729200 originally published online March 16, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M413729200

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