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GGA1-mediated endocytic traffic of LR11/SorLA alters APP intracellular distribution and amyloid-β production

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Abstract: Proteolytic processing of the amyloid-β precursor protein (APP) and generation of amyloid-β peptide (Aβ) are key events in Alzheimer’s disease (AD) pathogenesis. Cell biological and genetic evidence has implicated the low-density lipoprotein and sorting receptor LR11/SorLA in AD through mechanisms related to APP and Aβ production. Defining the cellular pathway(s) by which LR11 modulates Aβ production is critical to understanding how changes in LR11 expression affect the development of Aβ pathology in AD progression. We report that the LR11 ectodomain is required for LR11-mediated reduction of Aβ and that mutagenesis of the LR11 Golgi-localizing, γ-adaptin ear homology domain, ADP-riboseylation factor (GGA)-binding motif affects the endosomal distribution of LR11, as well as LR11’s effects on APP traffic and Aβ production. Targeted small interfering RNA (siRNA) knockdown studies of GGA1, GGA2, and GGA3 indicate a surprising degree of specificity toward GGA1, suggesting that GGA1 is a candidate regulator of LR11 traffic. Additional siRNA knockdown experiments reveal that GGA1 is necessary for both LR11 and β-site APP-cleaving enzyme-1 (BACE1) modulation of APP processing to Aβ. Mutagenesis of BACE1 serine 498 to alanine enhances BACE1 targeting to LR11-positive compartments and nullifies LR11-mediated reduction of Aβ. On basis of these results, we propose that GGA1 facilitates LR11 endocytic traffic and that LR11 modulates Aβ levels by promoting APP traffic to the endocytic recycling compartment.

Introduction: The amyloid-β precursor protein (APP) and its derivative, amyloid-β peptide (Aβ), play central roles in Alzheimer’s disease (AD). Aβ accumulates in senile plaques, a pathological hallmark of AD (Masters et al., 1985), and single-gene mutations in APP cause familial AD by altering APP processing and Aβ production (Goate et al., 1991; Citron et al., 1992). To generate Aβ, APP is cleaved by β-secretase/β-site of APP-cleaving enzyme-1 (BACE1), resulting in secreted APPβ (APPβ) and a carboxyl-terminal fragment (CTFβ), and subsequent action of γ-secretase on CTFβ releases Aβ (Hussain et al., 1999; Sinha and Lieberburg, 1999; Vassar et al., 1999; Wolfe et al., 1999; Yan et al., 1999; Lin et al., 2000; Xia et al., 2000). Cell biological studies indicate that APP processing is determined by its intracellular traffic and exposure to secretase enzymes and that Aβ is produced in the endosomal compartment, as well as in the early secretory pathway (Sisodia, 1992; Koo and Squazzo, 1994; Hartmann et al., 1997; Xu et al., 1997).

Although specific gene mutations can cause familial AD, the causes of sporadic AD remain unclear. The best-established genetic risk factor is the ɛ4 allele of apolipoprotein E (APOE; Corder et al., 1993; Strittmatter et al., 1993). Moreover, members of the low-density lipoprotein receptor (LDLR) family, which bind ApoE, have been implicated in AD (Okuizumi et al., 1995; Kang et al., 1997; Ma et al.,...
Low-density lipoprotein receptor with 11 class A ligand-binding repeats (LR11)/SorLA is a multidomain LDLR family member that contains structural elements that also place it in the vacuolar protein sorting 10 protein (VPS10p) homology domain family of intracellular sorting receptors (Jacobsen et al., 1996). We first identified LR11 as a transcript that was down-regulated in microarray studies examining gene expression in patients with AD (Scherzer et al., 2004). Subsequently, we demonstrated preferential neuronal loss of LR11 protein in brain regions vulnerable to AD pathology in sporadic but not familial AD patients, suggesting that LR11 loss plays a specific role in the pathogenesis of sporadic AD (Scherzer et al., 2004; Dodson et al., 2006). More recent studies indicate early loss of neuronal LR11 in individuals with mild cognitive impairment, a condition that often represents prodromal AD (Sager et al., 2007). Single-nucleotide polymorphisms in noncoding regions of SORL1, the gene encoding LR11, have been linked to AD risk, implying that they influence disease by altering expression levels rather than by producing variant forms of LR11 (Rogaeva et al., 2007).

Several reports revealed insights into the mechanism by which LR11 may influence AD pathogenesis. Overexpression of LR11 results in a highly consistent, dose-dependent reduction in APP processing to Aβ. These results indicate that the LR11 ectodomain is required for LR11 endocytic traffic and that GGA1 is critical for BACE1 and LR11’s requirement for this motif with respect to LR11 traffic and its effects on endogenous Aβ production. On the basis of these studies, we propose that LR11 endocytic traffic, facilitated by GGA1, is critical for LR11 modulation of APP traffic and the generation of Aβ.

RESULTS

The LR11 ectodomain is required for LR11-mediated Aβ reduction

To assess the effect of the ectodomain or cytoplasmic tail of LR11 on Aβ production, we constructed two chimeras in which the ectodomain or cytoplasmic domain of LR11 was replaced with that of human LDLR, thus generating LDLR-LR11 and LR11-LDLR, respectively (Figure 1A). LDLR was chosen because it is in the same lipoprotein receptor family as LR11 and was reported to have no effect on APP processing or Aβ levels (Fryer et al., 2005). In addition, we generated a construct consisting of the LR11 transmembrane and cytoplasmic domains but lacking the entire ectodomain (LR11ΔED). All constructs were epitope tagged with V5 at the N-termini.

To investigate effects on APP processing and Aβ production, we transiently expressed LR11 constructs in human embryonic kidney (HEK) 293 cells. This cell line was chosen due to the ability to detect endogenous APP protein by immunoblot and secreted Aβ by enzyme-linked immunosorbent assay (ELISA), and our standard transfection efficiency is ~60% under these conditions. Cell extracts and conditioned media were assayed for endogenous full-length APP and α-secretase cleaved APPs, respectively, by immunoblotting, and secreted Aβ1-40 was detected by sandwich ELISA. None of the LR11 constructs significantly altered the levels of full-length, cell-associated APP or APPs in the conditioned media as compared with vector (pcDNA3.1; Figure 1B). Conversely, Aβ1-40 levels were reduced by 42% of controls in cells transfected with LR11; however, Aβ1-40 levels in conditioned media were similar in vector-, LDLR-, LR11–, and LR11ΔED-expressing cells (Figure 1C). Cells transfected with LR11-LDLR exhibited a 19% reduction in Aβ levels, which was significantly different from both vector- and LR11-transfected cells. These results indicate that the LR11 ectodomain is required for LR11 modulation of Aβ production, whereas the cytoplasmic domain is necessary to achieve the full effect. Control experiments demonstrate that LR11, but not LDLR, has an effect on Aβ1-40 levels, and the V5 tag did not alter this effect (Supplemental Figure S1).

Mutagenesis of the LR11 GGA-binding domain alters LR11’s effects on Aβ production and APP traffic

The observation that the LR11 ectodomain is essential for LR11-mediated Aβ reduction supports our hypothesis that LR11 directs APP traffic into a nonamyloidogenic pathway through lumenal domain interactions. Short sorting motifs in the LR11 cytoplasmic tail (or other single pass receptor) dictate binding to specific regulators of intracellular traffic. These include an acidic cluster that is required for AP-2 complex–dependent endocytosis and a GGA-binding domain found on proteins, such as sortilin and mannose 6-phosphate receptors, that shuttle between the TGN and endosomes (Jacobsen et al., 1996, 2001, 2002; Puertollano et al., 2001; Zhu et al., 2001; Doray et al., 2002a, 2002b; Nielsen et al., 2007).
GGA1 mediates LR11 effects on amyloid-β

To test whether these motifs affect LR11 effects on endogenous Aβ production, we used site-directed mutagenesis to substitute alanines for residues comprising the LR11 acidic cluster or GGA-binding domain (Figure 2A). The acidic cluster, responsible for

FIGURE 1: The LR11 ectodomain is required and the LR11 cytoplasmic tail is necessary for LR11-mediated reduction of Aβ. (A) Schematic representation of the N-terminal V5 epitope–tagged LR11 constructs. The V5 tag is indicated in red, and the transmembrane domain is green. The LR11 ectodomain and cytoplasmic tail are peach colored, and the LDLR ectodomain and cytoplasmic tail are blue. To analyze the effects of the LR11 ectodomain on Aβ production, we generated LDLR-LR11 and LR11ΔED, in which the LR11 ectodomain was replaced by that of LDLR or deleted, respectively. LR11-LDLR was generated to assess the requirement of the LR11 cytoplasmic domain, which was replaced with that of LDLR, on Aβ production. Not drawn to scale. (B) Effects of LR11 ectodomain and cytoplasmic domain on full-length, cell-associated APP and APPs. HEK293 cells were transfected with vector (pcDNA3.1), LR11, LDLR-LR11, LR11ΔED, or LR11-LDLR and harvested after 72 h. Media were conditioned for 48 h beginning 24 h after transfection. Equivalent volumes of conditioned media per sample and 50 μg of protein for cell lysate analyses were loaded for SDS–PAGE. Immunoblot analyses of conditioned media and cell lysate indicate similar levels of endogenous APPs and APP, respectively, among all samples. Immunoblot of cell lysate with V5 antibody indicated similar expression levels of all LR11 constructs. (C) Effects of LR11 ectodomain and cytoplasmic domain on endogenous secreted Aβ1-40. ELISA revealed that expression of LR11 reduced secreted Aβ1-40 levels by 44 and 37%, respectively, compared with vector control. Expression of LDLR-LR11 or LR11ΔED had no observable effect on secreted Aβ1-40. Values are shown as mean ± SEM (error bars). ***p < 0.001; **p < 0.01. Data shown are representative of three independent experiments.

LR11 endocytosis, comprises amino acids 2190, 2191, and 2194–2198 (DD–EDDED; Nielsen et al., 2007); these five aspartic acid and two glutamic acid residues were mutated to alanines, generating D2190-2198/A. The GGA-binding domain comprises amino acids 2207–2211 (DDVPM). The two aspartic acid residues
suggest that LR11–GGA interactions influence LR11 effects on APP processing to Aβ. However, the possibility that changes in Aβ levels are simply due to differences in LR11 and DD2207/8AA protein level cannot be ruled out.

GGA family proteins are sorting adaptors that regulate vesicle traffic between the TGN and endosomes (Boman et al., 2000; Dell’Angelica et al., 2000; Hirst et al., 2000; Poussu et al., 2000; Takatsu et al., 2000). Therefore we hypothesized that mutation of the LR11 GGA-binding domain would affect LR11 endosomal distribution. To test this, we transiently transfected HEK293 cells with LR11 or DD2207/8AA and 24 h later plated cells on coverslips for immunofluorescence and confocal microscopy analyses. We compared colocalization of LR11 and DD2207/8AA with the following organelle markers: Golgi (GM130), sorting/early endosomes (EEA1), recycling endosomal compartment (TfR), or late endosomes/lysosomes (CD63). (A, B) Representative images of LR11 and DD2207/8AA (LR11DD2207/8AA; green) and organelle markers (red), with colocalization (yellow) shown in the merged image. (C) Quantitative analyses of LR11 and DD2207/8AA staining. DD2207/8AA overlap with EEA1 (LR11, 40.9%; DD2207/8AA, 19.7%; p < 0.001) and TfR (LR11, 76.1%; DD2207/8AA, 30.8%; p < 0.001) was significantly reduced compared with LR11. DD2207/8AA colocalization with CD63 (LR11, 22.2%; DD2207/8AA, 14.6%) and GM130 (LR11, 6.6%; DD2207/8AA, 4.1%) was not significantly reduced. Values are shown as mean ± 1 SD (error bars). ***p < 0.001. Data shown are representative of three independent experiments. Scale bars, 10 μm.
To determine whether changes in LR11 traffic caused by mutations in its GGA-binding domain affect APP traffic, we analyzed the effect of LR11 or DD2207/8AA expression on endogenous APP distribution (Figure 4, A–C). LR11 expression increased APP colocalization with EEA1 and especially TIR (Figure 4D), suggesting that LR11 promotes APP traffic to the endocytic recycling compartment. To support these findings, we also examined APP colocalization with Rab11a (Supplemental Figure S3). In contrast, expression of DD2207/8AA substantially decreased APP colocalization with TIR in comparison to LR11 and vector transfected cells. There was no observed increase in APP overlap with EEA1 under DD2207/8AA expression, and APP colocalization with GM130 and CD63 was similar among vector-, LR11-, and DD2207/8AA-transfected cells. Together, these results reveal that the LR11 GGA-binding domain plays a role in LR11-mediated effects on Aβ production and APP traffic to early endosomes and the recycling endosomal compartment.

**GGA1 is a candidate regulator of LR11 traffic**

Three GGAs (GGA1, GGA2, and GGA3) are each ubiquitously expressed in human cells and tissues (Boman et al., 2000; Dell’Angela et al., 2000; Hirst et al., 2000; Poussu et al., 2000; Takatsu et al., 2000). The functions of each family member and possible redundancies are not well established; some reports indicate cooperativity between GGAs, and others suggest distinct roles. Yeast two-hybrid analysis identified GGA1 and GGA2 as putative interacting partners for LR11, and GGA1 and LR11 can be coimmunoprecipitated when they are overexpressed in Chinese hamster ovary cells (Jacobsen et al., 2002; Schmidt et al., 2007). On the basis of our observations in experiments using LR11 GGA-binding domain mutants, we sought to identify the specific GGA family member(s) that mediates these effects.

In transfected cells, immunoblots of cell extracts showed reduced band intensity of the GGA-binding mutant (DD2207/8AA) compared with wild-type LR11 (Figure 2B). This phenotype was used to screen for the GGA family member(s) responsible. We hypothesized that absence of the requisite GGA adaptor would lead to decreased LR11 protein levels comparable to those seen with the GGA binding mutant. Immunoblot analysis was used to evaluate endogenous LR11 levels after targeted siRNA knockdown of GGA1, GGA2, or GGA3. To this end, HEK293 cells were transfected with GGA1-, GGA2-, or GGA3-targeted or control (nontargeting) siRNA smart pools, and cells were collected after 96 h to assess the efficiency of knockdown and measure changes in LR11. As seen in Figure 5, GGA1 and GGA2 were reduced to 10 and 29% of controls, respectively, whereas GGA3 was reduced to 60% of controls. Endogenous LR11 protein level was reduced to 45% of controls in GGA1-depleted cells (Figure 5, A and B); however, no effect on LR11 was observed in cells transfected with siRNA targeted to GGA2 or GGA3 (Figure 5, C and D). Reduced LR11 protein level was confirmed after RNA interference (RNAi) depletion of GGA1 using individual short hairpin RNA (shRNA) sequences (Supplemental Figure S4, A and B). A previous study of GGA family members in HeLa cells indicated that knockdown of any one GGA partially reduced the level of the other GGAs (Ghosh et al., 2003). Therefore we blotted for GGA2 and GGA3 under GGA1-knockdown conditions to evaluate whether the observed effects on LR11 were due to combinatorial reductions of the GGAs. No effect on GGA2 or GGA3 was observed in cells depleted of GGA1 (Figure 5A). Although these experiments do not exclude the possibility that GGA2 and GGA3 play a role in LR11 biology, overall, the specificity of GGA1 effect on LR11 protein levels suggests that this adaptor plays a unique role in its regulation or traffic. Of note, treatment with inhibitors of

![Image](https://example.com/image)
lysosomal enzymes increased LR11 protein level by 32% in cells depleted of GGA1 (Figure 5E). These findings suggest that LR11 undergoes enhanced lysosomal degradation in the absence of GGA1.

On the basis of these data, we hypothesized that GGA1-mediated traffic of LR11 was necessary for LR11’s effects on APP processing. To test this hypothesis, we evaluated LR11-mediated reduction of Aβ after targeted siRNA knockdown of GGA1 in HEK293 cells. Cells were first transfected with GGA1, GGA2, or control (nontargeting) siRNA smart pools and subsequently transfected with LR11 or empty vector 24 h later. Media were conditioned for 48 h beginning 24 h after LR11 or vector transfection. The efficiency of GGA1 and GGA2 knockdown and endogenous full-length APP was analyzed by immunoblotting cell extracts, and secreted Aβ1-40 was detected by sandwich ELISA. The levels of GGA1 and GGA2 knockdown were similar to results described in Figure 5, A and C, and across all samples no observable changes in full-length APP were detected (Figure 6A). Aβ1-40 was reduced by 44% in cells transfected with control siRNA and LR11 (Figure 6B). Depleting cells of GGA1 reduced Aβ1-40 by 24%, and expressing LR11 after GGA1 knockdown produced no further effect on Aβ1-40 levels (27% reduction). These findings were confirmed after RNAi depletion of GGA1 using individual shRNA sequences (Supplemental Figure S4, C and D). Knockdown of GGA2 had no effect on levels of Aβ1-40, and expression of LR11 under these conditions lowered Aβ1-40 by 40%. These results indicate that depletion of GGA1 reduces Aβ production, and LR11’s effects on Aβ levels are disrupted after knockdown of GGA1 but not GGA2.

Given the biochemical and cellular observations with DD2207/8AA, we suspected that the loss of LR11’s ability to reduce Aβ production after GGA1 depletion was the result of aberrant LR11 endocytic traffic. To test whether depletion of GGA1 affects endocytic localization of LR11, we transduced HEK293 cells with lentivirus expressing LR11 and subsequently transfected them with plasmid expressing GGA1-targeted shRNA or scramble shRNA and green fluorescent protein (GFP). Cells were plated on coverslips 72 h after transfection and fixed for immunofluorescence and confocal microscopy 24 h later. Parallel samples were transfected for 96 h with indicated plasmids and harvested for immunoblot to analyze efficiency of GGA1 knockdown (Figure 6D). Confocal images reveal that colocalization of LR11 with TfR, a marker for the endocytic recycling compartment, is disrupted under GGA1 knockdown (Figure 6C). These results, coupled with the cell biological analyses of DD2207/8AA, suggest that endocytic traffic of LR11 is impaired in the absence of GGA1 binding.

**FIGURE 5:** GGA1 knockdown decreases LR11 protein level. To identify the GGA family member(s) potentially involved in LR11 traffic, we used immunoblot analyses to examine endogenous LR11 protein level after targeted siRNA knockdown of GGA1, GGA2, or GGA3. (A) Targeted siRNA knockdown of GGA1. HEK293 cells were transfected with GGA1 targeted or scramble (nontargeting) siRNA smart pools and harvested after 96 h. For analyses of cell lysate, 50 μg of protein was loaded per lane in SDS–PAGE. Immunoblot analyses indicated that GGA1 was reduced to 10% of scramble controls. (B) LR11 protein level was quantified by immunoblot analyses and showed significant difference in GGA1-knockdown cells (55% decrease in LR11) compared with scramble. Values are shown as mean ± SEM (error bars). ***p < 0.0001. (C) Targeted siRNA knockdown of GGA2. HEK293 cells were transfected with GGA2 targeted or scramble siRNA smart pools and harvested after 96 h. GGA2 was reduced to 29% of controls; however, no effect on LR11 was observed. (D) Targeted siRNA knockdown of GGA3. HEK293 cells were transfected with GGA3 targeted or scramble siRNA smart pools and harvested after 96 h. GGA3 was reduced to 60% of controls, and no effect on LR11 was observed. β-Actin was used as an immunoblot loading control. (E) Lysosomal enzyme inhibitors prevent degradation of LR11. HEK293 cells were transfected with GGA1 targeted or scramble siRNA smart pools, and 72 h posttransfection, cells were treated with leupeptin and pepstatin (Leu/Pep) for 24 h. Depletion of GGA1 reduced LR11 to 50% of controls, and incubation with Leu/Pep increased LR11 protein level by 32%. Calnexin was used as an immunoblot loading control. Data shown are representative of three independent experiments.

Mutagenesis of BACE1 serine 498 nullifies LR11’s effects on Aβ production

Previous studies established that BACE1 interacts with the three GGAas and that GGA1 promotes BACE1 traffic from early endosomes to late endocytic compartments or the TGN (He et al., 2002, 2003, 2005; von Arnim et al., 2004; Wahle et al., 2005). BACE1 cleavage of APP to CTFβ is optimum in acidic environments such as the lumen of endosomes and/or lysosomes (Haass et al., 1999; Lin et al., 1996; Sinha et al., 2004; Wahle et al., 2005). BACE1 expression of LR11 under these conditions lowered Aβ1-40 by 40%. Therefore we hypothesized that GGA1 influences BACE1’s effects on Aβ production, and disruption of GGA1–BACE1 interaction may contribute to the observed reduction in Aβ after GGA1 knockdown (Figure 6B). To test this hypothesis and investigate LR11-mediated reduction of Aβ under these conditions, we evaluated BACE1’s effects on endogenous Aβ production after siRNA knockdown of GGA1 in HEK293 cells. Cells were first transfected with siRNAs that targeted GGA1 or control (nontargeting) and subsequently transfected with empty vector plasmid, BACE1, or BACE1 plus LR11 24 h later. Media were conditioned for 48 h beginning 24 h after plasmid transfection. The efficiency of GGA1 knockdown and endogenous full-length APP was analyzed by immunoblotting, and secreted Aβ1-40 was detected by sandwich ELISA. The levels of GGA1 knockdown were similar to results described in Figures 5
and 6, and across all samples no observable changes in full-length APP were detected (Figure 7A). Expression of BACE1 increased Aβ1-40 levels by 51%, whereas coexpression of LR11 with BACE1 lowered Aβ1-40 levels to 27% of scramble siRNA transfected samples. Knockdown of GGA2 had no effect on secreted Aβ1-40 levels, and expression of LR11 mediated reduction of secreted Aβ1-40 by 101%, whereas coexpression of LR11 with BACE1S498A had no affect on Aβ1-40 levels (91% increase), indicating that LR11 action was precluded by the presence of BACE1S498A. Cotransfection of LR11 with BACE1S498A had no effect on Aβ1-40 levels (91% increase), indicating that LR11 action was precluded by the presence of BACE1S498A.

Previous reports demonstrated that BACE1S498A accumulates in early endosomes and exhibits enhanced endocytic recycling (Walter et al., 2001; Wahle et al., 2005). In this study, we show that LR11 promotes traffic of APP to the endocytic recycling compartment, and this is critical for LR11’s effects on Aβ production. To investigate whether the absence of LR11-mediated reduction of Aβ in BACE1S498A-expressing cells may be due to enhanced targeting of BACE1S498A to LR11-positive compartments, we detected colocalization between BACE1 or BACE1S498A and LR11 by immunofluorescence and confocal microscopy (Figure 7E). Analyses of confocal images revealed that BACE1S498A overlapped with LR11 was increased 45% compared with BACE1. Coupled with the biochemical analyses of Aβ1-40 levels described above, these results suggest that enhanced targeting of BACE1S498A to
LR11-containing compartments nullifies LR11-mediated reduction of Aβ. On the basis of these studies, we propose that LR11 promotes traffic of APP in a nonamyloidogenic pathway by shunting APP away from endosomes that are optimal for BACE1 enzymatic activity.

**DISCUSSION**

Previous studies reported insights into the pathways by which LR11, a multifunctional low-density lipoprotein and sorting receptor, may influence AD susceptibility. Although LR11 has multiple functional domains and potential mechanisms linked to AD pathogenesis, we hypothesize that LR11 is an endosomal chaperone that increases APP traffic in a nonamyloidogenic pathway (Scherzer et al., 2004; Andersen et al., 2005; Offe et al., 2006; Rogaeva et al., 2007; Dodson et al., 2008; Rohe et al., 2008). To elucidate these mechanisms, it is important to better understand the functional motifs of LR11 and the proteins that regulate LR11 modulation of APP processing. Our results indicate that the LR11 ectodomain is required for LR11-mediated reduction of Aβ, and the GGA-binding domain regulates LR11 distribution in endosomal compartments and its effects on APP processing. Targeted siRNA knockdown studies of GGA isoforms reveal effects on LR11 protein levels exclusively in cells depleted of GGA1, and additional knockdown experiments show that GGA1 contributes to modulation of Aβ levels by both LR11 and BACE1. Furthermore, disrupting BACE1–GGA1 interaction or altering BACE1 endocytic targeting precludes LR11-mediated reduction of Aβ. On the basis of these observations, we conclude that endocytic traffic of LR11, facilitated by GGA1, plays a critical role in LR11 regulation of APP traffic and processing.

An important question is how LR11 affects APP distribution and traffic. To address this question, we compared colocalization of endogenous APP with organelle markers under conditions of LR11 overexpression. Expression of LR11 dramatically increased APP overlap with the endosomal markers EEA1, TIR, and Rab11a (Figure 4, A and B, and Supplemental Figure S2). TfRs and LDLR family members are well-studied cargo proteins that are regulated by clathrin-dependent endocytosis, and recycling back to the plasma membrane after endocytosis seems to be the default pathway (reviewed in Maxfield and McGraw, 2004). Although often used as a marker of recycling endosomes, TIR staining is not exclusive to a single type of endosomes and overlaps with...
sorting and recycling compartments, as well as with clathrin-coated vesicles and even the TGN (Maxfield and McGraw, 2004). Of note, expression of LR11 induces an enlargement of TiR-positive compartments that is noticeably absent in cells expressing DD2207/8AA or in cells that are expressing LR11 and depleted of GGA1 (Figures 3, A and B, and 6C). Therefore it is likely that recruitment of GGA8s or other adaptor proteins to LR11-containing endosomes contributes to the observed morphological changes. Previous reports concluded that LR11’s effects on Aβ production occur in the perinuclear region in Golgi compartments (Spoelgen et al., 2006; Schmidt et al., 2007). However, our results, coupled with the analyses of Aβ secretion, suggest that it is LR11-directed traffic of APP to the endocytic recycling compartment that is critical for LR11 regulation of Aβ levels.

Previous studies examined a role for GGA1 in amyloidogenic processing of APP. APP and GGA1 were colocalized in cells overexpressing both proteins (von Arnim et al., 2006; Wahle et al., 2006), but glutathione S-transferase pull-down assays suggested that APP and GGA1 may not interact directly (Wahle et al., 2006). Furthermore, Wahle et al. (2006) showed that siRNA knockdown of GGA1 in H4 cells overexpressing APP results in marginally increased Aβ secretion. In contrast, our studies revealed reduced Aβ secretion under endogenous conditions after siRNA depletion of GGA1 (Figure 6B). Additional siRNA knockdown experiments indicate that BACE1 effects on Aβ depend on GGA1 (Figure 7B). GGA1 mediates traffic of BACE1 between endosomes and Golgi (He et al., 2002, 2003, 2005; von Arnim et al., 2004; Wahle et al., 2005), and therefore we hypothesize that the observed reduction of Aβ in cells depleted of GGA1 reflect impaired traffic to acidic compartments that favor BACE1 cleavage of APP to CTFβ. In addition to GGA1, BACE1’s GGA-binding domain mediates interaction with and putative sorting by GGA2 and GGA3 (He et al., 2002, 2003). Of note, GGA3 is believed to mediate lysosomal degradation of BACE1, and reduction of GGA3 by siRNA enhances BACE1’s effects on Aβ production (Tesco et al., 2007; Kang et al., 2010).

Our findings with DD2207/8AA, coupled with the results of GGA1-knockdown studies, provide strong evidence that GGA1 is necessary for LR11 endocytic traffic and LR11 modulation of APP processing to Aβ. These data highlight the possibility that GGA1 mediates traffic of BACE1 and LR11 and that each pathway culminates in distinct consequences related to APP processing to Aβ. We hypothesize that GGA1 facilitates LR11 traffic to recycling endosomes; however, it is possible that GGA2 also plays a role in this mechanism. Moreover, whether GGA-mediated sorting of LR11 is direct Golgi-to-endosome traffic or shuttling between endosomal compartments remains to be determined. Reduced LR11 protein levels observed after GGA1 knockdown or mutagenesis of the GGA-binding domain may indicate that in the absence of GGA1–LR11 interaction, LR11 is targeted to degradative pathways. Jacobson et al. (2002) showed in a yeast-two hybrid analysis that LR11 aspartic acids 2207 and 2208 were absolutely required for GGA1 binding. More recent studies suggest that GGA1 interaction with cargos, such as sortilin and LR11, are enhanced by phosphorylation of the serine residue that is immediately N-terminal to the cargo’s GGA-binding domain (Cramer et al., 2010). Our previous work demonstrated that ROCK2 phosphorylates LR11 serine 2206, which is located immediately N-terminal to the LR11 GGA-binding motif (Herskowitz et al., 2011). Moreover, we showed that mutagenesis of this residue abrogated LR11-mediated reduction of Aβ. Whether or not phosphorylation of serine 2206 contributes to GGA1 interaction with LR11 in mammalian cells remains to be demonstrated.

Defining the cellular mechanism(s) by which LR11 modulates Aβ production is critical to understanding how changes in LR11 expression affect the development of amyloid pathology in sporadic AD. This report demonstrates that the LR11 ectodomain is required for LR11-mediated Aβ reduction and that the LR11 GGA-binding domain is necessary for LR11’s effects on APP traffic. These data support our hypothesis that through interactions between the luminal domain of LR11 and APP, LR11 acts as an endosomal chaperone to promote APP traffic in a nonamyloidogenic pathway. Furthermore, our studies revealed that LR11-mediated reduction of Aβ is inhibited in the presence of BACE1S498A (Figure 7D), likely due to enhanced targeting of BACE1S498A to LR11-positive compartments (Figure 7E). We propose that after endocytosis, sorting of BACE1, LR11, and APP occurs in early endosomes, and LR11 reduces Aβ production by escorting APP to the endocytic recycling compartment (Figure 8). Through this activity, LR11 shunts APP away from early and late endosomes that are optimal for BACE1 enzymatic activity. GGA1 facilitates LR11 traffic to the ERC and mediates BACE1 traffic to late endosomes (late endosomal compartment [LEC]) and the TGN. PM, plasma membrane.

**FIGURE 8:** Proposed model of LR11-regulated APP traffic in a nonamyloidogenic pathway. We hypothesize that through luminal domain interactions, LR11 acts as an endosomal chaperone to promote APP traffic in a nonamyloidogenic pathway. After internalization from the cell surface, BACE1, LR11, and APP are sorted in early endosomes (endocytic sorting compartment [ESC]), and LR11 reduces Aβ production by escorting APP to the endocytic recycling compartment (ERC). Through this activity, LR11 shunts APP away from early and late endosomes that are optimal for BACE1 enzymatic activity. GGA1 facilitates LR11 traffic to the ERC and mediates BACE1 traffic to late endosomes (late endosomal compartment [LEC]) and the TGN. PM, plasma membrane.

**MATERIALS AND METHODS**

**Generation of expression vectors and LR11 and BACE1 mutants**

Human LR11 cDNA in pcDNA3.1 (Invitrogen, Carlsbad, CA) was a gift from Chica Schaller (Zentrum für Molekulare Neurobiologie, Universität Hamburg, Hamburg, Germany), and the LDLR cDNA in a pCMI-based vector was generously provided by David Russell (Department of Molecular Genetics, University of Texas Southwestern Medical Center at Dallas, Dallas, TX). Additional constructs were designed and generated based on these cDNAs, and all were epitope-tagged with V5 at the amino terminus. All cDNA was amplified using AccuPrime Pfx DNA polymerase Supermix (Invitrogen).

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LR11 cDNA was amplified using a sense primer with an XbaI site (5′-ATA TTC TAG AAG CGC TGC CCT GCA GCC CGA-3′) and an antisense primer introducing an Xhol site (5′-AAT ACT CGA GTC AGG CTA TCA CCA TGG GGA-3′). The LR11ΔED cDNA was amplified with a sense primer that introduced an XbaI site (5′-CCG TTC TAG ATC TAG GGA GAG TGT TGC TG TGT-3′) and an antisense primer containing an Xhol site (5′-AAT ACT CGA GTC AGG CTA TCA CCA TGG GGA-3′). To amplify full-length LDLR, the following primers were used: 5′-AGT CTC TAG GGG GGA CAG ATG CGA AAG-3′, introducing an XbaI site, and 5′-ATG CGG CGG CTC ACC AGC CGT CAT CCT CCA-3′, introducing a NotI site. TheLR11 ectodomain was amplified with the sense primer 5′-ATG TCT TAG AAG CGC TG CCT GCA GCC CGA-3′, containing an XbaI re site, and the antisense primer 5′-GAG CGA ATT CTC TGG CAG CCT GCG TTG CAG-3′, introducing an EcoRI site. The LDLR transmembrane and cytoplasmic domains for the LR11-LDLR chimera were amplified using a sense primer with an EcoRI site (5′-GAG CGA ATT CAG CGT GAG GGC TCT GTC GTC CAT-3′) and an antisense primer introducing a NotI site (5′-ATG CGG CCG CTC ACC AGG CCA CGT CAT CCT CCA-3′). The LDLR ectodomain was amplified with the sense primer 5′-AGT CTC TAG GGT GGA CAG ATG CGA AAG-3′, containing an XbaI site, and the antisense primer 5′-CGC CCT GCA GAG TGG GGT GCT TCT TCT CAT TTT-3′, containing an Xhol site. To generate the cytoplasmic tail of the LDLR-LR11 chimera, the amplified LR11 transmembrane and cytoplasmic domains using the sense primer 5′-CCG CCT CCT GCA GTC TAG GGA GAG TGT TGC TG TGT-3′, introducing an Xhol site, and the antisense primer 5′-ATG CGG CCG CTC AGG CTA TCA CCA TGG GGA-3′, introducing a NotI site. PCR products were cut using the appropriate restriction enzymes and ligated into modified pCDNA3.1 that contained either the LDLR signal sequence for sorting/early endosomes and GM130 antibody for Golgi (monoclonal from Transduction Laboratories/BD Biosciences, San Jose, CA). Equivalent amounts of cells were plated and transduced with the indicated constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. GGA1, GGA2, and GGA3 ON-TARGETplus SMARTpool (Dharmacon, Lafayette, CO) and ON-TARGET Non-targeting Pool siRNA (Dharmacon) were transfected using DharmaFECT 1 siRNA Transfection Reagent (Dharmacon) according to the manufacturer’s instructions. HEK293 cells were transfected with lentivirus expressing N-terminal V5-tagged LR11 at a multiplicity of infection of 1. For lysosomal enzyme inhibition, 0.1 μg of leupeptin and 0.05 μg of pepstatin A (both from Sigma-Aldrich, St. Louis, MO) were added per milliliter of media for 24 h (Nielsen et al., 2007).

**Antibodies**

For detecting the V5 tag on LR11 constructs, a polyclonal V5 antibody (Sigma-Aldrich) was used for immunocytochemistry, and a monoclonal antibody to V5 (MCA1360; AbD Serotec, Raleigh, NC) was used for immunoblot. For the organelle markers in immunocytochemical experiments, the following antibodies were used: EEA1 for sorting/early endosomes and GM130 antibody for Golgi (monoclonals from Transduction Laboratories/BD Biosciences, San Jose, CA), KDEL for endoplasmic reticulum (monoclonal from Assay Designs, Ann Arbor, MI), TIR for the recycling endosomal compartment (monoclonal from Zymed/Invitrogen, San Francisco, CA), and CD63 for late endosomes/lysosomes (monoclonal from BD Biosciences PharMingen, San Diego, CA). For endogenous LR11 immunoblot, monoclonal antibody (611860) from Transduction Laboratories/BD Biosciences was used. For immunoblot and immunofluorescence of GGA1, GGA2, and GGA3, the following antibodies were used: rabbit polyclonal antiserum to GGA1 were generated as described previously (Boman et al., 2000), GGA2 rabbit polyclonal (ab10552) from Abcam (Cambridge, MA), and GGA3 monoclonal (612310) from Transduction Laboratories/BD Biosciences. For immunofluorescence and immunoblot of full-length, cell-associated APP and immunoblot of secreted α-secretase cleaved APP (monoclonal 6E10; Signet, SIG-39320-200; Covance, Berkeley, CA) was used.
used. For immunoblot loading controls, the following antibodies were used: calnexin polyclonal (SPA-860; Assay Designs, Ann Arbor, MI) and β-actin monoclonal (ab6276; Abcam). For immunofluorescence and immunoblot of BACE1 and BACE1S498A, a polyclonal BACE1 antibody (ABH0281; BioSource International, Camarillo, CA) was used. For immunoblot of biotinylated transferrin, a polyclonal goat biotin antibody (600-131-098; Rockland Immunochemicals, Gilbertsville, PA) was used.

Cell harvest and immunoblotting
Cells were lysed as previously described (Offe et al., 2006; Herskowitz et al., 2010) in phosphate-buffered saline (PBS) plus protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), Halt phosphatase inhibitor cocktail (Pierce, Rockford, IL), and lysis buffer containing 0.5% NP-40, 0.5% deoxycholate, 150 mM sodium chloride, and 50 mM Tris, pH 7.4. Immunoblotting was performed as previously described (Offe et al., 2006). To load equivalent amounts of soluble lysate per sample for immunoblot analyses, we determined protein concentration by bicinchoninic acid method (Pierce). Images were captured using an Odyssey Image Station (Li-Cor, Lincoln, NE), and band intensities were quantified using Scion Image. Statistical analysis was performed using Student’s t test for independent samples.

Aβ1 measurements
HEK293 cells were transiently transfected with siRNA and/or indicated plasmids, and media were conditioned for 48 h beginning 24 h after final transfection. Conditioned media were collected and cells were harvested for immunoblot analyses as described. Endogenous Aβ1-40 was detected using a sandwich ELISA for human β amyloid 1-40 (Millipore, Billerica, MA) following the manufacturer’s instructions. Plates were read at 450 nm on a Spectra Max Plus plate reader (Molecular Devices, Sunnyvale, CA). Cell extracts and conditioned media samples were blotted for indicated proteins as described in the text. Experiments were performed in triplicate.

Immunocytochemistry and confocal microscopy
For immunocytochemistry experiments, cells were plated onto Matrigel extracellular matrix (BD, Franklin Lakes, NJ)–coated coverslips in 12-well culture dishes 24 h after transfection. Cells plated on coverslips were fixed for 30 min in 2% paraformaldehyde and rinsed several times with PBS containing 0.5% normal horse serum, 0.5% normal goat serum, and 0.05% saponin (rinse buffer). The cells were blocked and permeabilized for 30 min with PBS containing 5% normal horse serum, 5% normal goat serum, 1% bovine serum albumin, and 0.05% saponin (blocking buffer) plus 0.05% Triton X-100. Cells were rinsed several times with rinse buffer and incubated in primary antibody diluted in blocking buffer overnight at 4°C. For double and triple labeling, two primary antibodies were included in the incubation. The next day cells were rinsed and incubated for 1 h with both fluorophore-conjugated anti-rabbit and mouse secondary antibodies (Jackson ImmunoResearch, West Grove, PA) in blocking buffer. For triple labeling, the secondary antibodies used were goat Fab fragments (Jackson ImmunoResearch). This was followed by a block with unconjugated goat anti-mouse or anti-rabbit monoclonal Fab fragments. Cells were then incubated with the third primary overnight at 4°C. The next day, cells were rinsed and incubated with secondary antibody for 1 h at room temperature. After rinsing, cell nuclei were stained with Hoechst 333258 (Molecular Probes, Invitrogen, Carlsbad, CA) for 5 min, mounted onto slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and sealed. Control conditions included omission of one or more primary antibodies to test incomplete block, nonspecific secondary antibody binding, and bleedthrough. Controls showed negligible staining. Images were captured using an LSM510 confocal microscope (Zeiss, Thornwood, NY) and processed with Photoshop (Adobe, San Jose, CA) by manually tracing a single cell per image, as well as a 20 x 20 pixel region of the nucleus. Quantification of colocalization was performed using Matlab R2006 software (The MathWorks, Natick, MA). For each channel of the RGB image, the average pixel intensity plus 1 SD of the outlined nuclear region was measured and defined as background. The average pixel intensity plus 1 SD of the pixels in the outlined cell that were exceeding background intensity was then measured, and the threshold was set at this value. For pixels with intensities greater than threshold, the percentage of pixels in one channel overlapping with those in the other channels was calculated. The data were analyzed with two-way analysis of variance and Bonferroni post hoc tests using Prism software (GraphPad Software, San Diego, CA). The data are presented as means ± 1 SD. Experiments were performed in triplicate.

Transferrin recycling assay
HEK293 cells were plated in 60-mm dishes and subsequently transfected with indicated constructs. Twenty-four hours later the cells were split into 12-well dishes. The next day, the cells were serum starved for 1 h and then incubated with bovine serum albumin–supplemented media containing 10 μg/ml of iron-saturated biotinylated transferrin (Sigma-Aldrich) for 1 h at 37°C. Unbound and surface-bound biotinylated transferrin were removed by placing the cells on ice and washing twice with cold PBS, once with low-pH wash buffer (150 mM NaCl, 10 mM acetic acid pH 3.5), and finally twice with cold PBS. To measure transferrin recycling, we returned the cells to 37°C and incubated them in medium containing 1 mg/ml unlabeled transferrin (Sigma-Aldrich). Medium was collected at 0, 5, 10, 20, and 40 min, and the cells were harvested for immunoblot analyses as described. Recycled and cell-associated biotinylated transferrin were quantified as previously described (Kachhap et al., 2007). Briefly, a fraction of lysate and corresponding media was processed for immunoblot. At each time point recycled transferrin was expressed as a percentage of total transferrin.

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REFERENCES


Nielsen MS et al. (2007). Sorting by the cytoplasmic domain of the amyloid precursor protein binding receptor SorLA. Mol Cell Biol 27, 8462–8481.


