J. Cell. Mol. Med. Vol 12, No 6B, 2008 pp. 2665-2674

C-terminal 37 residues of LRP promote the amyloidogenic processing of APP independent of FE65

Madepalli K. Lakshmana, Eunice Chen, Il-Sang Yoon, David E. Kang *

Department of Neurosciences, University of California, San Diego, Gilman Drive, La Jolla, CA, USA

Received: January 15, 2008; Accepted: March 21, 2008

Abstract

The major defining pathological hallmark of Alzheimer's disease (AD) is the accumulation of amyloid β protein (Aβ), a small peptide derived from β- and γ-secretase cleavages of the amyloid precursor protein (APP). Recent studies have shown that the Low-density lipoprotein receptor-related protein (LRP) plays a pivotal role in the trafficking of APP and generation of Aβ. In particular, we recently showed that the soluble cytoplasmic tail of LRP (LRP-ST) without a membrane tether was sufficient to promote Aβ generation. In this study, we demonstrate that the last 37 residues of LRP cytoplasmic tail (LRP-C37) lacking the NPxY motifs and FE65 binding mediate the core pro-amyloidogenic activity of LRP-ST. Moreover, we show that the conserved dileucine motif within the LRP-C37 region is a key determinant of its Aβ promoting activity. Finally, results from a yeast two-hybrid screen using LRP-C37 region as bait reveal four new LRP-binding proteins implicated in intracellular signalling and membrane protein trafficking. Our findings indicate that the LRP-C37 sequence represents a new protein-binding domain that may be useful as a therapeutic target and tool to lower Aβ generation in AD.

Keywords: LRP • APP • FE65 • amyloid • Alzheimer • Snapin • RanBPM • SH3 • Filamin

Introduction

Amyloid β protein (Aβ) accumulation in the brain is an early toxic event in the pathogenesis of Alzheimer's disease (AD). Aβ is produced by proteolytic processing by β- and γ-secretase cleavages of the amyloid precursor protein (APP). The low-density lipoprotein receptor-related protein (LRP) is a large type I transmembrane (TM) protein that functions as a multi-functional endocytosis receptor for a diverse array of extracellular ligands, including lipoproteins, proteases, proteinase inhibitor complexes, matrix proteins, bacterial toxins, viruses, intracellular proteins and growth factors [1]. Within the central nervous system (CNS), LRP is highly expressed in neuronal cell bodies and dendritic processes. LRP is synthesized as a 600-kD precursor protein that is subsequently cleaved in the *trans*-Golgi compartment by furin to generate a large 515-kD α-chain and a smaller 85-kD TM β-chain that remain non-covalently linked [2].

Several lines of studies implicate a role for the LRP pathway in AD pathogenesis. First, LRP is genetically associated with late-onset AD in the majority of Caucasian and Asian populations examined [3–6]. Second, three of its key ligands, apoE, α2-macroglobulin and APP are also genetically associated with AD and found in senile plaques in the brains of AD patients along with LRP [7–10]. Third, LRP mediates the binding and clearance of Aβ complexes bound to apoE or α2-macroglobulin in cultured cells and in the brain [11, 12]. LRP also directly associates with Aβ and plays a crucial role in brain efflux of Aβ isoforms at the blood-brain barrier [13]. Finally, circulating secreted LRP can function as a 'peripheral sink' to reduce Aβ levels in brain [13, 14]. These findings therefore support a model in which LRP plays an important role in extracellular Aβ uptake and removal.

In an opposing effect, however, LRP also influences APP trafficking and cellular distribution such that processing to Aβ and its extracellular release is enhanced [15–19]. The loss of LRP or treatment of receptor-associated protein (RAP), an antagonist of all known LRP ligands, substantially reduces Aβ release, a phenotype that is reversed when full-length (LRP-FL) or truncated LRP is transfected in LRP-deficient cells [16, 17]. Specifically, LRP-CT lacking the extracellular ligand binding regions but containing the TM domain and the cytoplasmic tail (CT) is capable of rescuing amyloidogenic processing of APP and Aβ release in LRP deficient cells [16]. Moreover, LRP soluble tail (LRP-ST) lacking the TM domain and only containing the CT of LRP is sufficient to enhance

---

*Correspondence to: David E. KANG,
Department of Neurosciences, UC San Diego,
MC0691, 9500 Gilman Drive, La Jolla, CA 92093, USA.
Tel.: +858-822-6484
Fax: +858-822-1021
E-mail: dekang@ucsd.edu

doi:10.1111/j.1582-4934.2008.00320.x

Aβ secretion [18]. This activity of LRP-ST is achieved by promoting APP/Beta APP clearing enzyme 1 (BACE1) interaction and facilitating the targeting of APP to lipid rafts, cholesterol-rich membrane microdomains enriched in both β- and γ-secretase activities [19]. Similar to APP, LRP also undergoes BACE1-mediated ectodomain cleavage and presenilin-dependent intramembrane proteolysis to release the LRP intracellular domain (LICD) [20, 21]. Thus, the pro-amyloidogenic action LRP-ST not only reflects an activity of a physiological product but also may in part underlie the manner in which LRP normally promotes Aβ generation.

The short 100 amino acid CT contains two NPxY endocytosis motifs and binds a number of cytoplasmic adaptor and scaffold proteins, such as FE65, Disabled-1 (Dab1), Shc, and JIP-1 and -2, probably through the second NPxY motif [22–24]. Of particular significance is FE65, which constitutes a physical link between APP and LRP in regulating sAPP release [24–26]. Initially, we had hypothesized that one or more NPxY domains in LRP-ST might underlie the pro-amyloidogenic processing of APP, since all known LRP-interacting cytoplasmic adaptor proteins bind to NPxY-based motifs. In this study, however, we show that the last 37 C-terminal residues of LRP (LRP-C37) robustly promote Aβ production independent of FE65 and constitute a protein-binding domain for several new LRP-binding proteins.

Materials and methods

DNA constructs

The LRP-ST-6x Myc variants were subcloned into pLHCX vector (Clontech, Palo Alto, CA, USA). FE65 was a kind gift from Dr. Tom Sudhof [27]. pLHCX LRP-ST1-97, ST45-97 and ST61-97 constructs were generated by PCR amplification. Myc-tagged LRP-ST mutants, such as ST1-97Δ1Δ2, ST61-97ΔELL, ST61-97ΔDEKR and LRP-ΔC37 lacking the last 37 residues of LRP were generated by site-directed mutagenesis (Quikchange, Stratagene, La Jolla, CA, USA). All of these cDNAs were sequenced and protein expression confirmed prior to use.

Chemicals and antibodies

The polyclonal antibody 1704 recognizes the cytoplasmic domain of human LRP [16]. The polyclonal antibodies CT15 (against C-terminal 15 residues of APP), 63d (against APP ectodomain) and anti-FE65 have been described previously [18, 19, 26, 29]. Monoclonal antibodies 9E10 (against Myc; Calbiochem, San Diego, CA, USA) and 6E10 (against 1–17 of Aβ; Covance Research, Emeryville, CA, USA) were purchased from the indicated vendors. All secondary antibodies were purchased from Jackson ImmunoResearch laboratories (West Grove, PA, USA). All antibodies were diluted in 5% non-fat milk in Tris buffered saline with 0.1% Tween-20 (TBS-T) buffer. Minimal SD base, Minimal SD agar base and DO-Ade/-His/-Leu/-Trp supplement were all purchased from BD Biosciences (Palo Alto, CA, USA), X-α-Gal (5-bromo-4-chloro-3-indolyl-α-D-galactoside) from Research products International (Mt. Prospect, IL, USA), kanamycin solution from Teknova (Hollister, CA, USA) and antimouse IgG and anti-rabbit IgG agarose beads from American Qualex International.

Cell cultures and transient transfections

Human embryonic kidney (HEK) 293FT cells were grown in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum, 2 mM L-glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin. Experiments involving transient transfections were performed using lipofectamine 2000 (Invitrogen) and Opti-MEM I (Invitrogen, Carlsbad, CA, USA). Equal amounts of empty vectors were included to keep the overall DNA quantity constant for all the experimental groups. Twenty-four hours after the transfection, conditioned media were collected for Aβ detection.

Cells were lysed in buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.02% sodium azide, 400 nM Microcystin-LR, 0.5 mM sodium vanadate and 1% Nonidet P-40 with complete protease inhibitor mix (Sigma, St. Louis, MO, USA). Protein concentrations were measured by the micro bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Proteins were separated by electrophoresis on 4–15% SDS-polyacrylamide gel electrophoresis and analysed by immunoblotting. Antigens were detected by their corresponding primary and secondary antibodies, followed by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

Yeast two-hybrid screen

To subclone the bait constructs for use in the two-hybrid system [30], PCR-amplified cDNA-encoding human LRP-C37 region (LRP-ST61-97, the last 37 amino acids of LRP-ST) and LRP-ST1-97 were ligated into EcoRI-BamHI restriction sites of pGBKT7 bait plasmid (Clontech, Palo Alto, CA, USA) in frame with Gal4 DNA-binding domain resulting in pGBKT7-LRP-ST61-97 and pGBKT7-LRP-ST. The expression of LRP-ST1-97 and LRP-ST61-97-Gal4 fusion proteins were confirmed by immunoblotting. Initially, LRP-ST1-97 and LRP-ST61-97 constructs were tested for self-activation of the *His3* reporter gene in the absence of prey by plating transformed yeast on selective dropout plates lacking leucine and tryptophan (SD-LT). A high-stringency protocol was used to screen the cDNA library from 17-day-old mouse embryo fused with the Gal4 transactivation domain constructed in the pACT2 plasmid (Clontech). The yeast two-hybrid screening was performed in AH109 *Saccharomyces cerevisiae* that contains three reporters ADE2, HIS3 and MEL1. The bait plasmid was initially transformed into AH109, and growth was selected in SD dropout plates lacking leucine (SD-L). This yeast strain expressing LRP-ST61-97 was then used for sequential transformation of the 100 μg of cDNA library and plated them on SD-dropout plates lacking adenine, histidine, leucine and tryptophan [30]. Yeast was allowed to grow for 72 hrs at 30°C before His^+ cells were scored and an X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) overlay assay was performed. Colonies that grew under histidine (His^+) were then tested for β-galactosidase expression. Colonies that were positive for both His^+ and LacZ were selected as first round positives. The interactions were then verified by recovering prey plasmids from positive colonies, transforming them into yeast strains expressing LRP-ST61-97 bait and reconfirming the HIS^+ and $LacZ^+$ phenotype. The plasmid DNAs from the yeast were shuttled to bacteria by standard methods and subjected to endonuclease restriction digest analysis to sort out both different and identical cDNA library plasmids. Different sizes of cDNA prey inserts from yeast that grew under selection were sequenced. Identities of prey inserts were determined by BLAST comparison against the National Center for Biotechnology (NCBI) database.

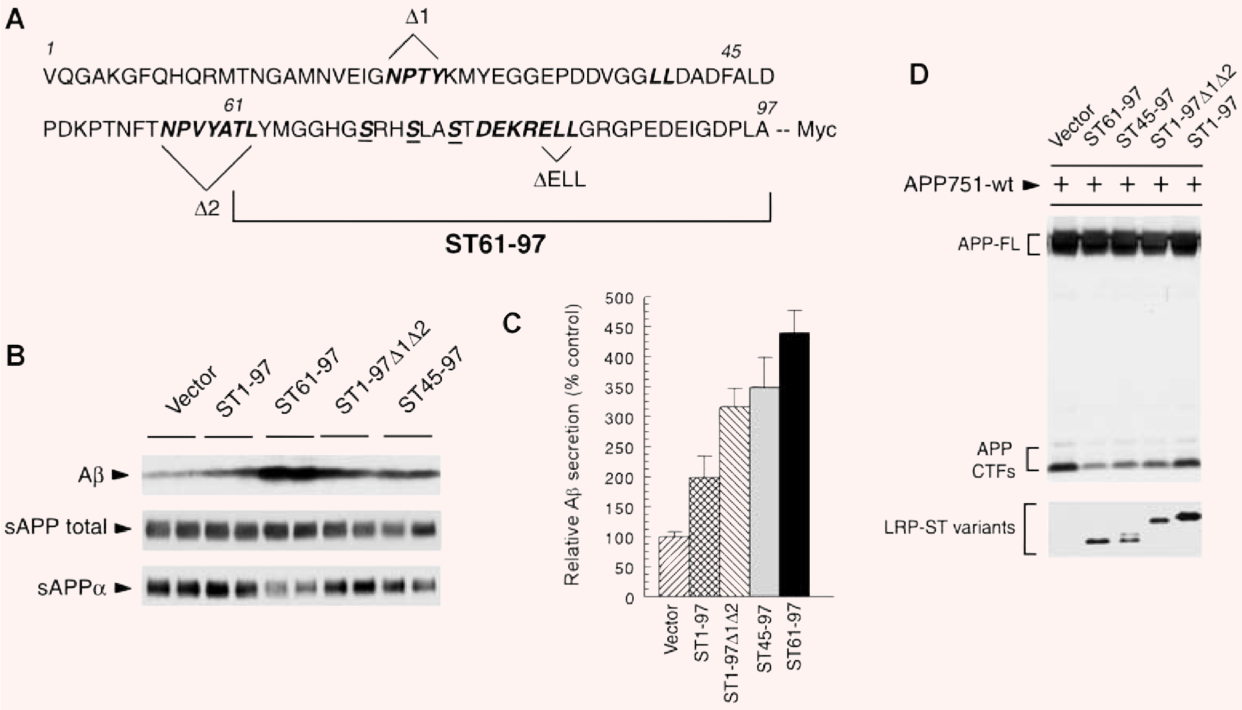

Fig. 1 LRP-ST61-97 lacking NPxY motifs is sufficient to robustly enhance amyloidogenic processing of APP. (**A**) Schematic illustration of amino acid sequence of LRP-ST1-97 to highlight the C37 region, NPxY-based motifs and dileucine motif. All LRP-ST variants were C-terminally fused with 6x myc tag. (**B**) HEK293FT cells were transiently cotransfected with APP751 and myc-tagged LRP-ST variants. Aβ (6E10), sAPPα (6E10) and sAPP total (63d) from the conditioned media were detected with their respective antibodies by straight Western blotting (for sAPPα & sAPP total) or by immunoprecipitation followed by Western blotting (for Aβ). Note the large increase in Aβ, reduction in sAPPα and no change in sAPP total by ST61-97. (**C**) Graph shows the mean Aβ levels (*n* = 6 each group) normalized to vector control from densitometric quantitations of blots using the Image J software. Error bars represent SEM. (**D**) HEK293FT cells were transiently cotransfected with APP751 and myc-tagged LRP-ST variants, and equal amount of lysates were immunoblotted for full-length APP (CT15), APP CTFs (CT15) and LRP-ST variants (9E10). Note the marked reduction in APP CTFs by ST61-97 (lane 2) and ST45-97 (lane 3) compared to vector control (lane 1) without altering APP-FL. The lower panel shows expression of various deletion mutants of LRP-ST.

Results

The C-terminal 37 residues of LRP-ST (ST61-97) are sufficient to robustly enhance Aβ production

We previously reported that deletion of the proximal or distal NPxY domains alone had no effect on the capacity of LRP-ST to promote Aβ production in stably transfected CHO cells. At the same time, the second half of LRP-ST (residues 45–97) was sufficient to enhance Aβ production, whereas the first half (residues 1–44) had no activity [18]. These results suggested the presence of another important domain distinct from the canonical NPxY motif (Fig. 1A) that mediates this pro-amyloidogenic activity, perhaps in concert with one or more NPxY motif(s). Therefore, we tested several more deletion mutants of LRP-ST to further dissect the minimal region(s) required to promote Aβ production. LRP-ST variants were fused with a C-terminal 6x Myc tag (Fig. 1A) and transiently cotransfected with APP751 in HEK293T cells. As previously shown in stably transfected cells [18], full-length ST1-97 promoted Aβ secretion by approximately twofold in transient cotransfection experiments (Fig. 1B and C). Surprisingly, deletion of both NPxY motifs (ST1-97Δ1Δ2) demonstrated that NPxY motifs are not required for LRP-ST-mediated Aβ promotion (Fig. 1B and C). In fact, the ST1-97Δ1Δ2 mutant elevated Aβ levels beyond that of LRP-ST1-97, suggesting an inhibitory effect of the NPxY motifs in the context of LRP-ST. ST45-97, which contains the second NPxY motif, also elevated Aβ levels as effectively as ST1-97Δ1Δ2 (Fig. 1B and C). Finally, ST-61-97, the C-terminal 37 residues of LRP (LRP-C37) lacking both NPxY motifs, was sufficient to robustly elevate Aβ secretion by an average of more than fourfold beyond vector controls in multiple experiments (Fig. 1B and C). The increase in Aβ by ST61-97 was accompanied by a reduction in sAPPα without altering total sAPP levels (Fig. 1B), indicating an increase in β-secretase at the expense of α-secretase cleavage. Interestingly, ST61-97 transfection also led

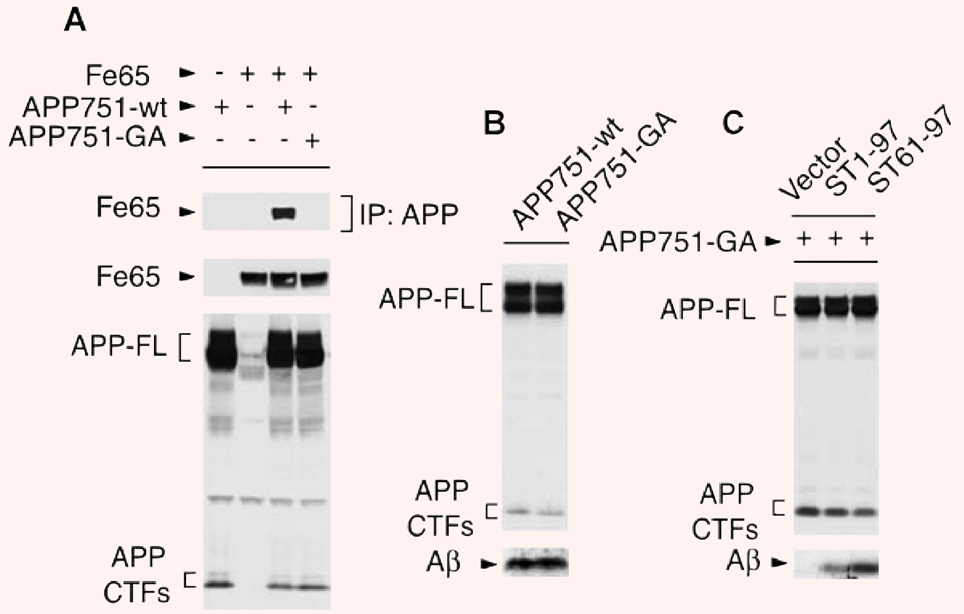

Fig. 2 FE65/APP binding is not required for LRP-ST-mediated amyloidogenic processing of APP (**A**) HEK293FT cells were transfected with combinations of FE65, APP751-wt or APP751-G737A mutant. Equal protein amounts were immunoprecipitated for APP (IG7). In the lower two panels, equal amounts of lysates were immunoblotted for FE65 (anti-FE65) APP-FL (CT15) and APP-CTFs (CT15). Note that FE65 does not bind to APP751-G737A mutant and APP-CTF levels were also unchanged. (**B**) HEK293FT cells were transiently transfected with either APP751-wt or APP751-G737A mutant and Aβ (6E10) detected in the conditioned media was not altered. Lysates were immunoblotted for APP-FL and APP-CTFs (CT15). (**C**) HEK293FT cells were cotransfected with APP-751-GA mutant along with empty myc-vector, myc-LRP-ST1-97 or LRP-ST61-97. Both LRP-ST1-97 and LRP-ST61-97 increased Aβ in the conditioned medium similar to that seen in APP-wt. Upper panel shows equal amounts of APP-FL while the levels of APP-CTFs were reduced by LRP-ST61-97.

to a consistent decrease in the level of APP-C-terminal fragment (CTFs) (Fig. 1D). Similar to that seen with full-length LRP-ST [19], overexpression of LRP-ST61-97 also led to increased localization of both full-length APP and CTFs to lipid rafts (not shown). Taken together, these results clearly demonstrate that the soluble LRP-ST61-97 (C37) constitutes the core Aβ promoting sequence of LRP-ST and functions to either activate or recruit as yet unknown interacting protein(s) to mediate the pro-amyloidogenic activity.

FE65/APP binding is not required for LRP-ST-mediated increase in Aβ

Our finding that the NPxY motifs are not required for LRP-ST-mediated pro-amyloidogenic activity was surprising, in part, because all known LRP-interacting cytoplasmic proteins are thought to bind through the NPxY motifs [22–24]. Of particular significance, FE65 was shown to enhance the interaction between APP and LRP, as the N- and C-terminal domains of FE65 can bind to APP and LRP independently [24–26]. To test whether FE65 is required for LRP-ST-mediated effect on Aβ production, we employed the APP-GA mutant in which the Gly737 was mutated to alanine. This mutant was previously shown neither to alter APP endocytosis nor Aβ secretion [31]. However, the APP-GA mutant could not bind FE65 and thus completely failed to transactivate the APP intracellular domain (AICD) in a GAL4-based nuclear reporter assay [32]. Indeed, we confirmed that the APP-GA mutant neither binds FE65 nor alters Aβ production in transient transfection experiments (Fig. 2A and B). Likewise, cotransfection of APP-GA mutant with ST1-97 or ST61-97 showed a similar elevation in Aβ secretion as seen with wild-type APP (Fig. 2C). Thus, these data clearly demonstrate that LRP-ST neither requires the NPxY motifs nor FE65/APP binding to promote Aβ production.

The dileucine-based signal within LRP-ST61-97 promotes Aβ production

Although the ST61-97 sequence does not contain NPxY motifs, a dileucine-based signal with the consensus sequence D/ExxxLL is present. This motif in LRP is completely conserved between all species examined and is thought to be involved in endosomal-lysosomal targeting of various membrane proteins [33]. To test whether the conserved dileucine sequence might underlie the pro-amyloidogenic activity of ST61-97, we deleted the sequences DEKR and glutamate-leucine-leucine (ELL) from ST61-97. In transient transfection experiments, the loss of ELL nearly completely abrogated the ST-61-97-mediated Aβ increase (Fig. 3A), indicating that the dileucine motif is a key determinant of the Aβ increasing activity. The deletion of the DEKR sequence only minimally reduced Aβ generation by approximately 25% (not shown). At the same time, however, deletion of the ELL sequence had no effect on the reduction in APP CTFs by ST61-97 (Fig. 3B), suggesting the presence of other protein binding motif(s) within LRP-ST61-97 important for different aspects of APP trafficking.

Identification of LRP-ST61-97 (C37) interacting proteins *via* yeast two-hybrid screen

The Aβ promoting action of LRP-ST61-97 is likely achieved by the recruitment of one or more as yet unknown interacting proteins. Thus, we next conducted a yeast two-hybrid screen of a mouse E17 cDNA library using the LRP-ST61-97 sequence as bait. Although two-hybrid screen is a very powerful system to identify novel protein interactions [30], LRP-ST has been shown to self-activate in a yeast two-hybrid screen [23, 24], and therefore, it is not suitable to be used as bait in this assay. Thus, we initially tested whether LRP-ST61-97 might also self-activate. As expected, cotransformation of LRP-ST1-97 and empty prey plasmid pGAD resulted in growth of blue colonies in the presence of X-gal, indicating self-activation (Fig. 4A). However, colonies cotransformed with LRP-61-97 and empty pGAD did not turn blue in the presence of X-gal (Fig. 4B), demonstrating that LRP-ST61-97 does not self-activate and is suitable for use as bait. A conventional two-hybrid screen results in many positive clones, many of which might be false-positives. Therefore, we used a stringent protocol using quadruple dropout plates to screen the cDNA library to minimize false-positive detection. Out of 2×10^6 clones screened, we identified 18 colonies that grew in quadruple dropout plates and turned blue in the presence of X-gal. Sequence analysis revealed four partial cDNA inserts fused in frame with Gal4, all of which were represented in multiple colonies. These included Filamin A, a previously uncharacterized SH3/dbl repeat RhoGTPase domain containing protein (shortened as SH3 protein here; Accession: AK030242), Ran-binding protein M (RanBPM) and Snapin. Because it is possible that more than one plasmid may be present in a given yeast colony, the results were reconfirmed after isolation of the plasmids from the yeast. Indeed cotransformation of the aforementioned isolated prey plasmids with LRP-ST61-97 bait plasmid all resulted in growth of colonies with high β-gal activity in quadruple dropout plates in the presence of X-gal. However, yeast cotransformed with the prey plasmid and empty bait plasmid failed to grow or show β-gal activity under identical conditions (Fig. 5A and B), indicating the specificity of these interactions in the yeast two-hybrid system.

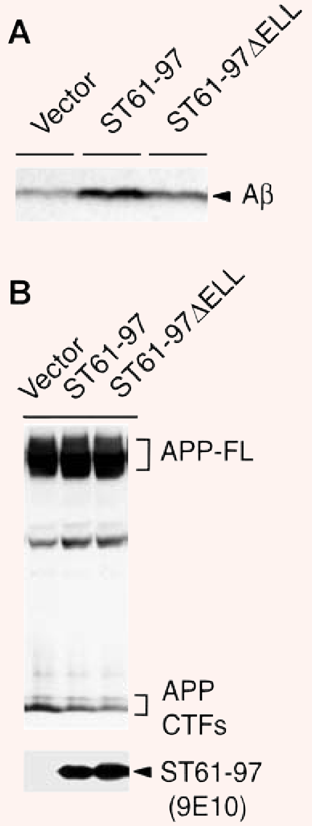

Fig. 3 The dileucine motif mediates LRP-ST61-97-induced amyloidogenic processing of APP. (**A**) Conditioned media from cells in Figure 3B were immunoprecipitated and immunoblotted for Aβ (6E10). Note that ST61-97ΔELL mutant nearly abrogates the increase in the secretion of Aβ by ST61-97, indicating the dileucine motif within ST61-97 is crucial for the generation of Aβ. (**B**) HEK293FT cells were transiently cotransfected with APP751 and myc-vector, myc-ST-61-97 or myc-ST61-97ΔELL, and lysates were subjected to immunoblotting to verify the expression levels of APP-FL, APP-CTFs and LRP-ST variants.

Coimmunoprecipitation of LRP-ST61-97-(C37)-binding proteins to LRP in cultured cells

To demonstrate that the clones identified by two-hybrid screens can interact with LRP in cultured cells, we initially transfected myc-Filamin A or myc-SH3 protein (AK030242) in HEK293T cells, and 1% NP40 lysates were subjected to immunoprecipitation for myc (9E10 antibody). Indeed, 9E10 immune complexes specifically

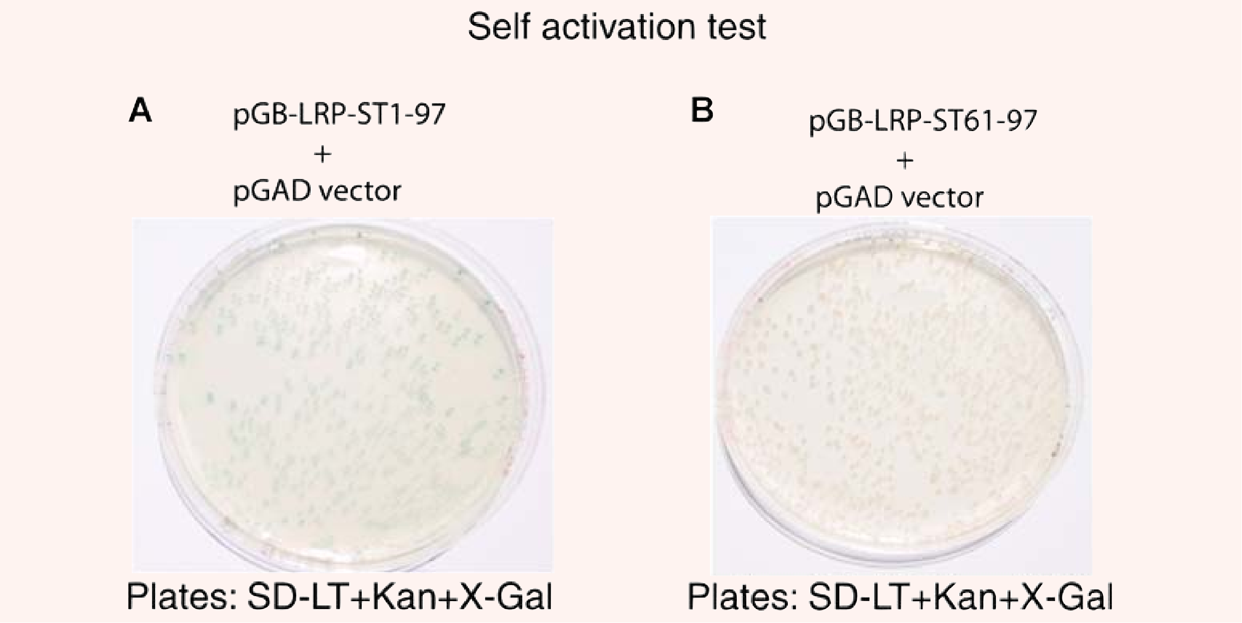

Fig. 4 LRP-ST61-97 sequence does not self-activate unlike LRP-ST in the yeast two-hybrid system. (**A**) Yeast transformed with pGB-LRP-ST1-97 and pGAD vector and plated onto synthetic dropout medium lacking leucine and tryptophan but containing kanamycin and X-gal (SD-LT + Kan + X-gal) turned blue indicating that the ST1-97 self-activates in this assay. (**B**) But yeast transformed with pGB-LRP-ST61-97 (LRP-C37) and pGAD vector and plated onto the same synthetic dropout medium does not turn blue indicating that LRP-C37 does not self-activate and therefore can be used in the yeast two-hybrid screens.

contained not only exogenously transfected LRP-CT but also endogenous LRP β-chain only when myc-Filamin A or myc-SH3 protein cDNAs but not when myc empty vector were cotransfected (Fig. 5C and D). Thus, coimmunoprecipitation experiments confirmed that the clones identified by two-hybrid screens are indeed true binding partners of LRP-C37 region. These results taken together demonstrate that the LRP-C37 region is a novel protein–protein interaction domain of LRP CT that regulates Aβ generation.

Discussion

Accumulation of Aβ is a critical event in the pathogenesis of AD. LRP and three of its key ligands, APP, α2-macroglobulin and ApoE are genetically associated or linked to AD [4, 7, 8, 10]. LRP is known to play an important role in extracellular Aβ uptake and removal [11–14]. At the same time, LRP has been shown to promote Aβ generation by altering the trafficking and processing of APP. Like APP, LRP also undergoes ectodomain cleavage by BACE1 and a phorbol ester-activated protease [20, 21]. This, in turn, leads to presenilin-dependent intramembrane proteolysis and release of the LICD [20, 21], a polypeptide that is essentially identical to LRP-ST. Previously, we showed that LRP soluble cytoplasmic tail (LRP-ST) was sufficient to enhance Aβ generation by promoting APP localization to lipid rafts [19]. The CT of LRP has been shown to interact with a number of adapter proteins, including Dab, FE65, JIP-1/2, PSD-95, SEMCAP-1, OMP25 and Shc [22–24]. With the exception of FE65 that can bind to both NPxY-based motifs in LRP [26], all other aforementioned proteins only interact with the second NPxY motif. Because LRP CT self-activates in yeast two-hybrid assays, most of these interactions were validated by GST-fusion protein pull-down assays based on yeast two-hybrid screens of homologous CTs of other LDL receptor family members [23, 24]. We had initially hypothesized that NPxY motifs within LRP-ST are responsible for increased Aβ generation, since all the known adaptor proteins bind to these motifs. Among all the known LRP-interacting adaptor proteins, FE65 is the only protein that has clearly been demonstrated to simultaneously bind to both LRP and APP and functionally bridge their interaction [24, 26]. However, we found that neither the NPxY motifs nor FE65 binding to APP were required for LRP-ST-mediated increase in Aβ generation. Previous studies showed conflicting results on whether FE65 increases Aβ generation. Overexpression of FE65 or FE65L increased Aβ secretion in Madin-Darby Canine Kidney (MDCK)

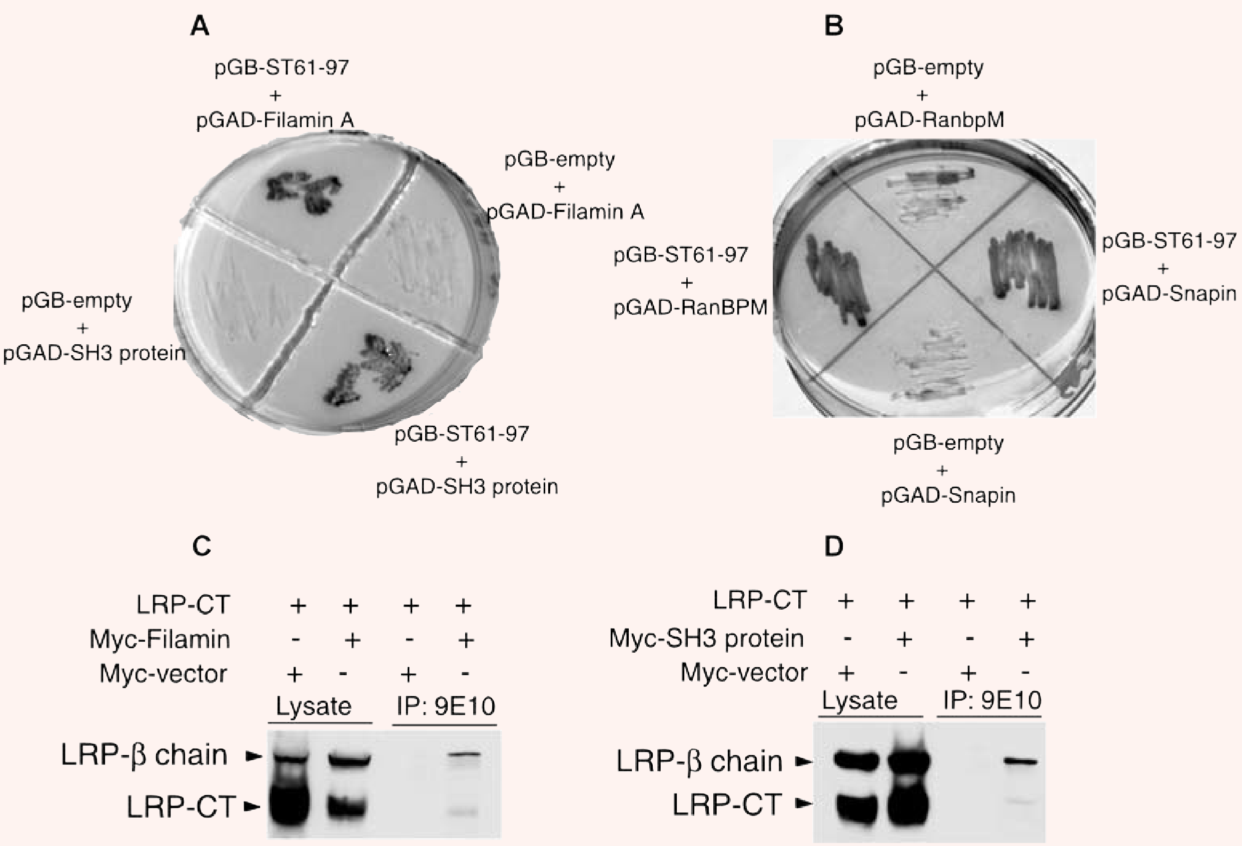

Fig. 5 LRP-ST61-97 sequence (C37 region) represents a novel protein interaction region with multiple binding partners. (**A**) pGB-ST61-97 or pGB-empty vector were cotransformed with pGAD-Filamin A or pGAD-SH3 protein into AH109 yeast strain and the resulting colonies were re-streaked onto high-stringency plates lacking histidine, adenine, leucine and tryptophan, but containing kanamycin and X-gal as a substrate for the target gene β-galactosidase. The robust growth of the colony and the change in colour to blue indicated the physical interaction between LRP-ST61-97 and Filamin A or SH3 protein (Accession: AK030242). However, the colonies failed to grow on the quadruple drop out plates when ST61-97 was replaced with either empty vector or vector expressing lamin as control. (**B**) Same as panel (A) but pGB-ST61-97 or pGB-empty vector were cotransformed with pGAD-Snapin or pGAD-RanBPM, demonstrating specific interactions of LRP-ST61-97 also with Snapin and RanBPM. (**C**) HEK293FT cells were cotransfected with combinations of LRP-CT, myc-Filamin A or myc-vector and lysates were subjected to immunoprecipitation for myc-Filamin A (9E10). Both the endogenous LRP (upper band) and the exogenously expressed LRP-CT (anti-LRP, 1704) were specifically pulled-down only when myc-Filamin was cotransfected but not with the myc vector. (**D**) Similar experiment as in panel (**C**), but cells were transfected with SH3 protein (AK030242).

cells stably transfected with APP [34]. In HEK293T cells, however, conflicting data were reported as to whether FE65 increases or decreases Aβ generation [35, 36]. Another study showed that expression of human FE65 in a mouse model of AD led to reduced Aβ load as well reduced APP CTFs and sAPPα [37]. While these studies demonstrated that overexpression of FE65 can alter APP processing in different ways, our study clearly indicated that FE65 binding to either APP or LRP-ST are not required for LRP-ST-mediated increase in Aβ production. Instead, we demonstrated that LRP-ST61-97 lacking NPxY motifs constitute the core Aβ promoting region of LRP-ST and that the D/ExxxLL dileucine motif is a key determinant of its pro-amyloidogenic activity.

Within the C37 region of LRP, it has been shown that the dileucine motif plays a minor role in LRP endocytosis [38]. The triple serine phosphorylation sequence (SRHSLAS) within the C37 region is known to influence the affinity of adaptor proteins to the second NPxY motif [39]. Aside from the preceding observations, we are not aware of any other data regarding the LRP-C37 region. Unlike previous studies that used self-activating sequences surrounding the second NPxY motif as bait [23, 24], we used non-self-activating LRP-C37 (ST61-97) sequences lacking NPxY motifs as bait in a yeast two-hybrid screen. Our yeast two-hybrid screening results clearly demonstrated that the LRP-C37 sequence is a protein—protein interaction domain that potentially has many biologically significant binding partners.

Table 1 New LRP-C37-binding proteins

Protein ID	Amino acids	Domains & motifs	Potential functions
Snapin	136	PKA phosphorylation site	Binds SNARE complexes & regulates exocytosis of secretory vesicles
		Coiled coil region	
		Nuclear-targeting sequence	
		Kid repeat motif	
Filamin A	2520	Actin-binding domain	Scaffold for cell motility
		Calphonin homology domain	Mutated in periventricular heterotopia & other disorders
RanBPM	729	Proline-rich domain	Binds to cytoplasmic tails of various surface receptors, activates Ras-ERK, & inhibits neurite outgrowth
		SPRY domain	
		LIS1 homology domain	
		CTLH domain	
SH3 protein (AK030242)	536	SH3 domain	Signalling & trafficking
		BAR domain	Membrane curvature
		RhoGEF domain	Vesicle excision
		Dbl homology domain	Activation of Rho proteins

Specifically, we identified four new LRP-binding proteins, all of which have been implicated or predicted to play a role in intracellular signal transduction and/or protein trafficking. The SH3/dbl repeat RhoGTPase domain containing protein (Accession: AK030242) possesses an N-terminal RhoGEF domain, mid portion BAR domain and a C-terminal SH3 domain. In particular, the presence of the BAR domain has been shown to be involved in lipid binding and driving membrane curvature, suggesting a role in membrane sorting events (Table 1). RanBPM appears to be a multi-functional protein that can bind a number of different cell surface receptors, including β2-integrin and Met receptor tyrosine kinase, and mediating their signal transduction events (Table 1) [40, 41]. Filamin A is a homodimeric F-actin cross-linking protein involved in organization of the cytoskeleton [42], and therefore, may be involved scaffolding the attachment of LRP to the cytoskeleton (Table 1). Interestingly, mutations in Filamin A result in a variety of human developmental diseases, including neuronal migration disorders [42]. Snapin was originally discovered as a SNAP-25 interacting protein in SNARE complexes that promotes the association of SNAP-25 with synaptotagmin *via* PKA-mediated phosphorylation [43]. It also interacts with another related SNARE protein expressed in both neuronal and non-neuronal cells, SNAP-23 [44]. Thus, Snapin has been proposed to function in membrane fusion events from ER to plasma membrane and in exocytosis of secretory vesicles (Table 1). Whether any of these aforementioned proteins mediate the LRP-ST-induced Aβ generation requires further detailed investigation. Nonetheless, the specific interactions of LRP to these proteins suggest a new role of the LRP-C37 region in signal transduction and protein trafficking both in the context of the TM receptor as well as the soluble intracellular domain.

The cellular and biochemical mechanisms by which Aβ are generated are critical for designing therapeutic strategies for AD. Inhibiting β- or γ-secretase activities are obvious therapeutic strategies. γ-secretase inhibitors are highly effective in lowering Aβ but also have unintended side effects, such as inhibition of Notch cleavage, an important activity for neurogenesis and maintenance of multiple stem cell populations [45]. Highly specific β-secretase inhibitors are not yet available for therapeutic purposes, and the potential side effects are uncertain, especially in light of new observations that BACE1 controls the myelination of CNS and peripheral axons [46]. In this study, we found that the LRP-C37 polypeptide itself (ST61-97) exerts profound effects on Aβ generation. Thus, it may be possible to reduce Aβ generation by therapeutically targeting the LRP-C37 region from interacting with potential pro-amyloidogenic proteins. Alternatively, it may be possible to generate peptide variants and peptidomimetics based on sequences from the LRP-C37 region that could potentially inhibit Aβ secretion.

Acknowledgements

We thank Dr. Thomas Sudhof for the pCMV-FE65 construct and Dr. Edward Koo for antibodies 1704, CT15, 63d and anti-FE65. This work was supported in part by the American Health Association (A2007-05, D.E. Kang) and NIA (AG 005131-24S1, D.E. Kang). We declare that we have no conflict of interest.

References

1. **Herz J, Strickland DK.** LRP: a multifunctional scavenger and signaling receptor. *J Clin Invest.* 2001; 108: 779–84.
2. **Herz J, Hamann U, Rogne S, Myklebost O, Gausepohl H, Stanley KK.** Surface location and high affinity for calcium of a 500-kd liver membrane protein closely related to the LDL-receptor suggest a physiological role as lipoprotein receptor. *EMBO J.* 1988; 7: 4119–27.
3. **Lambert JC, Wavrant-De Vrieze F, Amouyel P, Chartier-Harlin MC.** Association at LRP gene locus with sporadic late-onset Alzheimer's disease. *Lancet.* 1998; 351: 1787–8.
4. **Kang DE, Saitoh T, Chen X, Xia Y, Masliah E, Hansen LA, Thomas RG, Thal LJ, Katzman R.** Genetic association of the low-density lipoprotein receptor-related protein gene (LRP), an apolipoprotein E receptor, with late-onset Alzheimer's disease. *Neurology.* 1997; 49: 56–61.
5. **Hollenbach E, Ackermann S, Hyman BT, Rebeck GW.** Confirmation of an association between a polymorphism in exon 3 of the low-density lipoprotein receptor-related protein gene and Alzheimer's disease. *Neurology.* 1998; 50: 1905–7.
6. **Wavrant-DeVrieze F, Lambert JC, Stas L, Crook R, Cottel D, Pasquier F, Frigard B, Lambrechts M, Thiry E, Amouyel P, Tur JP, Chartier-Harlin MC, Hardy J, Van Leuven F.** Association between coding variability in the LRP gene and the risk of late-onset Alzheimer's disease. *Hum Genet.* 1999; 104: 432–4.
7. **Blacker D, Wilcox MA, Laird NM, Rodes L, Horvath SM, Go RC, Perry R, Watson B Jr, Bassett SS, McInnis MG, Albert MS, Hyman BT, Tanzi RE.** Alpha-2 macroglobulin is genetically associated with Alzheimer disease. *Nat Genet.* 1998; 19: 357–60.
8. **Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving N, James L, Mant R, Newton P, Rooke K, Roques P, Talbot C, Pericak-Vance M, Roses A, Williamson R, Rossor M, Owen M, Hardy J.** Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature.* 1991; 349: 704–6.
9. **Rebeck GW, Harr SD, Strickland DK, Hyman BT.** Multiple, diverse senile plaque-associated proteins are ligands of an apolipoprotein E receptor, the alpha 2-macroglobulin receptor/low-density-lipoprotein receptor-related protein. *Ann Neurol.* 1995; 37: 211–7.
10. **Schmechel DE, Saunders AM, Strittmatter WJ, Crain BJ, Hulette CM, Joo SH, Pericak-Vance MA, Goldgaber D, Roses AD.** Increased amyloid beta-peptide deposition in cerebral cortex as a consequence of apolipoprotein E genotype in late-onset Alzheimer disease. *Proc Natl Acad Sci USA.* 1993; 90: 9649–53.
11. **Kang DE, Pietrzik CU, Baum L, Chevallier N, Merriam DE, Kounnas MZ, Wagner SL, Troncoso JC, Kawas CH, Katzman R, Koo EH.** Modulation of amyloid beta-protein clearance and Alzheimer's disease susceptibility by the LDL receptor-related protein pathway. *J Clin Invest.* 2000; 106: 1159–66.
12. **Narita M, Holtzman DM, Schwartz AL, Bu G.** Alpha2-macroglobulin complexes with and mediates the endocytosis of beta-amyloid peptide *via* cell surface low-density lipoprotein receptor-related protein. *J Neurochem.* 1997; 69: 1904–11.
13. **Deane R, Wu Z, Sagare A, Davis J, Du YS, Hamm K, Xu F, Parisi M, LaRue B, Hu HW, Spijkers P, Guo H, Song X, Lenting PJ, Van Nostrand WE, Zlokovic BV.** LRP/amyloid beta-peptide interaction mediates differential brain efflux of Abeta isoforms. *Neuron.* 2004; 43: 333–44.
14. **Sagare A, Deane R, Bell RD, Johnson B, Hamm K, Pendu R, Marky A, Lenting PJ, Wu Z, Zarcone T, Goate A, Mayo K, Perlmutter D, Coma M, Zhong Z, Zlokovic BV.** Clearance of amyloid-beta by circulating lipoprotein receptors. *Nat Med.* 2007; 13: 1029–31.
15. **Kounnas MZ, Moir RD, Rebeck GW, Bush AI, Argraves WS, Tanzi RE, Hyman BT, Strickland DK.** LDL receptor-related protein, a multifunctional ApoE receptor, binds secreted beta-amyloid precursor protein and mediates its degradation. *Cell.* 1995; 82: 331–40.
16. **Pietrzik CU, Busse T, Merriam DE, Weggen S, Koo EH.** The cytoplasmic domain of the LDL receptor-related protein regulates multiple steps in APP processing. *EMBO J.* 2002; 21: 5691–700.
17. **Ulery PG, Beers J, Mikhailenko I, Tanzi RE, Rebeck GW, Hyman BT, Strickland DK.** Modulation of beta-amyloid precursor protein processing by the low density lipoprotein receptor-related protein (LRP). Evidence that LRP contributes to the pathogenesis of Alzheimer's disease. *J Biol Chem.* 2000; 275: 7410–15.
18. **Yoon IS, Pietrzik CU, Kang DE, Koo EH.** Sequences from the low density lipoprotein receptor-related protein (LRP) cytoplasmic domain enhance amyloid beta protein production *via* the beta-secretase pathway without altering amyloid precursor protein/LRP nuclear signaling. *J Biol Chem.* 2005; 280: 20140–7.
19. **Yoon IS, Chen E, Busse T, Repetto E, Lakshmana MK, Koo EH, Kang DE.** Low-density lipoprotein receptor-related protein promotes amyloid precursor protein trafficking to lipid rafts in the endocytic pathway. *FASEB J.* 2007; 21: 2742–52.
20. **May P, Reddy YK, Herz J.** Proteolytic processing of low density lipoprotein receptor-related protein mediates regulated release of its intracellular domain. *J Biol Chem.* 2002; 277: 18736–43.
21. **von Arnim CA, Kinoshita A, Peltan ID, Tangredi MM, Herl L, Lee BM, Spoelgen R, Hshieh TT, Ranganathan S, Battey FD, Liu CX, Bacskai BJ, Sever S, Irizarry MC, Strickland DK, Hyman BT.** The low density lipoprotein receptor-related protein (LRP)

is a novel beta-secretase (BACE1) substrate. *J Biol Chem.* 2005; 280: 17777–85.

22. **Barnes H, Ackermann EJ, van der GP.** v-Src induces Shc binding to tyrosine 63 in the cytoplasmic domain of the LDL receptor-related protein 1. *Oncogene.* 2003; 22: 3589–97.
23. **Gotthardt M, Trommsdorff M, Nevitt MF, Shelton J, Richardson JA, Stockinger W, Nimpf J, Herz J.** Interactions of the low density lipoprotein receptor gene family with cytosolic adaptor and scaffold proteins suggest diverse biological functions in cellular communication and signal transduction. *J Biol Chem.* 2000; 275: 25616–24.
24. **Trommsdorff M, Borg JP, Margolis B, Herz J.** Interaction of cytosolic adaptor proteins with neuronal apolipoprotein E receptors and the amyloid precursor protein. *J Biol Chem.* 1998; 273: 33556–60.
25. **Kinoshita A, Whelan CM, Smith CJ, Mikhailenko I, Rebeck GW, Strickland DK, Hyman BT.** Demonstration by fluorescence resonance energy transfer of two sites of interaction between the low-density lipoprotein receptor-related protein and the amyloid precursor protein: role of the intracellular adapter protein Fe65. *J Neurosci.* 2001; 21: 8354–61.
26. **Pietrzik CU, Yoon IS, Jaeger S, Busse T, Weggen S, Koo EH.** FE65 constitutes the functional link between the low-density lipoprotein receptor-related protein and the amyloid precursor protein. *J Neurosci.* 2004; 24: 4259–65.
27. **Cao X, Sudhof TC.** A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. *Science.* 2001; 293: 115–20.
28. **Takeda T, Yamazaki H, Farquhar MG.** Identification of an apical sorting determinant in the cytoplasmic tail of megalin. *Am J Physiol Cell Physiol.* 2003; 284: C1105–13.
29. **Koo EH, Squazzo SL, Selkoe DJ, Koo CH.** Trafficking of cell-surface amyloid beta-protein precursor. I. Secretion, endocytosis and recycling as detected by labeled monoclonal antibody. *J Cell Sci.* 1996; 109: 991–8.
30. **James P, Halladay J, Craig EA.** Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics.* 1996; 144: 1425–36.
31. **Perez RG, Soriano S, Hayes JD, Ostaszewski B, Xia W, Selkoe DJ, Chen X, Stokin GB, Koo EH.** Mutagenesis identifies new signals for beta-amyloid precursor protein endocytosis, turnover, and the generation of secreted fragments, including Abeta42. *J Biol Chem.* 1999; 274: 18851–6.
32. **Cao X, Sudhof TC.** Dissection of amyloid-beta precursor protein-dependent transcriptional transactivation. *J Biol Chem.* 2004; 279: 24601–11.
33. **Bonifacino JS, Traub LM.** Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annu Rev Biochem.* 2003; 72: 395–447.
34. **Sabo SL, Lanier LM, Ikin AF, Khorkova O, Sahasrabudhe S, Greengard P, Buxbaum JD.** Regulation of beta-amyloid secretion by FE65, an amyloid protein precursor-binding protein. *J Biol Chem.* 1999; 274: 7952–7.
35. **Ando K, Iijima KI, Elliott JI, Kirino Y, Suzuki T.** Phosphorylation-dependent regulation of the interaction of amyloid precursor protein with Fe65 affects the production of beta-amyloid. *J Biol Chem.* 2001; 276: 40353–61.
36. **Tanahashi H, Tabira T.** Molecular cloning of human Fe65L2 and its interaction with the Alzheimer's beta-amyloid precursor protein. *Neurosci Lett.* 1999; 261: 143–6.
37. **Santiard-Baron D, Langui D, Delehedde M, Delatour B, Schombert B, Touchet N, Tremp G, Paul MF, Blanchard V, Sergeant N, Delacourte A, Duyckaerts C, Pradier L, Mercken L.** Expression of human FE65 in amyloid precursor protein transgenic mice is associated with a reduction in beta-amyloid load. *J Neurochem.* 2005; 93: 330–8.
38. **Li Y, Marzolo MP, Van Kerkhof P, Strous GJ, Bu G.** The YXXL motif, but not the two NPXY motifs, serves as the dominant endocytosis signal for low density lipoprotein receptor-related protein. *J Biol Chem.* 2000; 275: 17187–94.
39. **Ranganathan S, Liu CX, Migliorini MM, von Arnim CA, Peltan ID, Mikhailenko I, Hyman BT, Strickland DK.** Serine and threonine phosphorylation of the low density lipoprotein receptor-related protein by protein kinase Calpha regulates endocytosis and association with adaptor molecules. *J Biol Chem.* 2004; 279: 40536–44.
40. **Denti S, Sirri A, Cheli A, Rogge L, Innamorati G, Putignano S, Fabbri M, Pardi R, Bianchi E.** RanBPM is a phosphoprotein that associates with the plasma membrane and interacts with the integrin LFA-1. *J Biol Chem.* 2004; 279: 13027–34.
41. **Wang D, Li Z, Messing EM, Wu G.** Activation of Ras/Erk pathway by a novel MET-interacting protein RanBPM. *J Biol Chem.* 2002; 277: 36216–22.
42. **Feng Y, Walsh CA.** The many faces of filamin: a versatile molecular scaffold for cell motility and signalling. *Nat Cell Biol.* 2004; 6: 1034–8.
43. **Ilardi JM, Mochida S, Sheng ZH.** Snapin: a SNARE-associated protein implicated in synaptic transmission. *Nat Neurosci.* 1999; 2: 119–24.
44. **Buxton P, Zhang XM, Walsh B, Sriratana A, Schenberg I, Manickam E, Rowe T.** Identification and characterization of Snapin as a ubiquitously expressed SNARE-binding protein that interacts with SNAP23 in non-neuronal cells. *Biochem J.* 2003; 375: 433–40.
45. **Harper JA, Yuan JS, Tan JB, Visan I, Guidos CJ.** Notch signaling in development and disease. *Clin Genet.* 2003; 64: 461–72.
46. **Hu X, Hicks CW, He W, Wong P, Macklin WB, Trapp BD, Yan R.** Bace1 modulates myelination in the central and peripheral nervous system. *Nat Neurosci.* 2006; 9: 1520–5.