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The mechanism of the low-density lipoprotein receptor-related protein (LRP) in the production of amyloid-[Beta] peptide

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The mechanism of the Low-density lipoprotein receptor-related protein (LRP) in the production of Amyloid-β Peptide

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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2008
This thesis of Eunice Chungyu Chen is approved and it is acceptable in quality and form for publication on microfilm:

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University of California, San Diego

2008
This is dedicated to my family and friends for always encouraging me when times were tough and celebrating with me when things went well.

I would like to thank the Kang and Koo labs for providing me with an opportunity to explore the world through my research and teaching me what it means to be a scientist.
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ABSTRACT OF THE THESIS

The mechanism of the Low-density lipoprotein receptor-related protein (LRP) in the 
production of Amyloid-β Peptide

by

Eunice Chungyu Chen

Master of Science in Biology
University of California, San Diego, 2008

Professor Gentry Patrick, Co-Chair
Professor David E. Kang, Co-Chair

Alzheimer’s disease (AD) is the most common form of neurodegenerative disorder affecting the elderly, presenting symptoms such as memory impairment and dementia. AD is pathologically characterized by the development of extracellular senile plaques and intracellular neurofibrillary tangles (NFT). The plaques are composed of amyloid-β peptide (Aβ) and the NFTs are composed of a hyperphosphorylated form of the tau protein. Aβ is formed by sequential proteolytic processing of the amyloid precursor protein (APP) by β-, and γ-secretase. Accordingly, alterations in APP processing result in increased Aβ generation. The low-density lipoprotein receptor-related
protein (LRP) is a large endocytic protein involved in diverse biological functions. It has been hypothesized that LRP plays a dual role in AD, playing a role in both the clearance and the production of Aβ. Previous studies have shown that the cytoplasmic tail alone is able to promote Aβ generation and promote APP processing.

This study seeks to determine the area of the cytoplasmic tail responsible for pro-amyloidogenic activity and how it occurs. Our findings indicate that the last 37 amino acids of the tail, containing a dileucine motif, are sufficient. Additionally, LRP facilitates the generation of Aβ by trafficking APP and BACE1 to the lipid raft domains. This function of LRP may be altered due to the presence of a Kunitz protease inhibitor (KPI) domain on APP. The results of our study have therapeutic potential to reduce β-amyloid by understanding the function of LRP in the amyloidogenic processing of APP.
INTRODUCTION

Alzheimer’s disease

Alzheimer’s disease (AD) is a progressive neurodegenerative disease characterized by neuron loss and gradual cognitive decline. It is the most common cause of dementia in the elderly, and it is estimated that approximately 25 million people worldwide are affected [1]. Less than 5% of the cases are genetically inherited, through an autosomal dominant pattern, and are classified as familial early-onset AD (FAD) [2]. The majority of cases are considered sporadic or late-onset cases.

AD is pathologically characterized by the accumulation of extracellular plaques and intracellular neurofibrillary tangles (NFT). The plaques consist of a dense core composed of amyloid-beta (Aβ) peptides arranged in β-sheets while NFT are formed from aggregates of hyperphosphorylated tau protein, a microtubule associated protein [3].

APP

Aβ is formed by the sequential cleavage of the amyloid precursor protein (APP) by a variety of secretases. APP is a type I transmembrane protein composed of a large hydrophilic N-terminal extracellular domain, a single hydrophobic transmembrane domain, and a small C-terminal cytoplasmic domain [4]. The APP gene is composed of approximately 18 exons [5] and is located on chromosome 21 [2]. Through alternative splicing of the gene, different isoforms are formed. The main Aβ-producing isoforms are APP 695, APP 751, and APP 770 which contain 695 amino acids, 751 amino acids, and 770 amino acids, respectively. Both APP 751 and APP 770 are commonly expressed in many tissues and contain a Kunitz Protease Inhibitor (KPI) domain [6]. In contrast, APP 695 does not contain a KPI domain and is primarily expressed in neurons [7].
APP Processing

The cleavage of APP can occur in two different pathways, a non-amyloidogenic pathway, which prevents the formation of Aβ, and an amyloidogenic pathway, which releases Aβ. These pathways occur via the activity of three secretases: α-, β-, and γ-secretase. In the non-amyloidogenic pathway, α-secretase cleaves APP within the Aβ domain, between the Lys 16 and Leu 17 bond, [8] and prohibits Aβ production. This cleavage releases two peptides, the APP ectodomain (sAPPα) and a membrane bound carboxyl-terminal fragment known as α-CTF. sAPPα is believed by many to possess neuroprotective effects [1,8] and may have a role in neuronal plasticity and survival [9]. Thus many researchers focus on α-secretase activity as a possible therapeutic target against the neurodegenerative effects of AD.

Researchers have identified α-secretase as a member of the ADAM (a disintegrin and metalloproteinase) family. The ADAMs are type I integral membrane proteins composed of many different domains. ADAM 9, 10, and 17 have been identified as candidates of α-secretase activity. Initially ADAM17, also known as TACE (tumor necrosis factor- α converting enzyme), was believed to be the α-secretase responsible [10]. However, functional redundancy among family members suggests the involvement of multiple ADAM secretases in APP processing.

Alternatively, APP can be cleaved by β-secretase. Similar to α-secretase, cleavage by β-secretase releases an APP ectodomain (sAPPβ) and a membrane bound carboxyl-terminal fragment known as β-CTF. Because β-secretase cleaves at the start site for Aβ and leads to Aβ generation, this is considered the amyloidogenic pathway. Researchers have identified β-secretase as BACE1 (β-site APP cleaving enzyme). BACE1 is an
aspartyl proteinase from the pepsin family and mainly located within neurons [11]. It preferentially cleaves APP in lipid rafts [12] and has optimum activity within acidic environments [8]. BACE1 is synthesized as a large precursor, proBACE1, and modified by glycosylation before being cleaved by a furin-like endoprotease to make mature protein [13, 14]. Interestingly, BACE1 is a homolog of another secretase, BACE2, which contains \( \alpha \)-secretase activity [15]. Upregulation or downregulation of BACE1 activity affects APP cleavage and \( \text{A\textbeta} \) generation providing evidence for the importance of BACE1.

The last step in either pathway is \( \gamma \)-secretase cleavage, which occurs after \( \alpha \)- and \( \beta \)-secretase activity. This cleavage occurs within the membrane bound \( \alpha \)-CTF or \( \beta \)-CTF APP fragments and releases either p3 or \( \text{A\textbeta} \), respectively. The \( \gamma \)-secretase cleavage site within \( \beta \)-CTF is variable and produces the wide variety of \( \text{A\textbeta} \) peptides that range in size from 39 to 43 amino acids, as counted from position 1 of the \( \beta \)-secretase cut site. The cleavage also generates a peptide known as the intracellular APP domain (AICD). The AICD molecule migrates to the nucleus upon release [1] and is believed to play a role in gene regulation [16].

The \( \gamma \)-secretase molecule is composed of at least four different proteins: presenilin 1 (PS1) or presenilin 2 (PS2), nicastrin (Nct), anterior pharynx-defective-1 (APH-1) and presenilin-enhancer 2 (PEN-2). Each member is required for \( \gamma \)-secretase activity, but presenilin is believed to be the catalytic component [8]. This hypothesis is supported by PS knockout experiments. The \( \gamma \)-secretase complex also cleaves other membrane-bound fragments in a similar manner, including APLP1 and 2, Notch 1-4, ErB-4, E-cadherin, LRP, Nectin 1-\( \alpha \), Delta, Jagged, and CD44 [1].
Figure 1: APP proteolytic processing by α-, β-, and γ-secretase


In the non-amyloidogenic pathway, α-secretase cleaves APP to release a soluble ectodomain, sAPP$_{\alpha}$ and a membrane-bound C-terminal fragment, α-CTF. In the amyloidogenic pathway, β-secretase cleaves APP in a similar manner to release sAPP$_{\beta}$ and β-CTF. The CTFs can then be further cleaved by γ-secretase to generate the APP intracellular domain (AICD) as well as p3 and Aβ from α-CTF and β-CTF, respectively.
**Lipid rafts**

Endocytic processing of APP is believed to be responsible for Aβ generation by trafficking APP to regions enriched in β- and γ-secretase activity [17, 18]. A possible area this occurs is in the lipid raft microdomains, a subpopulation of cellular membranes that are insoluble in nonionic detergents at 4°C and heavily enriched in cholesterol, sphingomyelin, and glycolipids [19, 20]. Additionally, lipid raft microdomains are known to be highly enriched with BACE and presenilin activity [21-23].

**Genetic Factors**

Early-onset or familial AD (FAD) constitutes only 5% of all cases, with the majority of cases being late-onset or sporadic AD. Scientists have identified three genes in which mutations have been linked to development of FAD: the APP gene, located on chromosome 21, and the PS1 and PS2 genes, located on chromosomes 14 and 1, respectively. Because the characteristic clinical features of FAD are indistinguishable from sporadic AD, many scientists study these genes in an attempt to understand the mechanisms of the disease [8].

The APP gene is a logical candidate due to its role in the development of Aβ peptides, and thus was the first AD gene to be identified [24]. Many missense mutations have been identified in the APP gene and they are located around the α-, β-, and γ-secretase cut sites. Because the mutations result in an increase in Aβ, scientists believe they alter the processing of APP [25]. There have been more than 100 autosomal dominant point mutations identified in PS which cause aggressive early-onset AD [1]. All tested FAD-linked PS mutations increase the ratio of Aβ42/Aβ40 [26, 27], accounting for the accelerated amyloid deposition seen in these cases. As PS is an integral component of
γ-secretase, the mutations are believed to alter γ-secretase activity and disturb APP proteolysis [28].

Late-onset AD is also associated with many risk genes including apolipoprotein E (apoE), the low-density lipoprotein receptor-related protein (LRP) gene, and α2-macroglobulin (α2M). The APOE gene is located on chromosome 19 and immunohistochemical staining shows the presence of apoE in senile plaques [29, 30]. The gene is inherited in three common coding sequence polymorphisms known as ε2, ε3, and ε4. Heterozygosity for the ε4 allele increases the risk for AD by three-fold as compared to the more common homozygous ε3 genotype, while the ε2 allele decreases the risk for AD by varying degrees depending on heterozygosity or homozygosity [31]. However, the mechanism by which this allele leads to increased AD susceptibility is unclear.

The LRP gene, a neuronal apoE receptor, has also been implicated as a possible gene in late-onset AD. A silent polymorphism, known as C776T, exists within exon 3 of the LRP gene and is associated with an increased risk for late-onset [32]. AD patients carry the C allele at a higher frequency than unaffected people, although it is unknown how the patients are affected by the alteration as it does not alter the coding region of the protein. There is also a tetranucleotide repeat in the 5’ region of LRP that is suspected to play a role, but the data is not consistent among studies [33, 34].

Lastly, there are two polymorphisms located within α2M, a circulating proteinase inhibitor and LRP ligand, which seem to be associated with late-onset AD [35, 36]. The first is a pentanucleotide deletion near an intronic splice site and the second is an exchange of isoleucine for valanine at position 1000 near the active site [37]. Although
some studies have shown that the polymorphisms are risk factors for AD, the data is controversial as other studies have shown conflicting results [38, 39].

**Aβ**

There are two major Aβ peptides known as Aβ40 and Aβ42, where the numbers refer to their respective lengths as counted from the cut site. The predominant form, Aβ40, is produced during normal metabolism of APP [24] and is present at ten times the amount of the fibrillogenic form, Aβ42 [40]. Aβ42 aggregates easier [41] and is significantly more neurotoxic and is the main constituent of plaques. The peptides are predominantly produced at plasma membranes and then travel to the extracellular space where they are deposited as senile plaques [42]. Soluble Aβ can be found in the body fluids of various species [43].

**LRP**

The low-density lipoprotein receptor-related protein (LRP) is a large endocytic receptor that is highly expressed in virtually all neurons in the brain and in the liver. LRP is involved in many different biological functions including lipid metabolism, homeostasis of proteinases and proteinase inhibitors, cellular entry of viruses and toxins, activation of lysosomal enzymes, cellular signal transduction, and neurotransmission [44]. It is synthesized as a precursor protein that is further 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 [45, 46]. The extracellular α-chain contains multiple epidermal growth factor and growth factor repeats as well as four ligand-binding clusters, as seen in Figure 2. Researchers believe the ligand-binding-type repeats are responsible for the ability of LRP to bind to a wide
variety of ligands, many of which are involved in AD [44]. Each repeat contains sequence variability within the short loop region [47] which results in functional duplication [48]. The cytoplasmic tail of LRP contains two NPxY motifs, which act as docking sites for endocytosis machinery and cytoplasmic adaptor and scaffolding proteins involved in signaling [49]. Additionally, many proteins interact with the tail itself [44].

**Apolipoprotein E**

Apolipoprotein E (apoE) is a small protein composed of two domains: a helical domain that binds hydrophobic substances and a receptor domain that binds members of the low-density lipoprotein family [50]. apoE clearly plays a role in AD, as shown by immunohistochemical staining of apoE in senile plaques [29, 30] and the genetic risk data for late-onset AD mentioned earlier. As a LRP ligand, apoE/Aβ complexes can be cleared by LRP resulting in a drop in Aβ levels [54, 57, 58].

**α2-Macroglobulin**

α2-Macroglobulin (α2M) is a tetrameric complex that acts as a pan-protease inhibitor. Through a conformational alteration, it becomes a ligand known as α2M* that can interact with LRP. It has been demonstrated that α2M* can bind Aβ [51] and alter its likelihood of being fibrillogenic [52]. Similar to apoE, an Aβ/α2M* complex can be metabolized and cleared by an LRP-mediated process [54, 57, 58].
**Figure 2:** LRP ligand-binding clusters and their ligands and the cytoplasmic tail (Herz, J. et al. *J. Clin. Invest.* (2001) **108**, 779-784) [44].

LRP recognizes a wide variety of ligands by four clusters of ligand-binding repeats. Clusters II and IV bind a majority of the currently known ligands of LRP, although ApoE binds clusters II, III, and IV. The two NpxY motifs in the cytoplasmic tail serve as docking sites for endocytosis machinery.
Ligand-Binding Cluster

I

NH₂

II

ApoE, α₂M, tPA, prourokinase, tPA:PAI-1, uPA:PAI-1, TFPI, lactoferrin, LPL, factor VIII

III

ApoE

IV

ApoE, tPA, prourokinase, tPA:PAI-1, uPA:PAI-1, TFPI, lactoferrin, LPL, factor VIII

COOH

* NPxY motif

EGF precursor homology domain

EGF repeat

Transmembrane domain

Ligand-binding-type repeat
**LRP and AD**

The link between LRP and AD is substantial. An increase in LRP is seen in reactive astrocytes and activated microglia, both of which are associated with AD [55]. As mentioned previously, LRP and its ligands, apoE, α2M, and APP, are genetically associated with AD and are present in senile plaques [2, 32, 35, 56]. LRP also mediates the binding and clearance of Aβ complexed to apoE or α2M in cell culture and in the brain [54, 57, 58]. In addition, LRP may play a role in brain efflux of Aβ across the blood-brain barrier (BBB) [59] and circulating secreted LRP can function as a ‘peripheral sink’ to reduce Aβ levels in the brain [60, 61]. Recent work suggests that LRP can play a dual role in AD, involved in both production and clearance of Aβ [62]. LRP can bind the longer, KPI domain-containing APP isoforms directly which alters APP processing and results in an increase in Aβ production [63, 64].

It has previously been shown that LRP positively regulates the amyloidogenic processing of APP to increase Aβ levels. The loss of LRP or treatment of receptor-associated protein (RAP), an antagonist of LRP ligands, results in a decrease of Aβ release; however, this phenotype can be reversed by the overexpression of full-length (LRP-FL) or truncated LRP [64, 65]. Specifically, the LRP cytoplasmic tail (LRP-CT), lacking ligand binding domains but retaining the transmembrane domain and cytoplasmic tail, is able to restore amyloidogenic processing of APP and Aβ generation in LRP deficient cells [65]. The LRP soluble tail (LRP-ST), containing only the cytoplasmic tail and no transmembrane domain, alone is also sufficient to rescue Aβ generation [66].

In this study, we have further narrowed down the pro-amyloidogenic region of LRP-ST and explored the mechanisms by which LRP-ST, and endogenous full length
LRP, alter APP processing and Aβ metabolism. We report here that the last 37 residues lacking the NPxY motif but containing an essential dileucine motif is sufficient to robustly promote Aβ production. Furthermore, we show evidence that the cytoplasmic domain of LRP forms a tripartite complex with APP and BACE1 to promote the trafficking of APP to lipid rafts and enhance Aβ generation. We also propose a mechanism by which full length LRP may differentially regulate the generation and clearance of Aβ.
MATERIALS AND METHODS

Cell culture

All cell lines were cultured in Dulbeco’s modified Eagle’s medium (Mediatech/Cellgro) with 10% fetal bovine serum (Omega Scientific). The Chinese hamster ovary (CHO) cells were stably transfected with APP695 (CHO695) or APP751 (CHO APP751). The LRP-deficient murine neuroblastoma N2a cells also expressed APP695.

To generate stable cell lines expressing our mutated LRP constructs, VSV-G (glycoprotein of the vesicular stomatitis virus) pseudo-typed retroviruses were packaged and used to infect the CHO cells [56]. The cells were selected using hygromycin (500 µg/ml), and antibiotic-resistant cells were expanded. Further culture was maintained with hygromycin selection at 250 µg/ml.

cDNA constructs

Transient LRP knock down was accomplished by custom synthesizing a small interfering RNA (RNAi) targeting human LRP1 (Dharmacon). The RNAi was transfected into CHO APP751 or CHO APP751_swe cells with Lipofectamine 2000 (Invitrogen). The transfection occurred twice to ensure effective knock-down by the siRNA.

The mutant constructs were made using PCR and QuikChange site-directed mutagenesis techniques (Stratagene) and then subcloned into a pLHCX vector (Clontech). The constructs were then transiently transfected into the LRP-deficient cells using Lipofectamine 2000 transfection reagent, as mentioned above. After an overnight the cells were selected and maintained with Hygromycin B (Invitrogen).
Stable LRP-deficient CHO APP695 and CHO APP751 lines were generated by subcloning a short hairpin RNA (shRNA) specifically targeting the 5’ region of the endogenous LRP mRNA into the pSuper-puro retroviral shRNA vector (OligoEngine). After transfection with Lipofectamine 2000, the cells were selected using puromycin, and antibiotic-resistant clones were expanded. To reintroduce LRP, a minireceptor encoding the fourth binding domain to the C-terminal end of LRP (LRP-L4) was stably transfected into puromycin-resistant pools and individual clones of LRP-deficient cell lines. The LRP-L4 construct was a kind gift from Dr. Marilyn Farquhar. The minireceptor contains an HA epitope tag following the signal peptide and is capable of binding various ligands ensuring proper function.

**Lipid raft isolation**

Cell extracts from 10 cm plates were fractionated by discontinuous sucrose density gradient ultracentrifugation in order to retrieve lipid raft fractions. The confluent plates were washed with PBS on ice, scraped in TNE buffer (25 mM Tris, pH 7.4, 150 mM NaCl, and 2 mM EDTA) containing protease inhibitor mix (Sigma), and pelleted by low speed centrifugation. The cell pellets were disrupted by hand with a 25-G needle in TNE buffer then mixed with an equal volume of TNE buffer containing 2% CHAPS and incubated on ice for 30 min. The CHAPS cell extracts were mixed with TNE buffer containing sucrose to yield a final concentration of 45% and placed beneath layers of 25% and 5% sucrose in an ultracentrifuge tube. The samples were spun at 4°C for 14-16 h at 44,000 rpm. Fractions were then collected in 0.5 ml volumes from top to bottom to
yield a total of 10 fractions. The buoyant lipid rafts settled at the interface between the 35 and 5% sucrose layers (fractions 2 and 3) as an amorphous white material.

A methanol/chloroform extraction was performed to concentrate the protein levels in the fractions. Equal volumes of each concentrated fraction were used in SDS-PAGE and immunoblotting.

**Antibodies**

The polyclonal antiserum 1704 recognizes the cytoplasmic domain of LRP1 and was used to determine LRP levels. The polyclonal antiserum CT15 recognizes the cytoplasmic domain of APP and was used to detect full-length APP and APP-CTFs. Monoclonal 6E10 antibody (Covance Research) and 16-9 antibody recognize N-terminal amino acids of the Aβ peptide and were used for immunoprecipitation as well as immunoblotting. The polyclonal antiserum 63D, in conjunction with 6E10 immunoprecipitation, was used to detect sAPPα levels from conditioned media. All polyclonal antibodies were diluted in 5% non-fat milk in TBST-T buffer and monoclonal antibodies were diluted in TBS-T buffer.

**Immunoprecipitation and immunoblotting**

To detect Aβ, 1 ml of conditioned media was collected after 24h or 48h and used for immunoprecipitation. For immunoblotting, cell extracts were prepared with 1% NP40 buffer or 1% CHAPS lysis buffer and 1 X protease inhibitor cocktail (Sigma). The prepared samples were denatured by SDS sample buffer and fractionated by SDS-PAGE in 4-12% NuPAGE Bis-Tris gels (Invitrogen) or 10% Tris-glycine gels. Gel loading was
normalized to total protein concentration using values determined by the micro BCA method (Pierce). Western blotting was carried out with the previously mentioned antibodies and detected by enhanced chemiluminescence (Pierce). All experiments were performed at least three times, and each experiment was done in duplicate.

**Aβ ELISA**

Conditioned media was collected from 12-well plates after 24h and stored at -80°C until ready for use. The cell lysates were collected in 1% NP40 buffer containing protease inhibitor cocktail (Sigma) and stored at -80°C for further biochemical analysis.

Aβ40 levels were measured from 100µl aliquots of the media in triplicate following an ELISA protocol. Samples from some cell lines were diluted, as necessary, to ensure they fell within the detectable range of the standards. The dilution was performed with the same media the cells were grown in. After capture with Ab9 antibody, detection with HRP-conjugated 13-1-1 antibody, and development with 1-step 1:1 TMB development solution (Pierce) the 96-well plate was read on a spectrophotometer. The optical density was read at 450 nm and the raw numbers used to calculate the Aβ40 concentration from a standard curve. As applicable, the raw concentrations were multiplied by the dilution factor to determine the final Aβ40 concentration.
RESULTS

**C-terminal 37 residues of LRP are sufficient to robustly promote Aβ production**

Previous research indicated that amino acids from position 48 to 97 of the soluble tail are sufficient to promote Aβ generation and that deletion of either the distal or proximal NPxY motif does not disturb the processing of APP [66]. This implies the importance of an additional motif to aid in the pro-amyloidogenic activity. To test this hypothesis, we generated mutations of LRP-ST and tested their effect on Aβ generation: 1) ST 1-97, the soluble tail containing the entire cytoplasmic domain, 2) ST 1-97 Δ1Δ2, the soluble tail with both NPxY motifs deleted, 3) ST 45-97, a truncated LRP molecule which contains the second NPxY motif, and 4) ST 61-97, also known as C37, which contains the last 37 amino acids in addition to a dileucine motif. The constructs were transiently co-transfected with APP751 into cells. As seen in Figure 3A and B, deletion of both NPxY motifs resulted in an elevation of Aβ levels, as compared to ST 1-97, while APP-FL, sAPP total and sAPPα levels remained approximately equal. This suggests that the NPxY motifs have an inhibitory effect on the soluble tail, and when removed allow for increased Aβ generation. Interestingly, the deletion of the NPxY motifs results in a decrease in APP-CTF levels.

ST 45-97, which contained the second half of the cytoplasmic domain and one NPxY motif, elevated Aβ levels as compared to ST 1-97. This suggests that within this region is a motif that is important to the amyloidogenic abilities of the cytoplasmic tail. Surprisingly, the ST 61-97 construct showed the greatest increase in Aβ of all the variants, elevating the Aβ level by almost four-fold as compared to the vector control. Along with the change in Aβ, there was also a decrease in sAPPα level, which occurred...
without a change in total sAPP levels. This suggests that the region has a preference for β-secretase processing at the expense of α-secretase. ST 61-97 also showed a decrease in APP-CTF level, as seen in Figure 3C. This data implicates the role of ST 61-97, or C37, as the main pro-amyloidogenic processing component.

**Dileucine motif is critical for the pro-amyloidogenic activity of the LRP C37 domain**

As mentioned earlier, the C37 domain contains a dileucine consensus motif, D/ExxxxLL. This motif is conserved in LRP between species and believed to be involved in the endosomal-lysosomal targeting of membrane proteins [67]. To investigate the effect of the dileucine motif, constructs with the ELL motif deleted from the ST 61-97 sequence were transiently co-transfected with APP 751 into cells and Aβ and APP levels were measured. As seen in Figure 4A, the deletion of the dileucine motif resulted in a drastic drop in Aβ levels, as compared to the wild-type ST 61-97 sequence, indicating the dileucine motif is critical to Aβ generation. However, APP-FL and APP-CTF levels remained unchanged indicating that ELL is not involved in APP trafficking (Fig 4B).

Figures 3 and 4 have been submitted for publication of the material as it may appear in the Journal of Cellular and Molecular Medicine, 2008, Lakshmana, MK; Chen, E; Yoon, IS; Kang, DE. The thesis author was the second author on this paper.

**LRP-ST interacts with APP and BACE1**

LRP has been shown to interact with APP through FRET-based techniques [68], however a physical interaction has not yet been shown by coimmunoprecipitation. To do so, APP751 was co-transfected with LRP constructs containing the first and second
ligand-binding domains (LRP-D1/2), LRP-CT, and EGFP fused LRP-ST (EGFP LRP-ST). EGFP was added to LRP-ST due to its instability on its own. When a pull-down assay was performed, all the various LRP constructs were able to pull down significant amounts of APP (Figure 5A and B). A small portion of APP was seen in cells transfected with APP alone, but this was attributed to binding by endogenous LRP. This data also shows that LRP-ST alone can pull down APP, and that the other domains of LRP are not necessary (Figure 5B). Because of this evidence, the next experiments were conducted with this construct to demonstrate the specific effects of the cytoplasmic tail.

Through FRET-based studies, it has been shown that the LRP light chain interacts with BACE1 on the cell-surface of lipid rafts [69]. Previous experiments in our lab have also shown that LRP-ST overexpressed with APP751 is able to increase Aβ and sAPPβ levels in cell culture in a manner which seems to favor the amyloidogenic β-secretase pathway. This suggests an interaction between LRP, APP, and BACE1 that promotes the amyloidogenic processing of APP. To show this interaction, cells were transiently co-transfected with combinations of EGFP-LRP-ST, Swedish APP751, and BACE1. Swedish APP751 was used because it is a better substrate for BACE1 as compared to wildtype APP. As seen in Figure 6, the presence of EGFP-LRP-ST, APP751_swe, and BACE1 elevated levels of Aβ and APP β-CTFs as compared to BACE1 and APP alone. Note that BACE1 levels and APP-FL levels are approximately equivalent between these samples. This suggests that LRP-ST promotes the processing of APP by physically associating it with BACE1.

**LRP-ST promotes APP trafficking to lipid rafts**
Lipid rafts are cholesterol-enriched microdomains that contain many molecules implicated in AD, including APP, PSEN1, and BACE1. Additionally, the lipid rafts have been implicated as a site of signal transduction, protein trafficking, and proteolytic processing [21]. Based on the previous association experiments we believe that the tripartite complex of APP, LRP-ST, and BACE1 travel to the lipid rafts where APP processing occurs. To illustrate this model, we transiently co-transfected cells with APP71swe, BACE1, and EGFP-LRP-ST, or EGFP for control. The cell extracts were lysed in 1% CHAPS buffer, so as not to solubilize the lipid rafts, and separated by discontinuous sucrose density gradient ultracentrifugation. This allows us to fractionate the lysate and isolate the lipid rafts, which are visible at the interface between the 5% and 35% sucrose layer.

As seen in Figure 7, EGFP-LRP-ST was strongly localized to the lipid raft fractions 2 and 3 as compared to EGFP control, indicating that LRP-ST can associate with the raft regions. The shift was mirrored by APP-FL and APP-CTF (α- and β-CTFs) levels with higher levels present in the raft fractions as compared to EGFP control. This was especially true for APP-CTF levels, which were present in lipid rafts more so than APP. This supports our hypothesis that LRP-ST delivers APP to the lipid raft domains to be proteolytically processed. As expected, BACE1 was present in all fractions in EGFP-LRP-ST and EGFP control. However, the presence of LRP-ST heavily shifted the amount of BACE1 present toward the raft fractions, as compared to control. This indicates that the cytoplasmic domain is facilitating the delivery of BACE1 to the lipid rafts. Note that the flotilin-1 levels remain approximately the same between EGFP-LRP-ST and EGFP-alone, indicating equivalent protein levels in the fractions. These experiments taken
together suggest that the cytoplasmic domain of LRP is able to form a complex with APP and BACE1 and facilitate the trafficking to the lipid raft microdomain for the amyloidogenic processing of APP.

**Endogenous LRP involved in APP processing**

The previous research was conducted by overexpressing the LRP cytoplasmic tail in cells, but this does not allow us to look at the role of LRP itself. To test the role of endogenous LRP in the processing APP, we transiently transfected a siRNA targeting endogenous LRP into CHO cells overexpressing APP751. A single stranded sense oligo was transfected into similar cells as control. The siRNA successfully knocked down LRP expression in a dose-dependent manner, as compared to control and decreased Aβ levels by approximately 82%. APP-FL levels remained unchanged (Figure 8 A and B).

Wildtype and endogenous LRP siRNA cell extracts were then lysed in 1% CHAPS buffer and subjected to discontinuous sucrose density gradient ultracentrifugation to separate the buoyant lipid raft fractions from the other fractions. LRP-β chain levels were present in all the fractions, although they were reduced in the more buoyant fractions of LRP siRNA cells, as expected (Figure 8C). Interestingly, when endogenous LRP levels were knocked down the levels of APP-FL and APP-CTF in the lipid rafts were also decreased (Figure 8C and 8D), as compared to control. Relative to fraction 10, the levels of APP-FL in raft fractions 2 and 3 were reduced by ~3-fold and the levels of APP-CTFs were reduced by ~10 fold when the siRNA was present. Nevertheless, caveolin-1 levels remained equivalent between LRP siRNA and control cells in all fractions. This data suggests that LRP is normally involved in the processing of APP and the generation of Aβ. As the LRP-ST mimicked endogenous LRP in APP and
APP-CTF trafficking to lipid rafts, we propose that the cytoplasmic domain plays an integral role in the pro-amyloidogenic activity of endogenous LRP.

Figures 5-8 are a reprint of the material as it appears in The FASEB Journal, 2007, Yoon, IS; Chen, E; Busse, T; Repetto, E; Lakshmana, MK; Koo, EH; Kang, DE. The thesis author was the second on this paper.

**APP695 vs. APP751**

The previous experiments were completed with APP751, although APP695 is known to be predominantly expressed in neurons [7] and the difference between the two isoforms is the presence of the KPI domain. Stable cell lines were generated with reduced endogenous LRP levels in CHO APP751 and CHO APP695 cells. The lines were stably transfected with a short hairpin RNA targeting endogenous LRP. The cell extracts were then lysed in 1% NP40 buffer and protein levels detected by immunoblotting. In CHO APP751 cells, the addition of the shRNA reduced LRP levels as well as Aβ levels, as expected and as seen earlier (Figure 9A). When the experiment was repeated in CHO APP695 cells, we expected to see the same results. However, when LRP levels were reduced by the shRNA there was an increase in Aβ levels as compared to wildtype (Figure 10A). This data was the complete opposite of what was seen in CHO APP751 cells and unexpected.

To ensure the results seen were due to the reduction in LRP, a LRP mini-receptor was transfected into shRNA cells to rescue levels. The LRP L4 mini-receptor contains the cytoplasmic tail to the fourth ligand binding domain and should retain the similar
functions and binding capabilities as the full-length LRP. The addition of the LRP-L4 mini-receptor to CHO APP751 shRNA cells successfully rescued LRP and Aβ levels, as expected (Figure 9B). The addition of the LRP-L4 mini-receptor to CHO APP695 shRNA cells re-elevated LRP levels but decreased Aβ levels (Figure 10B), once again the opposite of what was seen in CHO APP751 cells. To ensure the effects were not cell-line induced, the experiment was repeated in the murine neuroblastoma cell line N2a APP695. The cells are LRP-deficient, and when the LRP-L4 mini-receptor was stably transfected there was a decrease in Aβ levels as compared to wildtype control (Figure 10C). These results suggest that APP695 is being processed by LRP in a different manner than APP751.

To see if APP695 is also being trafficked differently by LRP, CHO APP695 and CHO APP751 cells stably transfected with LRP shRNA were extracted in 1% CHAPS buffer and subject to sucrose density gradient ultracentrifugation. Equal volumes of each fraction were run on a gel and subject to immunoblotting. As seen in the top panel of Figure 11, CHO APP751 cells behaved as mentioned earlier. The reduced LRP levels resulted in a decrease in the amount of APP751 present in the lipid raft fractions. However, wild type CHO APP695 cells showed very little APP in the raft fractions and the reduction of LRP levels by the shRNA did not significantly alter APP (bottom panel, Figure 11). This data suggests the lack of APP 695 processing by LRP at the lipid rafts.

**Extracellular domains of LRP in Aβ metabolism**

The KPI domain of APP and Aβ are able to interact directly with the ligand-binding domains II and IV of LRP [63, 70]. Thus we propose that the Aβ production and Aβ clearance mechanisms of LRP compete in a KPI-dependent manner. To test this
hypothesis, a construct containing only the extracellular domain of sAPP751 (Ex-751) was stably transfected into CHO APP695 cells. If our hypothesis were correct, the presence of the extracellular domain should be enough to inhibit the Aβ clearance mechanism of LRP and result in an increase in Aβ levels in APP695 cells. This was exactly the result that was seen; the transfection of Ex-751 resulted in an increase in Aβ as compared to Ex-695 control (Figure 12). This data suggest that when KPI levels are low, the clearance of Aβ is dominant over production. However, when sufficient levels of KPI are present, the production of Aβ is dominant as the clearance of Aβ is competitively blocked by the KPI-APP/LRP interaction.
Figure 3: The C37 domain alone is sufficient to promote Aβ generation

Various LRP constructs were transiently co-transfected with APP751 into cells to determine the specific LRP domain involved in APP processing. A) The LRP-ST 61-97 elevated Aβ almost four-fold, more than the LRP-ST 1-97, and showed decreased sAPPα levels with unchanged total sAPP levels. The LRP-ST 1-97Δ1Δ2, lacking both NPxY motifs, and LRP-ST 45-97, lacking the first NPxY motif, elevated Aβ levels above the vector control, but not much higher than ST 1-97. This confirms previous findings. B) The graph shows mean Aβ levels (n=6 in each group) normalized to the vector control. C) APP-FL and APP-CTF levels are shown for the various transient transfections. ST 61-97 and ST 45-97 showed decreased APP-CTF levels, as compared to vector control, although APP-FL levels were maintained.
**Figure 4:** The importance of the LRP C37 dileucine motif

The dileucine motif, ELL, was deleted from LRP-ST 61-97 constructs and co-transfected with APP751 into cells. A) Aβ levels obtained from conditioned media are shown for the various constructs. Deletion of the dileucine motif resulted in a decrease in Aβ level, as compared to ST 61-97. B) APP-FL and APP-CTFs levels are shown, as detected from cell lysates and immunoblotting.
**Figure 5:** The cytoplasmic tail of LRP can interact with APP

LRP constructs containing the cytoplasmic tail were transiently co-transfected with APP751. A) The assay shows that LRP-CT, containing the cytoplasmic tail and the transmembrane domain, and LRP-D1/2, containing the cytoplasmic tail and binding domains I and II, were able to physically interact with APP751. The lysates were extracted with 1% NP40 and detected by immunoblotting. B) LRP-ST was able to interact with APP751_{swe} to successfully pull it down. The LRP-ST construct was fused to EGFP to ensure stability.
**Figure 6:** LRP-ST promotes interaction of APP with BACE1

EGFP-LRP-ST, APP751_swe, and BACE1 were transiently co-transfected into cells. The lysates were extracted with CHAPS buffer and detected by immunoblotting. When EGFP-LRP-ST was present, APP and BACE1 were able to interact much more effectively resulting in higher levels pulled down. The presence of LRP-ST also resulted in an increase in Aβ generation while BACE1 levels were unaffected. This supports the hypothesis that the cytoplasmic domain of LRP mediates the processing of APP by BACE1.
Cells were transiently co-transfected with APP713w, BACE1, and EGFP-LRP-ST or EGFP-only. The cell extracts were collected in 1% CHAPS buffer and subject to discontinuous sucrose density gradient ultracentrifugation. Fractions were collected and equal volumes were loaded onto a gel. Protein levels for APP-FL, APP-CTFs, BACE1, flotillin-1, EGFP, and LRP were detected by immunoblotting.

**Figure 7:** LRP-ST promotes APP trafficking to lipid rafts
Figure 8: Endogenous LRP promotes APP trafficking

Endogenous LRP levels were reduced by transient transfection with siRNA. A) The siRNA reduces Aβ levels in a dose-dependent manner, as compared to a single-stranded sense control oligo. B) Aβ levels were reduced on average ~82% as compared to control cells (n=6 for each group). C) The cells were lysed in 1% CHAPS buffer and subject to discontinuous sucrose density gradient ultracentrifugation. Equal volumes were collected for each fraction and detected by immunoblotting for APP-FL, APP-CTFs, LRP-β chain, and caveolin-1. D and E) Graphs show quantitation of decrease in APP-FL and APP-CTF, respectively, as compared to fraction 10.
**Figure 9:** LRP shRNA knockdown and rescue in CHO APP751 cells

A) CHO APP751 cells were stably transfected with a shRNA targeting endogenous LRP levels. The addition of the shRNA resulted in decreased LRP-β chain and Aβ levels while APP-FL remained unchanged. Cell extracts were lysed in 1% NP40 and protein levels detected by immunoblotting. B) The addition of a LRP-L4 mini-receptor rescued both Aβ and LRP-β chain levels. Protein levels were detected by immunoblotting.
**Figure 10:** LRP knockdown and rescue in CHO APP695 and N2a cells

A) CHO cells overexpressing APP695 were stably transfected with a shRNA specifically targeting endogenous LRP levels. When LRP levels were reduced, Aβ levels were elevated while APP695 levels remained constant. The cells were lysed in 1% NP40 and protein levels were detected by immunoblotting. B) To restore LRP levels, a LRP-L4 mini-receptor was introduced. The addition of the mini-receptor rescued LRP β-chain levels but drastically reduced Aβ levels. Note that APP695 levels once again remained constant. The samples were prepared as mentioned above. C) The LRP-L4 mini-receptor was stably transfected into N2a cells, which contain no endogenous levels of LRP. As compared to control cells, there was a decrease in Aβ levels although APP695 levels remained unchanged. The samples were prepared as mentioned above.
Figure 11: Lipid raft isolation of CHO cells overexpressing APP695 and APP751

Equal volumes of each fraction were loaded into 4-12% gradient gels and detected with CT15 antibody. Fractions 2 and 3 are where the lipid rafts primarily appear. CHO APP751 cells saw a loss of APP levels in fractions 2 and 3 as LRP levels were reduced. Similar results were not duplicated in CHO APP695 cells.
**Figure 12:** Overexpression of KPI-sAPP increases Aβ levels in CHO APP695 cells

The Ex-751 construct, which contains only the extracellular binding domains of sAPP751, was stably transfected into CHO APP695 cells. The overexpression resulted in an increase in Aβ levels as compared to stably transfected Ex-695 control.
CONCLUSION

AD is pathologically characterized by the deposition of extracellular Aβ plaques in the brain. As such, much research has gone into understanding the process by which Aβ accumulates. LRP is one protein that has received much attention, as it and its ligands are genetically associated with AD and can be found within senile plaques. Additionally, cells that are deficient in LRP show a reduction in Aβ levels [64], indicating LRP is somehow involved in the production of Aβ. However, LRP seems to play a dual role as it also functions in the clearance of Aβ by mediating the clearance of Aβ complexed to apoE or α2M [54, 57, 58].

Previous research has shown that the cytoplasmic domain of LRP seems to be responsible for the proteolytic processing of APP and the generation of Aβ [66]. Additionally, the cytoplasmic tail of LRP has been shown to interact with a number of adaptor proteins. In this study we show that the last 37 amino acids of the cytoplasmic domain seem to contain the core of the pro-amyloidogenic capabilities of LRP and that a conserved D/ExxxLL dileucine motif may contribute to the activities. Indeed in other studies, we identified a novel scaffolding protein that specifically interacts with LRP-C37 region that mediates the pro-amyloidogenic activity of LRP-C37 (not shown). This area leaves out the two NPxY motifs present in the LRP cytoplasmic tail, however the data indicate that they function in an inhibitory manner and may not be necessary.

We have shown that the formation of a complex LRP-ST between APP and BACE1 results in increased APP processing causing elevated levels of Aβ, APP-FL, and APP-CTFs. As both APP and BACE1 are able to interact with the cytoplasmic domain of
LRP [68, 69], it seems as if the cytoplasmic domain is acting as a scaffold to hold the two proteins together.

We propose that the increased activity seen when the proteins interact occurs because LRP delivers the complex to lipid raft microdomains. The lipid rafts are a subpopulation of cellular membranes that are highly enriched in cholesterol, sphingomyelin, and glycolipids [19, 20]. Previous research has already shown that these areas contain BACE1 and presenilin activity, in addition to being sites of signal transduction, protein trafficking, and proteolytic processing [21-23]. Indeed, when LRP-ST was overexpressed with BACE1 and APP, we saw a shift in APP-FL, APP-CTF, and BACE1 levels towards the more buoyant raft fractions and away from the heavier fractions, as compared to transfected control. This data suggests that the cytoplasmic domain does facilitate the processing of APP by BACE1 by targeting them towards the lipid raft domains. As the cells for the coimmunoprecipitation experiments were lysed in 1% CHAPS, which does not solubilize the lipid rafts, this hypothesis explains the increased levels observed.

When endogenous LRP levels were selectively reduced with a transient RNAi strategy, there was a corresponding decrease of APP levels in the lipid raft fractions. As similar results were obtained with endogenous LRP and the LRP-ST construct, it is reasonable to assume that the cytoplasmic domain of endogenous LRP plays a critical role. This would suggest that the normal function of LRP is to promote the processing of APP and Aβ generation by facilitating it`s trafficking to the lipid raft domains.

Recent experiments have shown that the extracellular ligand-binding domains II and IV of LRP are able to bind directly to APP via the extracellular KPI domain [63, 70].
Additionally, LRP mini-receptors containing only the fourth-ligand binding domain and no cytoplasmic tail were able to successfully interact with the KPI-containing isoform, APP770 [70]. When we pursued the importance of the fourth-binding domain, we obtained interesting results. Although previous evidence states that APP processing is isoform-independent [65], our results show that the KPI domain may indeed play a role. The addition of a LRP mini-receptor, spanning from the fourth ligand-binding domain to the end of the cytoplasmic tail, into LRP-deficient cells rescued Aβ levels in the presence of APP751, but decreased Aβ levels in the presence of APP695. To check the unexpected results of the latter, the experiment was repeated in different cells, the naturally LRP-deficient murine neuroblastoma N2a cell line, and similar results were obtained. The processing of APP695 by LRP is also different than that of APP751, as demonstrated by lipid raft fractionation experiments. Additionally there was very little APP695 present in the rafts, even with wildtype cells. This suggests that APP695 is being processed differently by LRP.

It is therefore possible that LRP alters the processing of APP via both the extracellular and cytoplasmic domains. We propose that Aβ production and clearance mechanisms of LRP are competing in a KPI-dependent manner (Figure 13). When KPI levels are sufficient, as when APP751 is present, there are two interactions that occur. The extracellular domain of LRP interacts directly with the KPI domain of APP and the cytoplasmic tail of LRP interacts indirectly with the cytoplasmic domain of APP. The former means Aβ is unable to bind directly to LRP and thus Aβ clearance can not occur. However the latter results in Aβ generation, due to lipid raft trafficking, and thus results in an overall production of Aβ. In contrast, when the KPI domain is not present, as in
APP695, Aβ is able to bind directly to the extracellular domains of LRP and be cleared. Although there is still Aβ being generated due to the interaction between the LRP cytoplasmic domain and APP, the amount of Aβ present is greater than the amount of sAPP to compete. Thus there is more Aβ clearance than Aβ production in APP695. This model is supported by experiments where the extracellular binding domain of sAPP751 is overexpressed in CHO APP695 cells.

The understanding of how APP is processed to generate Aβ can be used to develop potential therapeutic methods to help reduce Aβ load in AD patients. Small peptide sequences derived from the LRP cytoplasmic domains may be used to block LRP-dependent deliver of AP to lipid rafts. Further studies of the KPI domain and the sequences they interact with may yield peptides for use as therapeutic drugs.
**Figure 13:** KPI domain critical in determining Aβ production or clearance

This schematic shows our hypothesized model for the role of the KPI domain and LRP expression on the production or clearance of Aβ. When APP695 and LRP are both present, Aβ metabolism favors clearance over production. However, when APP741 and LRP are both present, Aβ metabolism favors the production over clearance.
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