Experimental Neurology 225 (2010) 85–93

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

The role of low-density receptor-related protein 1 (LRP1) as a competitive substrate of the amyloid precursor protein (APP) for BACE1

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ARTICLE INFO

Article history:
Received 12 January 2010
Revised 17 May 2010
Accepted 20 May 2010
Available online 26 May 2010

Keywords:
Amyloid precursor protein (APP)
Beta-Site APP cleaving enzyme 1 (BACE1)
Low-density lipoprotein receptor-related protein 1 (LRP1)
Alzheimer's disease (AD)

ABSTRACT

Cleavage of APP by BACE1 is the first proteolytic step in the production of amyloid-beta (Aβ), which accumulates in senile plaques in Alzheimer's disease. Through its interaction with APP, the low-density receptor-related protein 1 (LRP1) enhances APP internalization. Recently, BACE1 has been shown to interact with and cleave the light chain (lc) of LRP1. Since LRP1 is known to compete with APP for cleavage by gamma-secretase, we tested the hypothesis that LRP1 also acts as a competitive substrate for β-secretase. We found that the increase in secreted APP (sAPP) mediated by over-expression of BACE1 in APP-transfected cells could be decreased by simultaneous LRP1 over-expression. Analysis by multi-spot ELISA revealed that this is due to a decrease in sAPPβ, but not sAPPα. Interaction between APP and BACE1, as measured by immunoprecipitation and fluorescence lifetime assays, was impaired by LRP1 over-expression. We also demonstrate that APP over-expression leads to decreased LRP1 association with and cleavage by BACE1. In conclusion, our data suggest that – in addition to its role in APP trafficking – LRP1 affects APP processing by competing for cleavage by BACE1.

Introduction

Amyloid-β (Aβ) is the major component of senile plaques, one of the histopathological hallmarks in Alzheimer's disease. Cleavage of the amyloid-β precursor protein (APP) by the type I transmembrane aspartyl protease β-site APP cleaving enzyme (BACE1) is the first proteolytic step in the production of Aβ (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999). Besides APP, other BACE1 substrates that have been identified include the APP homologues APLP1 and APLP2 (Li and Sudhof, 2004), a membrane-bound sialyltransferase (Kitazume et al., 2001), P-selectin glycoprotein ligand-1 (Lichtenthaler et al., 2003), neuregulin1 type III isoform (Willem et al., 2006), the β-subunits of voltage gated sodium channels (Wong et al., 2005) and the low-density lipoprotein receptor-related protein 1 (LRP1) (von Arnim et al., 2005).

LRP1 is a roughly 600 kDa type I integral membrane protein and three of its key ligands (apoE, α2-macroglobulin, and APP) are genetically associated with Alzheimer disease and are found in senile plaques. Within cells LRP1 is found on the cell surface and cycles between the cell membrane and endosomes. LRP1 mediates the binding and clearance of Aβ complexes bound to apoE or α2-macroglobulin in cultured cells and in the brain. LRP1 may also play a crucial role in brain efflux of Aβ isoforms at the blood–brain barrier. However, in addition to its role in Aβ clearance, LRP1 interacts directly with APP in two ways. First, KPI-containing isoforms of APP are among the ligands for the extracellular binding sites on LRP1's 515 kDa N-terminal domain (Herz and Strickland, 2001; Kounnas et al., 1995; Li et al., 2001). Second, the short cytoplasmic tail of LRP1's 85 kDa C-terminal domain contains two intracellular NPXY sites which, through the adaptor protein FE65, bind APP. This binding event leads to increased endocytosis of APP from the cell surface, (Kinoshita et al., 2001; Kounnas et al., 1995; Pietrzik et al., 2002; Rebeck et al., 2001; Trommsdorff et al., 1998; Ulery et al., 2000), which is important since BACE1 cleavage of APP occurs more or less exclusively within intracellular compartments (Kinoshita et al., 2003a,b; Koo and Squazzo, 1994).

LRP1 undergoes proteolysis in an interesting pattern that parallels APP in some ways. It undergoes γ-cleavage resulting in the release of the LRP1 intracellular domain (May et al., 2002). In doing so, LRP1 has been shown to compete with APP for this presenilin 1-dependent γ-secretase activity (Lleo et al., 2005). Ectodomain shedding of LRP1 (Quinn et al., 1999) and cleavage of LRP1 by matrix metalloproteases have also been reported (Rozanov et al., 2004; Higashi and Miyazaki, 2003). Furthermore – and as with APP – β-secretase cleavage of LRP1 leads to release of a secreted LRP1 (sLRP) domain (von Arnim et al., 2005). sLRP normally circulates in plasma (Quinn et al., 1997) and can bind Aβ (Sagare et al., 2007) leading to its clearance from the brain.

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doi:10.1016/j.expneurol.2010.05.017

Given that both APP and LRP1 are β-secretase substrates, we asked whether they compete to be cleaved by this protease. Since BACE1 has been postulated to be the "rate-limiting" step for APP cleavage, raising the possibility of pharmacologic down-regulation of β-secretase as a treatment for AD, the question of how BACE1 activity is modulated endogenously is of particular interest. We have shown previously that BACE1 and LRP1 interact and that LRP1 is a BACE1 substrate. In this study, we analyzed the impact of LRP1 co-expression on APP cleavage by BACE1 and, vice versa, the impact of APP co-expression on LRP1 cleavage by BACE1 and found that LRP1 and APP can compete for BACE1 cleavage.

Materials and methods

Antibodies

As displayed by corresponding numbers in Fig. 1, in Western blots LRP1 constructs were detected using (1) anti-c-Myc (9E10) (Mouse/M4439/Sigma). APP was detected by (2) anti-HA (Rabbit/H6908/Sigma), (3) anti-APP-N-terminal (22C11) (Mouse/MAB348/Millipore), (4) anti-APP-C-terminal (Rabbit/A8717/Sigma) and (5) anti-β-amyloid (6E10) (Mouse/SIG39320/Covance). BACE1 was detected by (7) Anti-GFP-N-terminal (Rabbit/G1544/Sigma). In immunocytochemistry, LRP1 constructs were detected by (8) anti-c-myc (Rabbit/C3956/Sigma) and APP by (6) anti-V5 (Mouse/R96025/Invitrogen). Endogenous FE65 was detected by anti-FE65 (Pietrzik et al., 2004).

Expression constructs

We utilized previously-described BACE1-GFP, APP695-V5, APP695-RFP, secreted alkaline phosphatase (SEAP)-APP and GFP-mRFP expression constructs (Kinoshita et al., 2003a,b; Lichtenthaler et al., 2003; von Arnim et al., 2004, 2005, 2008). We also utilized the truncated C-terminal forms of LRP1, LC (LRP1 light chain)-Myc and SEAP-LC_ β-Myc used in previous studies (von Arnim et al., 2005), which contains the transmembrane domain essential for interaction with and cleavage by BACE1 but lacks the extracellular domains needed to influence Aβ uptake and degradation. pEGFP-N3 (Clontech), pcDNA3.1-myc/pcDNA3.1-V5 (Invitrogen) and phCMV3 (Genlantis) were used as control vectors.

Cell culture and transient transfection

Neuro-2A mouse neuroblastoma cells (DSMZ no.: ACC 148) or HEK293 human embryonal kidney (DSMZ no.: ACC 305) were seeded on 35 mm dishes (Nunc) and maintained in DMEM (Gibco) with 10% newborn calf serum (NCS) (PAA) and 1× penicillin/streptomycin (P/S) (PAA). For microscopy, U373 MG human glioblastoma–astrocytoma cells (ECACC no.: 89081403) were seeded on 35 mm glass bottom dishes (MatTek) and incubated in RPMI 1640 Medium (10% NCS + 1× P/S). Transient transfection was performed 24 h later using Satisfection (Stratagene) (N2A/U373) according to the manufacturer's instructions or Calcium Phosphate transfection method (HEK). Cells were incubated 24 h to 36 h before experiments.

Lysis

Cells were lysed either in 1× Reporter lysis buffer (Promega) or brain extraction buffer (Bai et al., 2008) both supplemented with HALT protease/phosphatase inhibitor cocktail (Thermo) for 15 min on ice. Protein concentrations were determined by BCA measurements.

LDS-PAGE, Western blotting and detection

Cell lysates were electrophoresed under denaturing conditions using NuPAGE Novex Bis-Tris 4–12% gradient gels (Invitrogen) and MOPS and MES running buffer (Invitrogen). Proteins were transferred semi-dry on PVDF-membranes and blocked in 1× RotiBlock (Carl Roth) and 0.25% milk powder (Carl Roth) for 1 h. Incubation with primary antibody was performed at 4 °C overnight in blocking solution followed by HRP-coupled secondary antibodies (Molecular Probes) for 1 h. Detection was performed using Femtoglow ECL (PJK) and Intelligent DarkBox LAS-1000 (FujiFilm).

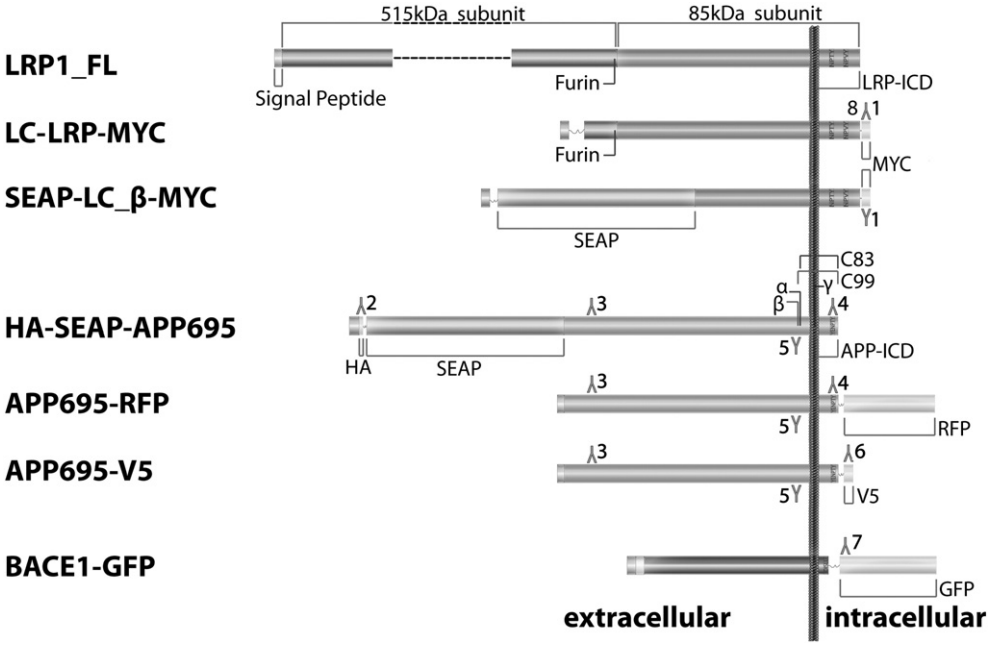


Fig. 1. Constructs and antibodies used in this study. Since full-length LRP1 is poorly expressed by transfected cells, we used truncated forms. The LC (light chain)-LRP-myc contains the last 81AA of the 515 kDa subunit and the complete 85 kDa subunit of LRP1 fused to a C-terminal myc tag. To avoid furin cleavage, SEAP-LC_ β-myc consists of SEAP (secreted alkaline phosphatase) fused to the last 408AA of the 85 kDa LRP1 subunit with a C-terminal myc tag. HA-SEAP-APP695 is a fusion construct of an N-terminal HA tag, SEAP and the last 678AA of APP695. APP695-V5 and BACE1-GFP are full-length proteins fused to the respective C-terminal tag. All constructs have an N-terminal export signal peptide.

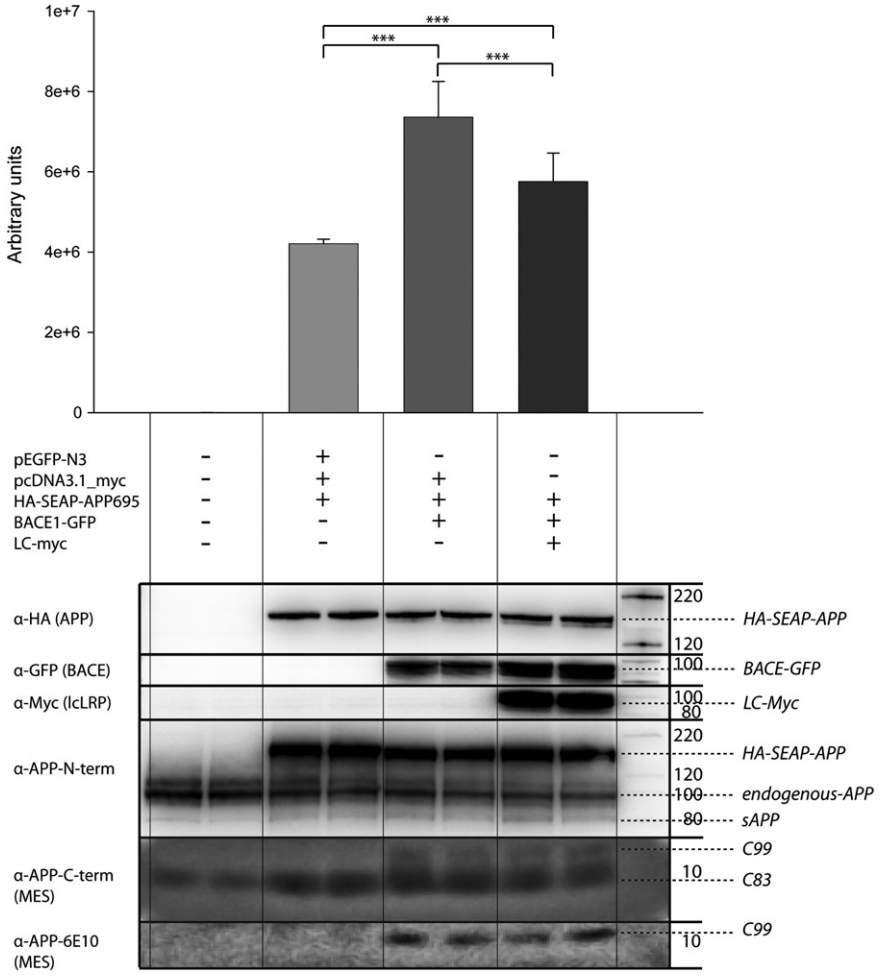


Fig. 2. APP cleavage assay. Untransfected N2A cells (lane 1), transfected with SEAP-APP and empty vectors (lane 2), SEAP-APP and BACE1-GFP and empty vector (lane 3) and SEAP-APP, BACE1-GFP and LC-LRP-myc (lane 4). Shown is the alkaline phosphatase activity in the conditioned medium 48 h after transfection. Given are the means and S.D. of a representative sample of 17 independent assays. Even protein expression was controlled by BCA and either Western blot or β-galactosidase activity, if β-gal vector was co-transfected. Both transfection and measurement were carried out in duplicate or triplicate.

Secreted alkaline phosphatase assay

For SEAP measurements, N2A or HEK293 cells were seeded and transiently transfected as described above. Measurement of SEAP activity in the conditioned media was carried out in triplicate by SEAP Reporter Gene Assay (Roche) according to the manufacturer's instructions 48 h after transfection. SEAP activity was normalized either by β-Galactosidase Enzyme Assay (Promega) or by assessment of equivalent expression levels with Western blots.

Aβ measurements

Aβ38, Aβ40 and Aβ42 levels were determined in the same cell culture supernatants utilized for the SEAP measurements, using the Multi-Spot Human (6E10) Aβ Triplex Assay and the SECTOR Imager 2400 (Meso Scale Discovery) according to the manufacturer's instructions.

sAPPα and sAPPβ measurements

sAPPα and sAPPβ levels were determined in the same cell culture supernatants utilized for the SEAP measurements, using the Multiplex sAPP Assay (Meso Scale Discovery) as described previously (von Arnim et al., 2008).

Immunocytochemistry

24 h after transient transfection with APP695-V5, APP695-V5 + BACE1-GFP or APP695-V5 + BACE1-GFP + LC-LRP-myc, N2A cells were fixed in 4% PFA/3% sucrose for 10 min, permeabilized by 0.2% TritonX-100 in PBS for 30 min and blocked with 2% normal goat serum in PBS/1× Roti-Immunoblock (Carl Roth) for 1 h. Cells were immunostained overnight with polyclonal rabbit anti-C-Myc (Sigma, 1:200) and monoclonal mouse anti-V5 (Invitrogen, 1:200) and for 1 h with secondary goat antibodies anti-mouse-Alexa555 and anti-rabbit-Alexa647 (both Invitrogen, 1:750). Cells were analyzed by confocal laser scanning microscopy with a Zeiss LSM 510 Meta.

Co-immunoprecipitation

N2A cells were lysed 24 h after transient transfection in pre-cooled lysis buffer (Miltenyi Biotec, Bergisch Gladbach, Germany), supplemented with HALT Protease & Phosphatase Inhibitor (Thermo Scientific, Rockford, IL). Cleared lysate supernatants were incubated on ice with anti-HA coated MicroBeads (Miltenyi Biotec) for 2 h and purified on μColumns (Miltenyi Biotec) following the manufacturer's instructions. Alternatively, lysates were incubated subsequently with 2 μg monoclonal mouse anti-myc (9E10, Sigma) antibody and protein G micro beads (Miltenyi Biotech) prior to purification. In each case, proteins were eluted with 60 μl pre-heated (70 °C) NuPAGE lithium

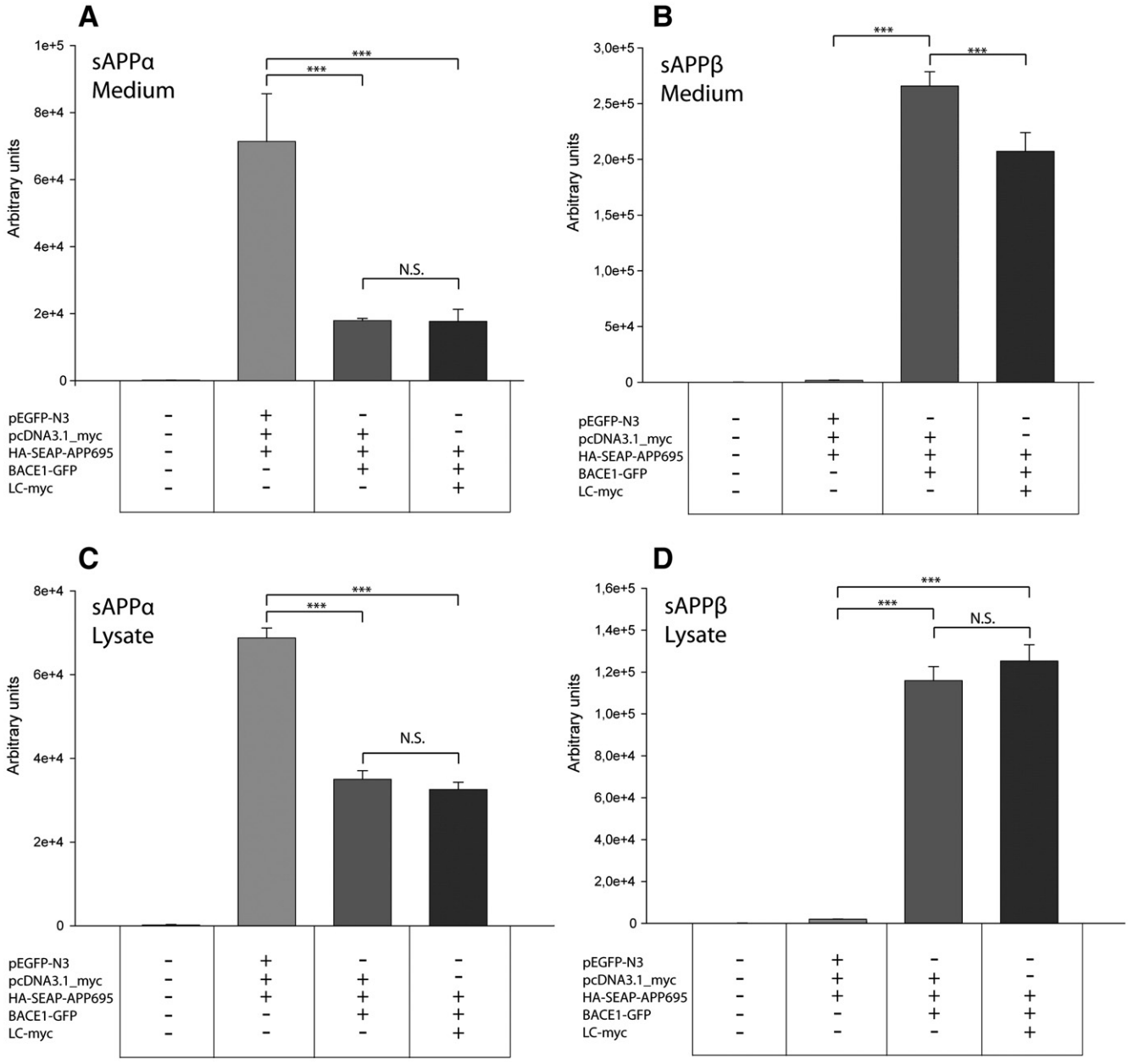


Fig. 3. sAPP-MesoScale. A + C: sAPPα levels in medium (A) and lysate (C) of untransfected N2A (lane 1), N2A co-transfected with SEAP-APP695 + empty vectors (lane 2), N2A co-transfected with SEAP-APP + BACE1-GFP + empty vector (lane 3), N2A co-transfected with SEAP-APP + BACE1-GFP + LC-LRP-myc. B + D: sAPPβ levels in medium (B) and lysate (D) of untransfected N2A (lane 1), N2A co-transfected with SEAP-APP695 + empty vectors (lane 2), N2A co-transfected with SEAP-APP + BACE1-GFP + empty vector (lane 3), N2A co-transfected with SEAP-APP + BACE1-GFP LC-LRP-myc. Given are the means and S.D. of one of 3 independent assays. Even protein expression was controlled by BCA and Western blot. Both transfection and measurement were carried out in triplicate.

dodecyl sulfate (LDS) sample buffer (Invitrogen) containing 100 mM dithiothreitol (DTT) and analyzed by LDS-PAGE and Western blotting.

Microscopic imaging and fluorescence lifetime analysis

Fluorescence decay kinetics of U373 cells were recorded 48 h after transfection and evaluated as described previously (von Arnim et al., 2008).

Statistical analysis

Statistical analyses were performed by ANOVA and Student–Newman–Keuls test. Results were considered significant if $p<0.05$ (*), $p<0.01$ (**) or $p<0.001$ (***).

Results

LRP1 co-expression decreases sAPPβ secretion

To analyze the effect of LRP1 over-expression on processing of APP by BACE1, we used the secreted alkaline phosphatase assay (SEAP). Production of sAPP was measured after cells were transfected either with β-galactosidase (β-gal) + HA-SEAP-APP695 + pEGFP-N3 + pcDNA3.1-myc (Fig. 2, lane 2), β-gal + HA-SEAP-APP695 + BACE1-GFP + pcDNA3.1-myc (Fig. 2, lane 3) or β-gal + HA-SEAP-APP695 + BACE1-GFP + LC-LRP-myc (Fig. 2, lane 4) and measured against empty N2A cells as control (Fig. 2, lane 1). Over-expression of HA-SEAP-APP695 led to the expected increase in sAPP secretion as measured by SEAP activity in the conditioned media (Fig. 2, lane 2). Co-expression of BACE1 further increased production of secreted APP

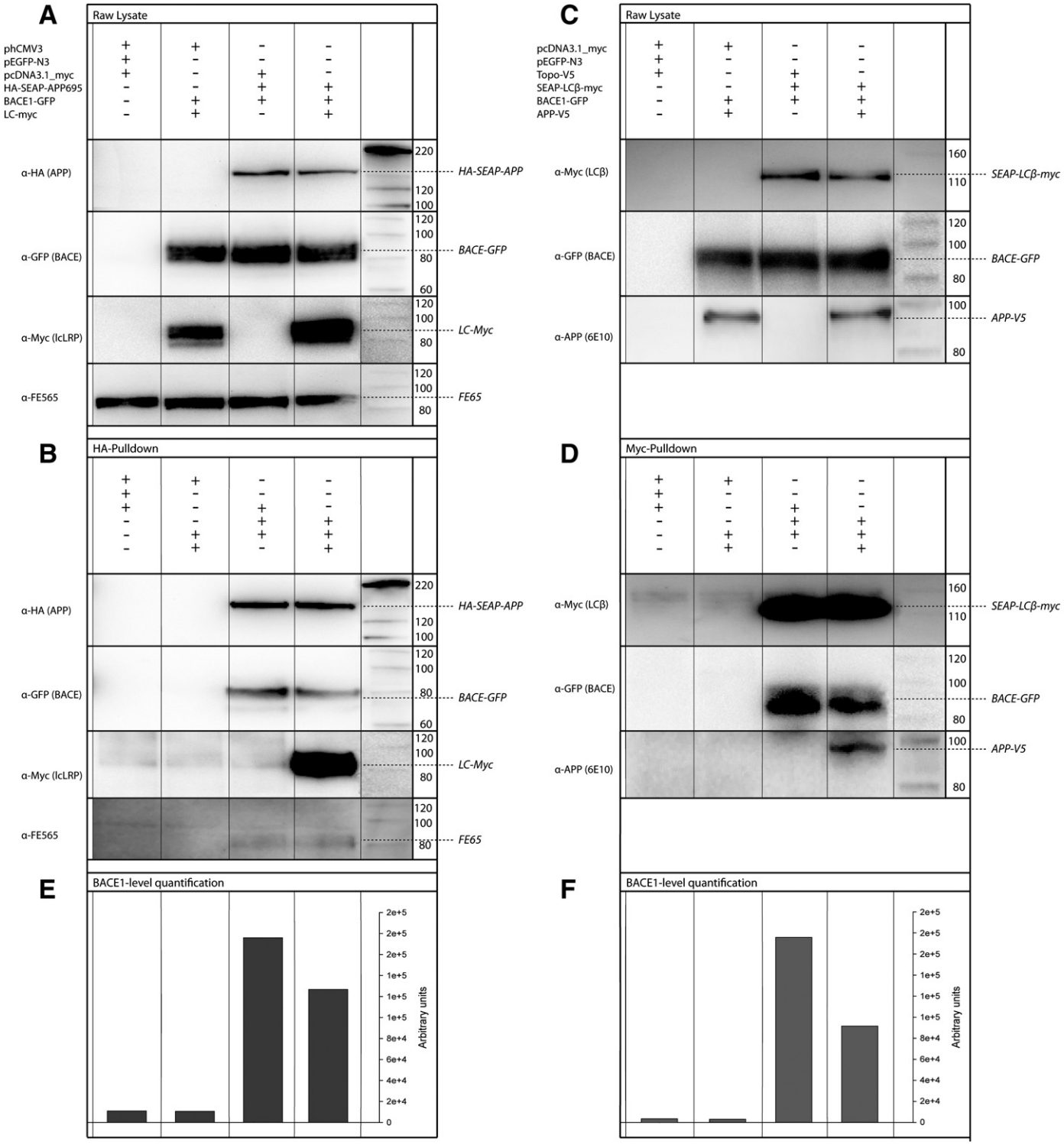


Fig. 4. Competition analysis by co-immunoprecipitation. N2A cells + empty control vectors (lane 1 A–D). LC-Myc + BACE-GFP + empty HA vector (lane 2 A + B). HA-SEAP-APP + BACE-GFP + empty-Myc vector (lane 3 A + B). HA-SEAP-APP + BACE-GFP + LC-Myc (lane 4 A + B). APP-V5 + BACE-GFP + empty-Myc vector (lane 2 C + D). SEAP-LCβ-Myc + BACE-GFP + empty V5 vector (lane 3 C + D). SEAP-LCβ-Myc + BACE-GFP + APP-V5 (lane 4 C + D). A + C: Raw lysates; B + D: immunoprecipitates; E + F: pixel based quantification of BACE1.

fragments (Fig. 2, lane 3) as seen previously (von Arnim et al., 2004). However, when the light chain of LRP1 was also over-expressed, we observed decreased sAPP production (Fig. 2, lane 4). However, as the SEAP-APP assay does not distinguish between α- and β-site cleavage, we analyzed the secreted fragments by an electrochemiluminescence based ELISA assay that quantitates α- and β-site cleaved secreted APP fragments (sAPPα and sAPPβ) within the same well. We used mouse neuroblastoma cells (N2A) as these cells do not secrete endogenous human sAPP (Figs. 3A and B, lane 1). When APP was over-expressed alone (Figs. 3A and B, lane 2), we detected mainly sAPPα indicating high levels of endogenous α-secretase but not β-secretase activity. Upon co-expression of BACE1 with APP, α-cleaved fragments decreased significantly, indicating a shift from α- to β-secretase pathway (Figs. 3A and B, lane 3). Additional co-expression of LRP1 did

not alter the amount of α-cleaved APP fragments (Fig. 3A, lane 4). However the amount of β-cleaved APP fragments decreased significantly, indicating that LRP1 is a competitive substrate for APP (Fig. 3B, lane 4).

Additionally we used the same assay to assess intracellular levels of sAPP. The amount of neither sAPPα (Fig. 3C, lanes 3 + 4) nor sAPPβ (Fig. 3D, lanes 3 + 4) within the cell increased significantly upon LC-LRP over-expression, proving that the decrease in sAPPβ secretion is not due to an intracellular accumulation of the fragments.

Interestingly we could not detect significant alterations in the amount of APP C-terminal fragments in Western blots after co-expression of LC-LRP-myc (Fig. 2).

Localization of BACE1, APP and LRP1 constructs

To evaluate whether LRP1 over-expression impairs BACE1 and APP695 co-localization through compartmental redistribution of APP695, we used confocal laser scanning microscopy to look for alterations in APP or BACE1 distribution upon LC-LRP co-expression in N2A cells transfected with BACE1-GFP and APP-V5. Over-expressed BACE1 showed a punctate distribution throughout the cell with the highest concentration in perinuclear compartments (presumably Golgi) where it co-localizes with APP (Supp.-Fig. 1a). Co-expression of LC-LRP did not cause redistribution of BACE1-GFP and APP-V5. Instead, we observed co-localization of all three proteins to the Golgi (Supp.-Fig. 1b).

Competition analysis by co-immunoprecipitation

We next investigated the hypothesis that LRP1 over-expression interferes with APP binding to BACE1 using co-immunoprecipitation. N2A cells expressing empty control vectors were used as negative controls (Fig. 4, lane 1 A–D). When expressed with an empty HA vector, neither LC-myc nor BACE-GFP were immunoprecipitated by anti-HA antibody (Fig. 4, lane 2 A + B). Similarly, BACE-GFP and APP-V5 were not immunoprecipitated by beads coated with anti-myc antibody (Fig. 4, lane 2 C + D). When HA-SEAP-APP was pulled down with anti-HA beads (Fig. 4, lane 3 A + B), BACE-GFP co-immunoprecipitated (Fig. 4, lane 3 B). Additional co-expression of LC-myc resulted in decreased BACE-GFP co-precipitation (Fig. 4, lane 4 B). In the converse experiment, pull down of SEAP-LCβ-myc with anti-myc beads (Fig. 4, lane 3 C + D) led to co-immunoprecipitation of BACE-GFP (Fig. 4, lane 3 D). Simultaneous over-expression of APP-V5 impaired BACE-GFP co-precipitation (Fig. 4, lane 4 D). APP and LRP1 are known to interact through binding of adapter proteins to their N-terminal NPXY-motifs, and we found LC-Myc in the HA-SEAP-APP pulldown and APP-V5 in SEAP-LCβ-Myc pulldown, respectively (Fig. 4, lane B + D). We also detected the adapter protein FE65 co-precipitated by HA-SEAP-APP (Fig. 4, lanes 3 + 4 B).

LRP1 co-expression disturbs BACE1 and APP proximity in fluorescence lifetime experiments

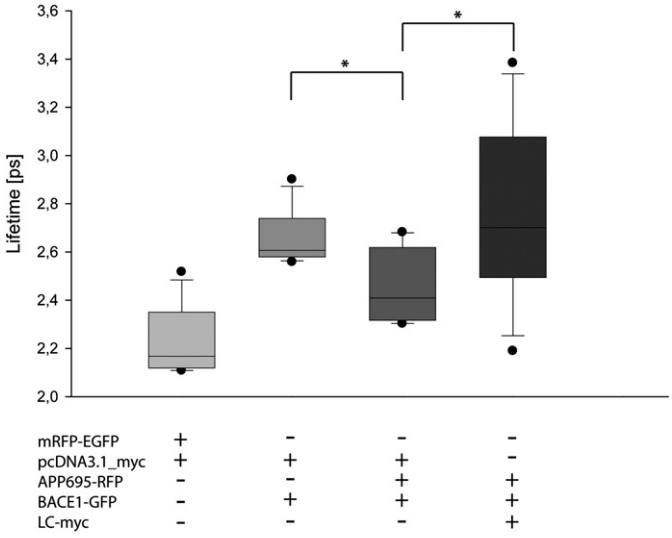
To confirm the data of the co-immunoprecipitation experiments we measured fluorescence lifetime kinetics in U373 cells transiently transfected with GFP-mRFP fusion protein (positive control, Fig. 5, lane 1), BACE1-GFP (Fig. 5, lane 2), BACE1-GFP + APP695-RFP (Fig. 5, lane 3) and BACE1-GFP + APP695-RFP + LC-LRP-myc (Fig. 5, lane 4). We observed a significant decrease in donor fluorescence lifetime (BACE1-GFP) upon co-expression of the fluorescence acceptor (APP695-RFP) (Fig. 5, lane 3) compared with the lifetime of the donor alone (Fig. 5, lane 2), indicating interaction between APP and BACE1. This effect was neutralized by additional co-expression of LC-LRP-myc (Fig. 5, lane 4).

Co-expression of APP decreases LRP1 cleavage by BACE1

Having found that LRP1 over-expression impaired APP processing by BACE1, we employed the SEAP assay to assess whether APP over-expression similarly reduced processing of LRP1 by BACE1. We measured the release of the extracellular domain of LRP1-β-chain by means of SEAP fused to its N-terminus (SEAP-LC_β-myc) as described in von Arnim et al. (2005). Cells were transfected either with β-gal + SEAP-LC_β-myc + pEGFP-N3 + pcDNA3.1-V5 (Fig. 6, lane 5), β-gal + SEAP-LC_β-myc + BACE1-GFP + pcDNA3.1-V5 (Fig. 6, lane 3) or β-gal + SEAP-LC_β-myc + BACE1-GFP + APP695-V5 (Fig. 6, lane 4) and measured against empty N2A cells as control (Fig. 6, lane 1). SEAP-LC_β-myc over-expression led to an increase in SEAP activity in the conditioned media (Fig. 6, lane 2). Upon co-expression of BACE1 with SEAP-LC_β-myc, we observed an increased SEAP activity in the conditioned media (Fig. 6, lane 3) relative to cells over-expressing SEAP-LC_β-myc alone. However, when APP was also over-expressed, we observed decreased cleavage and release of the extracellular domain of LRP1 as measured by SEAP activity (Fig. 6, lane 4). SEAP activity was either normalized to β-gal activity or equal protein expression was assured by Western blot. These experiments were also performed in U373 and HEK293 cells with comparable results (data not shown). In contrast to the SEAP-APP experiments, we also detected alterations in the levels of LC-myc C-terminal fragments. Upon co-expression of BACE1-GFP we observed an increase of LC-myc CTF's in the lysate. This effect is reversed by additional expression of APP-V5 (Fig. 6, lanes 2–4).

LRP1 co-expression does not alter Aβ production

Data concerning the influence of LRP1 on Aβ production is conflicting. We therefore measured Aβ levels in the conditioned



Donor/*Acceptor*	N	Median Lifetime (ps)	25%	75%	Probability compared to 2
1_GFP-*mRFP*	12	2,167	2,124	2,314	p<0,05
2_BACE-GFP	12	2,607	2,584	2,705	
3_BACE-GFP + *APP-RFP*	12	2,409	2,329	2,606	p<0,05
4_BACE-GFP + *APP-RFP* + LC-myc	12	2,701	2,555	2,996	NS

Fig. 5. Competition analysis by fluorescence lifetime measurement. As a FRET positive control GFP-mRFP fusion protein was expressed in U373 cells (lane 1). The negative control was the donor BACE1-GFP alone (lane 2). BACE1-GFP and APP-mRFP co-expression decreased donor lifetime significantly (lane 3) whereas triple-expression with LC-LRP-myc led to no significant alteration in donor lifetime compared with the negative control (lane 4).

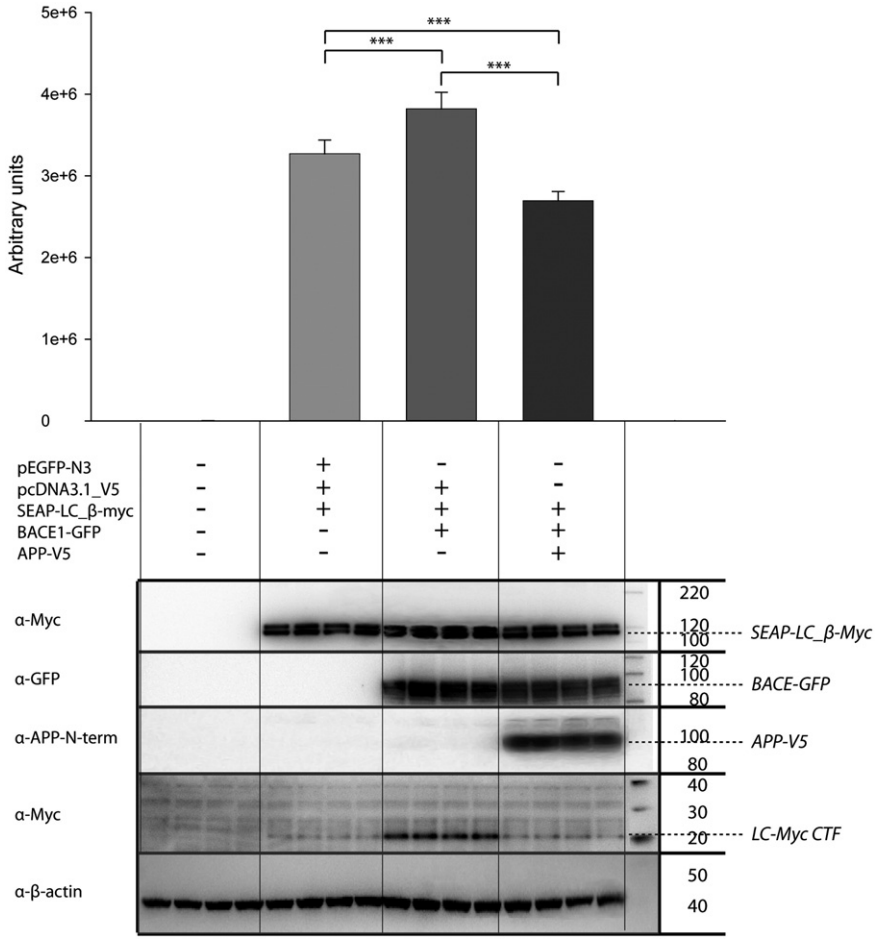


Fig. 6. LRP1 cleavage assay. Untransfected N2A cells (lane 1), transfected with SEAP-LC + empty vectors (lane 2), SEAP-LC + BACE1-GFP + empty vector (lane 3) and SEAP-LC + BACE1-GFP + APP-V5 (lane 4). Shown is the alkaline phosphatase activity in the conditioned medium 48 h after transfection. Given are the means and S.D. of a representative sample of 6 independent assays. Even protein expression was controlled by BCA and either Western blot or β-galactosidase activity, if β-gal vector was co-transfected. Each combination was transfected 12 times and measurement was carried out in duplicate.

medium from the previous experiments using a MesoScale triplex assay that quantitates human Aβ38, Aβ40 and Aβ42 within the same well. For these measurements, mouse N2A cells were again utilized since these mouse cells do not secrete endogenous human Aβ (Figs. 7A, B and C, lane 1). Upon over-expression of APP alone, we detected a moderate increase in secretion of all three Aβs (Figs. 7A, B and C, lane 2). Co-expression of BACE1 with APP significantly increased production of all three types of Aβ (Figs. 7A, B and C, lane 3). Additional co-expression of LRP1 did not alter the secretion of any Aβ isoform (Figs. 7A, B, and C, lane 4).

Discussion

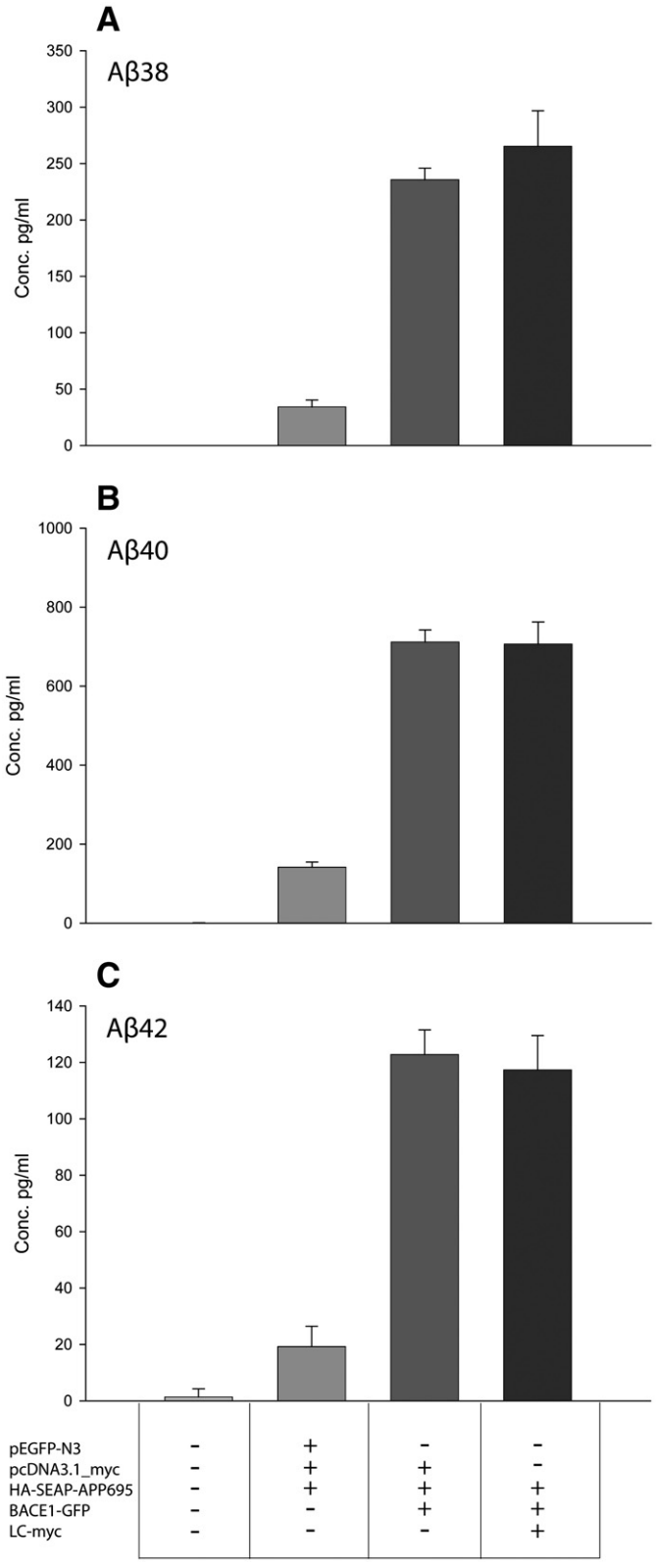
Since the identification of BACE1 as the APP β-secretase, multiple additional BACE1 substrates have been identified. However, it is not clear whether cleavage of APP and of non-APP BACE1 substrates is performed by the same β-secretase molecules in the same compartments and may therefore be subject to competitive enzyme kinetics. We here investigate these questions with regard to the BACE1 substrate LRP1.

When APP, BACE, and LRP1 light chain (LC) were coexpressed and sAPP was measured in conditioned media by SEAP assay, sAPP production was found to be reduced relative to over-expression of APP and BACE alone. As this assay could not distinguish whether LRP1 was influencing α- or β-cleavage of APP, we confirmed via ELISA assay that LRP1 over-expression decreased sAPPβ but not sAPPα. There was no intracellular sAPP accumulation to account for the reduced extracellular levels. Unexpectedly, LRP1 co-expression did not change production of APP CTFs. This finding may result from limitations in detection assays as the SEAP assay is extremely sensitive compared to Western blot. In addition, LRP1 increases APP-CTF stability through interaction of APP and LRP1 via Fe65 (Pietrzik et al., 2002).

To further investigate whether LRP1 competitively inhibits BACE1 cleavage of APP, we first confirmed with confocal microscopy that APP and BACE1 do not redistribute within the cell upon LRP1 co-expression but in fact co-localize within the same subcellular compartments (Supplementary data). However, co-immunoprecipitation experiments demonstrated decreased interaction between APP and BACE1 upon LC over-expression and, conversely, between LC and BACE1 upon APP over-expression (Fig. 4). To confirm that LC impaired the interaction of APP with BACE1, we performed fluorescence lifetime experiments involving a donor fluorophore attached to BACE1 and an acceptor fluorophore fused to APP. Decreased quenching of BACE1-GFP by APP-RFP in the presence of LC revealed decreased proximity between APP and BACE1 upon LC co-expression. Finally, we performed the reciprocal experiment to our SEAP assay for sAPP secretion and measured sLRP production in cells co-expressing SEAP-LC-LRP and BACE1 with or without APP. We found decreased sLRP in the conditioned media as well as reduced β-cleaved LC-CTF in lysate when APP was co-expressed (Fig. 6).

Taken together, we interpret this data to be consistent with the idea that LRP1 competes with APP for β-cleavage by BACE1 in addition to γ-cleavage by presinilin 1 (Lleo et al., 2005). This implies that both proteins – APP and LRP1 – are cleaved by the same population of β-

secretase. Interestingly, LC-myc expression driven by a strong CMV promoter has only a moderate effect on sAPP production, though HA-SEAP-APP is under the control of a weaker EF1-alpha promoter. Meanwhile, APP-V5 co-expression more or less completely abolishes LRP1-BACE1 processing even though expression of both proteins is controlled by the same CMV promoter. Overall, these observations suggest that APP is a more potent substrate for BACE1 than LRP1.



Reports addressing the influence of LRP1 on APP processing and Aβ production are conflicting. On one hand LRP1 over-expression leads to decreased Aβ40 and Aβ42 production in CHO cells over-expressing APP751 (Lleo et al., 2005). On the other hand LRP1 knock-out leads to decreased Aβ secretion (Ulery et al., 2000; Pietrzik et al., 2002; Yoon et al., 2007). There are also reports that Aβ secretion is not altered upon LRP1 expression in transgenic mice (Zerbinatti et al., 2006), in MEF's (Kang et al., 2000) and in CHO cells stably transfected with Swe-APP751 (Yoon et al., 2007). The variation in reported Aβ levels may be due to variation in the cell lines, APP isoforms, LRP1 constructs, or endogenous protein levels between experiments. However, we cannot completely exclude the possibility that the strong over-expression of the proteins may be contributing to the effects we observed.

Recent data show that sLRP seems to be a major peripheral Aβ binding protein (Sagare et al., 2007). In humans, sLRP normally binds ~70–90% of circulating Aβ and acts as an "endogenous sink agent" (Deane et al., 2008). In AD, decreased sLRP levels result in decreased binding of Aβ. In addition it was reported that LRP1 is directly involved in intracellular Aβ uptake and degradation (Narita et al., 1997; Deane et al., 2004; Zerbinatti et al., 2006). Our data show that increased levels of APP can lead to reduced sLRP production, which might impair Aβ clearance and thereby contribute to development of AD. Additionally, our data confirm that BACE1 can be targeted not only by pharmacological inhibitors but also by substances perturbing the balance of BACE1 cleavage of APP. BACE1 is a target of several pharmacological approaches and the first BACE1 inhibitors are already tested in clinical trials.

The number of known BACE1 substrates has grown rapidly and there likely remain more to be discovered. Here we provide novel evidence that LRP1 and APP compete for binding to and β-site cleavage by BACE1. The role of this competition at physiologic levels of protein expression and in pathological systems needs further examination using endogenously-expressed protein and siRNA knock-down of APP and LRP1. However, our work supports the idea that the expression level of LRP1 is an important modifier of APP processing and AD development and points to a new mechanism by which LRP1 exerts this influence.

Acknowledgments

This work was supported by a grant from Hirnliga e.V. and Heidelberger Akademie der Wissenschaften to C.v.A.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.expneurol.2010.05.017.

Fig. 7. Aβ-MesoScale. Conditioned medium from the SEAP assay transfection was also used for an Aβ-triplex MesoScale assay. A: Aβ38 levels in medium of untransfected N2A (lane 1), N2A co-transfected with SEAP-APP695 + empty vectors (lane 2), N2A co-transfected with SEAP-APP + BACE1-GFP + empty vector (lane 3), N2A co-transfected with SEAP-APP + BACE1-GFP + LC-Myc (lane 4). B: Aβ40 levels in medium of untransfected N2A (lane 1), N2A co-transfected with SEAP-APP695 + empty vectors (lane 2), N2A co-transfected with SEAP-APP + BACE1-GFP + empty vector (lane 3), N2A co-transfected with SEAP-APP + BACE1-GFP + LC-Myc (lane 4). C: Aβ42 levels in medium of untransfected N2A (lane 1), N2A co-transfected with SEAP-APP695 + empty vectors (lane 2), N2A co-transfected with SEAP-APP + BACE1-GFP + empty vector (lane 3), N2A co-transfected with SEAP-APP + BACE1-GFP + LC-Myc (lane 4). Given are the means and S.D. of one assay. Even protein expression was controlled by BCA and Western blot. Both transfection and measurement were carried out in duplicate.

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