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X11α impairs γ- but not β-cleavage of amyloid precursor protein

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Abstract

The phosphotyrosine binding domain of the neuronal protein X11α/mint-1 binds to the C-terminus of amyloid precursor protein (APP) and inhibits catabolism to β-amyloid (Aβ), but the mechanism of this effect is unclear. Coexpression of X11α or its PTB domain with APPswe inhibited secretion of Aβ40 but not APPsβswe, suggesting inhibition of γ- but not β-secretase. To further probe cleavage(s) inhibited by X11α, we coexpressed β-secretase (BACE-1) or a component of the γ-secretase complex (PS-1Δ9) with APP, APPswe, or C99, with and without X11α, in HEK293 cells. X11α suppressed the PS-1Δ9-induced increase in Aβ42 secretion generated from APPswe or C99. However, X11α did not impair BACE-1-mediated proteolysis of APP or APPswe to C99. In contrast to impaired γ-cleavage of APPswe, X11α or its PTB domain did not inhibit γ-cleavage of NotchΔE to NICD (the Notch intracellular domain). The X11α PDZ–PS.1Δ9 interaction did not affect γ-cleavage activity. In a cell-free system, X11α did not inhibit the catabolism of APP C-terminal fragments. These data suggest that X11α may inhibit Aβ secretion from APP by impairing its trafficking to sites of active γ-secretase complexes. By specifically targeting substrate instead of enzyme X11α may function as a relatively specific γ-secretase inhibitor.

Keywords: amyloid precursor protein, X11α, BACE, presenilin, notch, γ-secretase.

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Alzheimer's disease (AD) is pathologically defined by the density and distribution of amyloid plaques and neurofibrillary tangles in brain. The major components of amyloid plaques are β-amyloid (Aβ) peptides which are derived by β- and γ-cleavage of amyloid precursor protein (APP). Alternative catabolic pathways of APP including cleavage by β-secretases preclude Aβ generation. The products of β-secretase cleavage, mediated in brain by BACE-1 (Vassar *et al*. 1999), include a cell-associated C99 stub and secreted APPsβ. Subsequent γ-cleavage of C99 generates Aβ40 and Aβ42 and the C-terminal fragment γCTF (Selkoe 2001). γCTF may regulate gene transcription via its interaction with the transcription factor Tip60 and subsequent translocation to the nucleus (Kimberly *et al*. 2001). The γ-secretase complex consists of presenilin-1 or -2:nicastrin:pen-2:aph-1 (Li *et al*. 2000a; Li *et al*. 2000b; Xia *et al*. 2000; Lee *et al*. 2002; Steiner *et al*. 2002). The active site of this complex includes two Asp residues in transmembrane domains 8 and 9 of presenilin (Wolfe *et al*. 1999).

The cytoplasmic tail of APP contains an absolutely conserved –GYENPTY- motif that is essential for its proper targeting and trafficking, and therefore its metabolism (Lai *et al*. 1995). Adaptor proteins interact with this motif in the C-terminus of APP to modulate its cellular trafficking and metabolism (Nishimoto *et al*. 1993; Borg *et al*. 1996; Chow *et al*. 1996; Zheng *et al*. 1998; Howell *et al*. 1999; Kamal

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Abbreviations used: AD, Alzheimer's disease; APP, amyloid precursor protein; APPswe, Swedish mutation of APP; APPs, secreted N-terminal ectodomains of APP; APPsα, secreted α-cleavage product of APP; APPsβ, secreted β-cleavage product of APP; APPsαswe, secreted α-cleavage product of APPswe; APPsβswe, secreted β-cleavage product of APPswe; AICD, APP intracellular domain; Aβ, β-amyloid; BACE-1, β-secretase APP cleaving enzyme 1; ELISA, enzyme-linked immunosorbent assay; FAD, familial Alzheimer's disease; γCTF, γ-cleaved C-terminal fragment of APP or APPswe; NICD, Notch1 intracellular domain; NotchΔE, Notch1 with a deletion of the extracellular domain; PDZ, repeated sequences in post-synaptic density-95 (PSD-95), *D. melanogaster* septate junction protein *D*isks-large (Dlg), and epithelial tight junction protein Zona occludens-1 (ZO-1); PBS, phosphate-buffered saline; PS-1Δ9, familial AD mutation presenilin-1 with deletion of exon 9; PTB/PI, phosphotyrosine binding/protein interaction; RIP, regulated intramembranous proteolysis; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

et al. 2000; Matsuda *et al*. 2001; Zambrano *et al*. 2001; Russo *et al*. 2002; Scheinfeld *et al*. 2002; Tarr *et al*. 2002; Taru *et al*. 2002a; Taru *et al*. 2002b). The best-characterized of these APP–adaptor protein interactions are of those in the X11 (α, β, and γ) and Fe65 (Fe65, Fe65L1, and Fe65L2) families, which share conformational requirements for APP interaction (Borg *et al*. 1996) yet differentially affect APP metabolism. For example, X11α (also known as mint-1 and lin-10) delays maturation of APP and secretion of its catabolites (Borg *et al*. 1998b; Mueller *et al*. 2000; King *et al*. 2003), while Fe65L1 accelerates these processes (Guenette *et al*. 1999). X11α increases APP half-life (Borg *et al*. 1998a), while Fe65L1 decreases it (Guenette *et al*. 1999). X11α decreases Aβ secretion (Borg *et al*. 1998a; Sastre *et al*. 1998; Mueller *et al*. 2000), while Fe65 and Fe65L1 increase it (Sabo *et al*. 1999; Ando *et al*. 2001). X11α contains a munc interaction domain, a CASK interaction domain, a phosphotyrosine binding (PTB) domain and two PDZ (post-synaptic density-95, disks-large (Dlg), and ZO-1) domains. Fe65 contains a WW domain and two PTB domains. The distinct protein interaction networks of the X11 and Fe65 families likely mediate their differential effects on APP trafficking and metabolism and imply cellular functions of APP and its proteolytic fragments.

The PTB domain of X11α interacts with a -YENP-containing sequence in the cytoplasmic tail of APP (Borg *et al*. 1996; Zhang *et al*. 1997; King *et al*. 2003). The X11α PTB–APP interaction increases cellular APP levels, increases APP half-life, and decreases secretion of Aβ, total APPs, and APPsα (Borg *et al*. 1998a; Sastre *et al*. 1998). To inhibit Aβ secretion from APP, X11α may inhibit cleavage by either β- or γ-secretase, or both. The inhibitory effect may occur either by steric hindrance (impairing interaction of APP with protease), by impaired trafficking of APP to cellular compartments containing β- and γ-secretases, or both. To define the mechanism whereby X11α or its PTB domain inhibits APP catabolism to Aβ, we examined: (i) β- and γ-specific cleavage fragments with endogenous and exogenous (transfected) β- or γ-secretase activities; (ii) the specificity of X11α on γ-cleavage of APP and APPswe versus NotchΔE; and (iii) results obtained with intact cells versus a cell-free system to remove the variable of cellular trafficking.

Experimental procedures

Cell culture

Human embryonic kidney (HEK) 293 cells (American Type Culture Collection) were passaged in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing penicillin (100 U/mL) and streptomycin sulfate (100 μg/mL) supplemented with 10% fetal bovine serum (Atlanta Biological, Norcross, GA, USA).

DNA constructs

Myc-tagged X11α and its deletion constructs X11α ΔN, X11α ΔPDZ, and X11α PTB were cloned in pRK5 as described (Borg *et al*. 1996). APPswe$_{751}$ was cloned in pcDNA3 as described (Perez *et al*. 1999; Steinhilb *et al*. 2002). The Swedish mutation of APP (K651N/M652L APP, or APPswe, in the 751 isoform) was used to allow robust detection of Aβ40 and Aβ42 in conditioned media, and to mimic the more amyloidogenic APP metabolism found in neurons in a non-neuronal cell line (HEK 293) (Forman *et al*. 1998). The C99 construct was cloned into pCEP4 and included a signal peptide for proper orientation in the cell membrane (Lichtenthaler *et al.* 1999).

Cell transfection and protein extraction

Six cm plates were precoated with poly D-lysine (10 μg/mL) prior to use. HEK 293 cells were plated (7.5×10^5 cells/6-cm plate) 24 h prior to transfection. Fifteen microliters of LipofectAMINE 2000 (Invitrogen) per plate was used to transfect a total of 9 μg of DNA for each transfection according to the manufacturer's instructions. Cells were maintained in serum free OptiMem I (Invitrogen) for 6 h post transfection. At 6 h, OptiMem I containing 5% fetal bovine serum replaced the previous media and cells were further incubated for 24–48 h. Conditioned media was removed and centrifuged at 15 000 × *g* for 10 min at 4°C to remove cellular debris. After washing in phosphate-buffered saline (PBS), cells were lysed with NP40 lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS, 0.5 mM EDTA) supplemented with phenylmethylsulfonylfluoride (PMSF, Sigma, St Louis, MO, USA) and complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Cell lysates were centrifuged at 15 000 × *g* for 10 min at 4°C.

Immunoblotting

Proteins were separated by SDS–PAGE (Invitrogen) and transferred to polyvinyldiene difluoride (PVDF) membranes (Roche). Membranes were blocked in 5% milk in TBST. The monoclonal antibody 22C11 (MAB348, Chemicon, Temecula, CA, USA) raised to an N-terminal domain of APP (residues 61–88) detected APP. Anti-myc antibody (sc-40, Santa Cruz, Santa Cruz, CA, USA), anti-HA antibody (sc-805, Santa Cruz), and antipresenilin-1 antibody (sc-7860, Santa Cruz) were used to confirm X11α, BACE-1 and PS1-Δ9 expression, respectively, in cell lysates. Experiments requiring C-terminal fragment detection utilized 10–20% Tris-tricine SDS–PAGE (Invitrogen) and Calbiochem anti-APP C-terminal antibody for immunoprecipitation followed by detection with anti C-terminal antibody 369. Generation of the Notch1 intracellular domain (NICD) was detected by an antibody specific to Notch1 cleaved at Val 1744 (Cell Signaling Technology, Beverly, MA, USA) after separation by 4–12% Tris-glycine SDS–PAGE (Invitrogen). ECL Plus (Amersham, Piscataway, NJ, USA) detected horseradish peroxidase-conjugated secondary antibodies (Chemicon) on a PhosphorImager screen. Protein bands were quantitated with a STORM Scanner using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

Immunoprecipitation

Twenty-four hours after transfection cells were lysed in NP40 lysis buffer and immunoprecipitated overnight using Protein A-Sepharose beads (Sigma) and anti-APP antibody (44400, BioSource,

Camarillo, CA, USA) for APP/X11α/PS-1Δ9 experiments or anti-Notch1 (sc-6014, Santa Cruz) for NotchΔE experiments. After separating proteins by 8% Tris-glycine SDS–PAGE (Invitrogen) membranes were immunoblotted to detect PS-1Δ9 (Santa Cruz) and X11α or its deletion constructs (Santa Cruz). In Notch1 interaction experiments, NotchΔE was immunoprecipitated with anti-Notch1 antibody (sc6014, Santa Cruz), proteins were separated by 10–20% Tris-tricine SDS–PAGE (Invitrogen), and membranes immunoblotted with anti-myc (9E10, Santa Cruz) to detect myc-tagged NotchΔE, X11α, and X11α PTB.

Cell-free system

HEK 293 cells were plated onto poly D-lysine coated 15-cm dishes 24 h prior to transfection. Cells were transfected with 8 μg of each DNA construct, incubated, and harvested in ice-cold PBS as described above. All remaining steps were conducted at 4°C. Cells were pelleted and resuspended in 1 mL buffer A [20 mM HEPES, 250 mM sucrose, 50 mM KCl, 2 mM EDTA, 2 mM EGTA, and protease inhibitor cocktail (Roche)]. Cells were lysed by 10 strokes in a Dounce homogenizer and five passages through a 27-gauge needle. Nuclei and intact cells were pelleted at $1500 \times g$ for 10 min, resuspended once, and both supernatants combined. Supernatants were centrifuged at $100\,000 \times g$ for 1 h. Protein concentration in the resuspended microsomal pellet was determined by BCA analysis. Half of the suspension was lysed with 0.1% TritonX-100 while the remaining half was incubated for 90 min at 37°C prior to lysis. Proteins were separated by 10–20% gradient Tris-tricine SDS–PAGE, transferred as above, and APP C-terminal fragments detected by immunoblot using an anti-C-terminal APP antibody (Calbiochem).

Metabolic radiolabeling

For C99 experiments, 24 h after transfection, cells were incubated for 15 min in cysteine/methionine-free DMEM (Invitrogen) followed by DMEM containing [^{35}S]methionine (ICN) for 4 h for pulse-labeling. Cells were then rinsed with PBS, lysed, and proteins immunoprecipitated using an anti-APP C-terminal antibody (171610, Calbiochem). Isolated proteins were separated by 10–20% Tris-tricine SDS–PAGE and radiolabeled bands detected by autoradiography and quantitated by Phosphorimager analysis using a STORM Scanner with ImageQuant software (Molecular Dynamics). For NotchΔE experiments, cells were prepared as above except they were labeled for 20 min followed by 1 h chase. Lysates were then immunoprecipitated using anti-myc antibody (9E10, Santa Cruz) and separated on 9% tris-glycine gels for autoradiographic detection by exposure to a Phosphorimager screen (Molecular Dynamics).

Aβ40 and Aβ42 ELISA

Aβ40 and Aβ42 in conditioned media were detected by a sensitive and specific sandwich ELISA (Suzuki *et al*. 1994; Turner *et al*. 1996; Borg *et al*. 1998b; Mueller *et al*. 2000). BAN50 (Aβ 1–10) was used as the capture antibody and either horseradish peroxidase-coupled BA-27 or BC-05 as the detection antibody for Aβ40 or Aβ42, respectively. Standard curves were generated with Aβ40 and Aβ42 (Bachem).

APPsβswe ELISA

APPsβswe in conditioned media was detected by a sandwich ELISA using 931 antiserum as capture antibody (Steinhilb *et al*. 2001; Ho *et al*. 2002; King *et al*. 2003). This antiserum does not recognize full length APPswe, APPsαswe, endogenous wild-type APP, APPsα, or APPsβ, and is thus specific for the neoepitope in the C-terminus of APPsβswe.

Statistical analysis

Significant differences between means were determined by multiple analyses of variance using a Student's *t*-test.

Results

X11α inhibited Aβ40 but not APPsβswe secretion

X11α consistently inhibits secretion of Aβ40 and Aβ42 from cells coexpressing APP or APPswe (Borg *et al*. 1998a; Sastre *et al*. 1998; Mueller *et al*. 2000; King *et al*. 2003). Due to impaired trafficking to secretory and endocytic cellular compartments containing β- and γ-secretases (King *et al*. 2003), we hypothesized that X11α would inhibit both β- and γ-cleavage of APP and APPswe. To probe the specific cleavage(s) inhibited and to examine potential modulatory roles of the other protein interaction domains of X11α we coexpressed APPswe with X11α or its deletion constructs (Fig. 1c) in HEK 293 cells. In agreement with previous data with Aβ40 and Aβ42 (Mueller *et al*. 2000), Aβ40 secretion was inhibited by X11α, X11αΔN, and PTB but not by X11α ΔPDZ ($p = 0.05$) (Fig. 1a). In contrast, secretion of the β-secretase cleavage product APPsβswe was unaffected (Fig. 1b), suggesting that β-cleavage of APPswe was not impaired. Equivalent expression of the X11α constructs of the appropriate M_r was confirmed by immunoblot (Fig. 1d). In contrast to our original hypothesis, these data suggest specific inhibition of γ- but not β-cleavage of APPswe by X11α, X11α ΔN, and PTB. These results also support a model (Mueller *et al*. 2000) of inhibitory effects of the PTB and C-terminal PDZ domains on APP catabolism but a stimulatory effect of N-terminal X11α domains apparent only in the absence of dominant PDZ domains.

X11α suppressed the PS1Δ9-induced increase in Aβ42 secretion

These data indicated that X11α specifically inhibited endogenous γ- but not β-cleavage of APPswe. To further probe the specific inhibition of β- versus γ-secretase(s) by X11α, we coexpressed BACE-1 or PS-1Δ9 with APPswe, with and without X11α. Based on the data in Fig. 1, we hypothesized that X11α would suppress PS-1Δ9-mediated but not BACE-1-mediated APPswe catabolism. Similar to other PS-1 mutations found in pedigrees of familial AD, PS-1Δ9, a deletion mutation of exon 9, specifically promotes Aβ42 but not Aβ40 generation from APP and APPswe (Steiner *et al*. 1999). BACE-1 catalyzes β-cleavage of APP and APPswe and regulates Aβ generation by generating C99 for subsequent γ-cleavage (Vassar *et al*. 1999). APPswe is a 10-fold

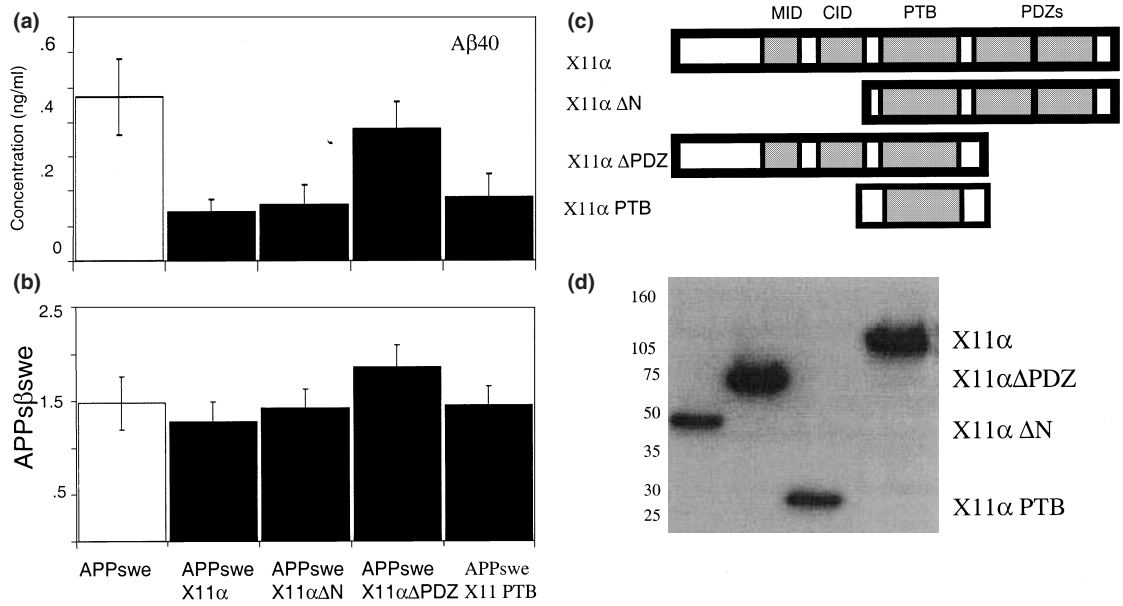

Fig. 1 X11α inhibited Aβ40 but not APPsβswe secretion. (a) Aβ40 (ng/mL) and (b) APPsβswe (relative absorbance) were measured in conditioned media by ELISA 48 h after transfection of HEK 293 cells with the constructs indicated. (c) Schematic drawing of X11α, its protein interaction domains, and the deletion constructs used in these studies. (d) Representative immunoblot of myc-tagged X11α deletion constructs. Data represent the mean ± SEM, $n = 5$.

better substrate for BACE-1 than APP, resulting in increased generation of both Aβ40 and Aβ42 (Citron *et al*. 1992; Felsenstein *et al*. 1994).

Coexpression of X11α increased (Fig. 2b, lanes 1 and 4) and PS-1Δ9 or BACE-1 decreased (Fig. 2b, lanes 1, 2, and 3) levels of cellular APPswe (Fig. 2a,b) suggesting that both secretases were expressed and active in catabolizing APPswe. Representative immunoblots of cell lysates confirmed equivalent expression of X11α, BACE-1, PS-1Δ9, and PTB (Fig. 2a). As expected (Mehta *et al*. 1998), coexpression of APPswe with PS-1Δ9 doubled Aβ42 (Fig. 2d, lanes 5 and 6) but not Aβ40 levels (Fig. 2c, lanes 5 and 6) in conditioned media and expression of X11α with APPswe inhibited Aβ40 and Aβ42 secretion (Fig. 2c,d, lanes 5 and 7). X11α suppressed the PS1-Δ9-induced increase in Aβ42 secretion (Fig. 2d, lanes 6 and 9; $p = 0.03$), suggesting inhibition of γ-cleavage and neutralization of the amyloidogenic effect of PS-1Δ9. The isolated PTB domain of X11α mimicked results obtained with full-length X11α (Fig. 2c,d, lanes 8 and 10), suggesting that other protein interaction domains of X11α were not essential for this inhibitory effect. In parallel experiments, X11α was cotransfected with BACE-1 and APPswe to determine if X11α may modulate β-cleavage of APPswe. However, our ELISA required intact Aβ that was undetectable in conditioned media with BACE-1 expression presumably due to additional cleavage sites within the Aβ sequence at glu11 and leu34 (Huse *et al*. 2002; Liu *et al*. 2002; Fluhrer *et al*. 2003).

X11α did not inhibit BACE-1-mediated generation of C99 from APPswe or APP

To further probe the regulation of β-cleavage of APPswe by X11α, we instead examined the generation of C99 from APPswe. C-terminal fragments of APPswe were immunoprecipitated from cell lysates, separated by Tris-tricine SDS–PAGE, and detected by immunoblot (Fig. 3a). As expected, coexpression of BACE-1 with APPswe significantly increased C99 levels in cell lysates (Fig. 3a, lanes 6, 9, and 10). When X11α or its PTB domain was coexpressed with BACE-1, C99 generation from APPswe was unaffected, suggesting that X11α did not impair β-cleavage (Fig. 3a,c). Consistent with previous data that X11α inhibits APPsα and total APPs secretion (Borg *et al*. 1998a), X11α coexpression consistently decreased the level of C83 generated by α-secretases (Fig. 3a,b, lanes 2 and 3). When BACE-1 was coexpressed with APPswe, potential C-terminal fragments generated by additional cleavage sites at glu11 and leu34 within the Aβ sequence (Fluhrer *et al*. 2003) were not apparent.

The APPswe mutation promotes BACE-1 cleavage at the β-cleavage site to generate C99 (Felsenstein *et al*. 1994). We hypothesized that similar to APPswe, X11α would not suppress BACE-1-mediated generation of C99 from wild type APP. In these experiments we detected C99 generated by β-cleavage at Asp1 of Aβ, C89 generated by β′ cleavage of APP (at Glu11 of Aβ), and C83 generated by α-secretases (Fig. 3d) (Huse *et al*. 2002; Liu *et al*. 2002). Consistent with

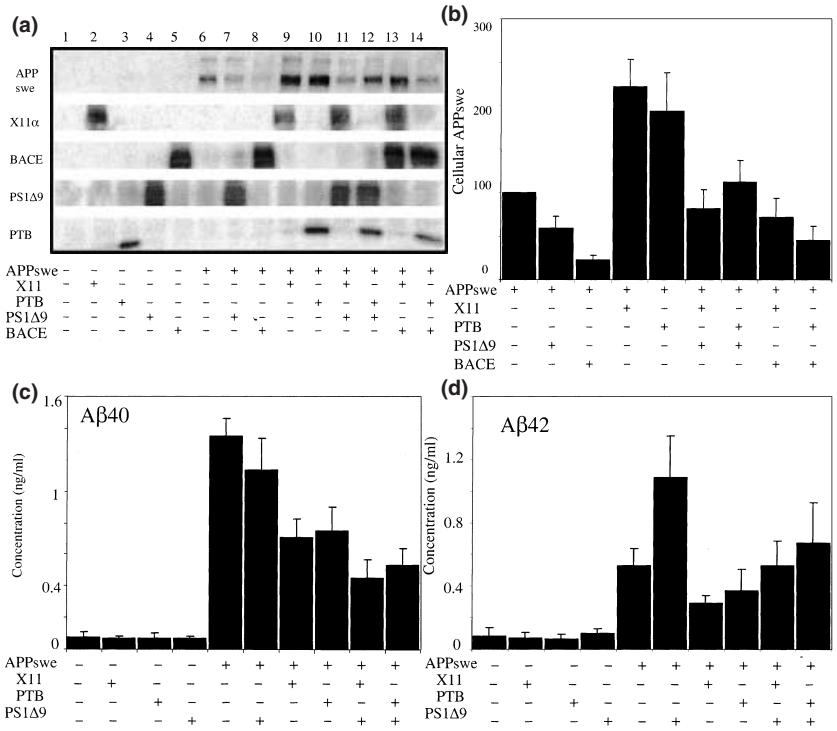

Fig. 2 X11α suppressed the PS-1Δ9-induced increase in Aβ42 secretion. (a) Representative immunoblots of proteins extracted from cell lysates confirmed equivalent expression of APPswe, X11α, BACE-1, PS-1Δ9, and PTB in transfected HEK 293 cells. (b) Relative levels of APPswe (mature plus immature) detected by immunoblot of proteins extracted from cell lysates and quantitated by PhosphorImager analysis. Each experimental data set was normalized to the level of APPswe expressed alone (set as 100%). Data represent the mean ± S.E.M, $n = 4$. (c) Aβ40 (ng/mL) and (d) Aβ42 (ng/mL) were detected by ELISA of conditioned media 48 h after transfection as indicated. Data represent the mean ± SEM, $n = 6$.

less secreted Aβ levels found with BACE-1 expression, C89 levels indicate significant β′-cleavage of APP at glu11 of Aβ (Liu *et al*. 2002). X11α coexpression diminished C83 levels (Fig. 3d, lanes 1 and 4), but not BACE-1 induced C99 and C89 levels (Fig. 3d, lanes 3 and 8, and Fig. 3e). In agreement with these data, X11α coexpression had no effect on C99 levels detected by metabolic radiolabeling, immunoprecipitation, and autoradiographs in cells transfected with BACE-1 and either APP or APPswe (data not shown).

In contrast to results obtained with APPswe, the PTB domain effectively diminished C99 generation from APP (Fig. 3d, lanes 3 and 9, and Fig. 3e), suggesting that the PTB domain may inhibit β-cleavage of APP. This difference may reflect the greater efficiency of BACE-1 cleavage for APPswe compared to wild type APP. Thus, β-cleavage of APP, a poor substrate for BACE-1 compared to APPswe, appears to be susceptible to inhibition by PTB coexpression.

X11α suppressed the PS1Δ9-induced increase in Aβ42 generation from C99

If X11α inhibits γ-cleavage of APP and APPswe to inhibit Aβ secretion, we hypothesized that X11α should also inhibit Aβ secretion from cells transfected with C99 (Lichtenthaler *et al*. 1999). These experiments remove of the variable of β-cleavage and the differential effects of APPswe versus APP since C99 generated by β-cleavage of either is identical. Thus, in contrast to previous experiments with APP and APPswe, we examined the effects of X11α on C99 metabolism. Compared to X11α which increased cellular levels of C99, X11α ΔN markedly increased and X11α ΔPDZ consistently decreased C99 levels in cell lysates (Fig. 4a), despite equivalent expression of X11α constructs of the appropriate M_r (data not shown). These data also support a model of inhibition of γ-cleavage of APP, APPswe, or C99 by X11α PDZ domains, but promotion of γ-cleavage by N-terminal domains apparent only in the absence of dominant PDZ domains (Mueller *et al*. 2000). Consistent with this model, X11α ΔN prolonged the half-life of C99 even greater than X11α (Fig. 4c). Secretion of Aβ40 and Aβ42 from C99-transfected cells was inhibited by X11α and X11αΔN, but not by X11α ΔPDZ or the PTB domain (Fig. 4b).

As X11α effects on C99 metabolism were similar to those found with APP, we hypothesized that γ-cleavage of C99 to Aβ would be impaired by X11α coexpression. As expected, PS-1Δ9 increased and X11α diminished Aβ42 levels in conditioned media of C99-transfected cells (Fig. 4d). Consistent

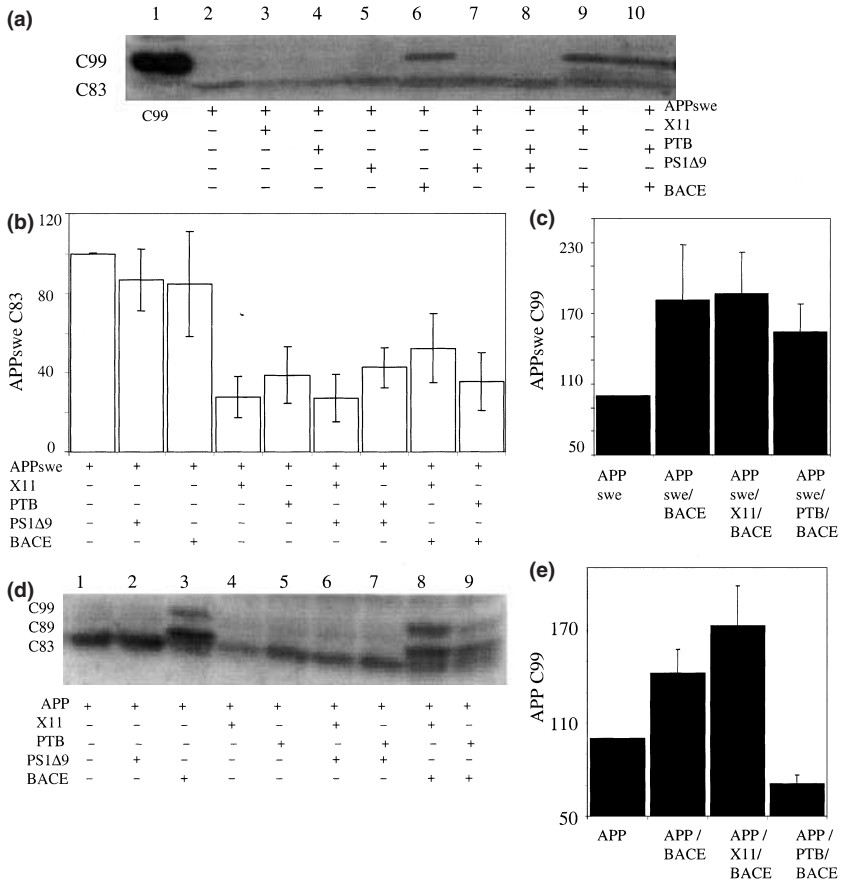

Fig. 3 X11α did not inhibit BACE-1-mediated C99 generation from APPswe or APP. (a) Representative immunoblot of C-terminal fragments of APPswe recovered by immunoprecipitation and detected by immunoblot from cell lysates of HEK 293 cells transfected as indicated. Relative levels of (b) C83 and (c) C99 were quantitated by Phosphorimager analysis, and normalized to the levels found with APPswe alone. (d) Representative immunoblot of C-terminal fragments of wild-type APP in cell lysates recovered by immunoprecipitation and detected by immunoblot from HEK 293 cells transfected as indicated. (e) Relative levels of C99 were quantitated by Phosphorimager analysis and each data set was normalized to levels of C99 found with APP expressed alone. The data represent mean ± SEM, $n = 6$.

with our previous results with APPswe, X11α suppressed the PS-1Δ9-induced increase in Aβ42 generated from C99. As found for APPswe, intact Aβ in conditioned media was not detected with BACE-1 coexpression presumably due to additional cleavage sites within the Aβ sequence (Fig. 4d) (Huse *et al*. 2002; Liu *et al*. 2002).

X11α did not impair γ-cleavage of NotchΔE to NICD. X11α may inhibit γ-cleavage of APP by its interaction with substrate (PTB–APP) or enzyme (PDZ–PS-1) (Lau *et al*. 2000). If the former, then X11α may function as a relatively specific γ-secretase inhibitor of APP, in contrast to non-specific or less specific small molecular-weight inhibitors that target enzyme and also inhibit Notch1 processing. Thus, we examined γ-cleavage of NotchΔE to NICD (Notch1 intracellular domain) in the presence and absence of X11α. Analogous to C99 and its γ-secretase fragment γCTF (also known as AICD or the APP intracellular domain), NotchΔE requires only γ-cleavage to generate NICD (Schroeter *et al*. 1998; De Strooper *et al*. 1999). Because PS-1 cleaves Notch1 more efficiently than PS-1Δ9 (Song *et al*. 1999; Nakajima *et al*. 2000; Chen *et al*. 2002) we expressed PS-1 in these experiments. Equivalent expression of myc-tagged X11α, X11α PTB, NotchΔE, and PS-1 were confirmed in cell lysates (Fig. 5a).

Immunoprecipitation of Notch1, followed by immunoblot with anti-myc, revealed that X11α, but not its PTB domain, interacted with NotchΔE (Fig. 5b), presumably via X11α PDZ domains since they are relatively promiscuous. Notch1 coexpression with PS-1 revealed a low level of NICD generation in cell lysates, as detected by an antibody that specifically detects the neoepitope of γ-cleaved Notch1 (Cell Signaling Technologies) (Pheil *et al*. 2003). Specificity of this NICD antibody was confirmed by preincubation with the antigen (cleaved Notch1 peptide beginning with Val1744) or with uncleaved Notch1 peptide beginning with Met1727. Preincubation with the cleaved peptide blocked detection of NICD, while preincubation with the full-length peptide did

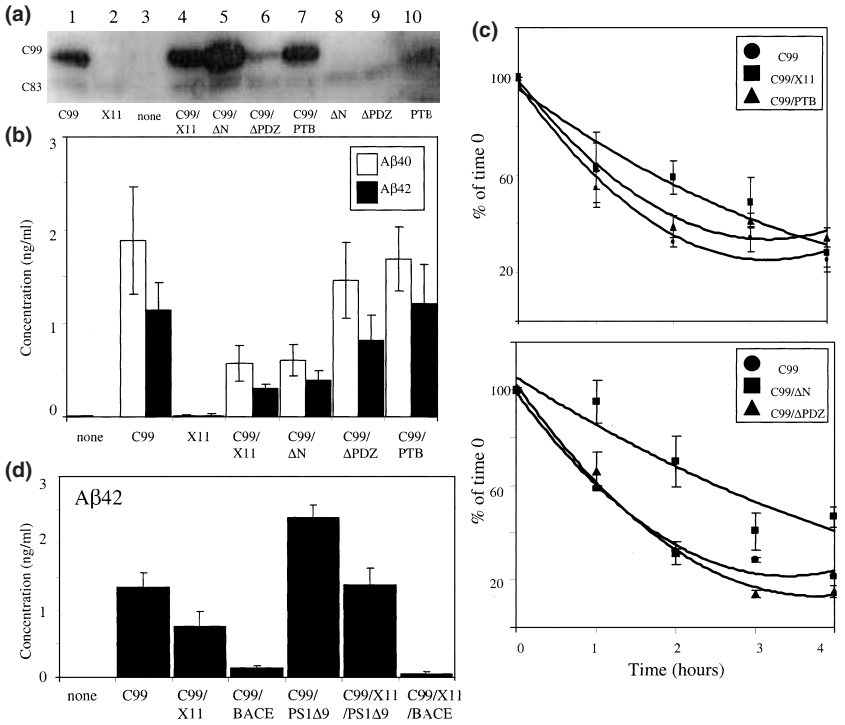

Fig. 4 X11α suppressed the PS-1Δ9-induced increase in Aβ42 generation from C99. (a) Representative immunoblot of APP C-terminal fragments extracted from HEK 293 cells transfected as indicated. (b) Aβ40 and Aβ42 (ng/mL) levels measured by ELISA from conditioned media of cells transfected as indicated. (c) Pulse-chase analyses of C99 from [^{35}S]methionine-labeled HEK 293 cells transfected with the constructs indicated. Radiolabeled C99 was immunoprecipitated from cell lysates, detected by autoradiography, and quantitated by Phosphorimager analysis. Data for (b) and (c) represent mean ± SEM, $n = 5$. (d) Aβ42 (ng/mL) levels measured by ELISA from conditioned media of cells transfected as indicated. Data represent mean ± SEM, $n = 6$.

not (data not shown). As expected, NICD generation was inhibited by incubation with the γ-secretase inhibitor L-685, 458 (Calbiochem) at 10 μM. In these experiments NotchΔE expression was confirmed by immunoblot of cell lysates (data not shown). NICD generation from NotchΔE was robust with endogenous levels of γ-secretase activity and was unaffected by coexpression of X11α or its PTB domain (Fig. 5c). Pulse-chase experiments confirmed that coexpression of X11α or its PTB domain did not impair PS-1-mediated cleavage of NotchΔE to NICD (Fig. 5d). After correction for NotchΔE expression, Phosphorimager quantitation of autoradiographs from multiple experiments revealed no inhibitory effect of X11α expression on NICD generation (Fig. 5e). Collectively, these data suggest that due to the specific PTB–APP interaction, X11α may function as a relatively specific γ-secretase inhibitor. In contrast, X11α PDZ interaction with PS-1 appear to be irrelevant to γ-cleavage activity.

X11α may impair γ-cleavage by alteration of APP trafficking

The binding of X11α to the cytoplasmic tail of APP, APPswe, or C99 may preclude their interaction with the γ-secretase complex (Xia *et al*. 1997; Verdile *et al*. 2000; Xia *et al*. 2000), impair their trafficking to cellular compartments containing active γ-secretase complex, or both. For example, X11α may interfere with the direct interaction of nicastrin with the APP C-terminus (Yu *et al*. 2000). Alternatively, the X11α PDZ domains interact with PS-1 (Lau *et al*. 2000) and this may impair its catalytic activity or its ability to form a functional γ-secretase complex. However, because the PTB domain of X11α is sufficient to inhibit Aβ secretion (Fig. 1c) and suppress PS-1Δ9-induced elevations in Aβ42 from APPswe (Fig. 2c), the X11α PDZ–PS-1 interaction appears to have no effect on γ-cleavage activity.

We tested the hypothesis that X11α may impair the direct interaction of the PS-1/γ-secretase complex with APP (Xia *et al*. 1997). However, coimmunoprecipitation of APPswe, X11α, and PS-1Δ9 revealed that a heterotrimeric complex formed when all three proteins were coexpressed. When APPswe was immunoprecipitated, X11α did not affect recovery of PS-1Δ9 and vice versa (Fig. 6a). Likewise, binding of the X11α PTB domain to APPswe did not affect PS-1Δ9 recovery (data not shown). These data argue against a competitive APP–X11α versus APP–γ-secretase complex interaction as the inhibitory mechanism of X11α.

To further probe the potential role of the X11α PDZ–PS-1 interaction on the inhibitory effect on γ-cleavage, we coexpressed X11α or its deletion constructs with APPswe and PS-1Δ9 and measured Aβ42 in conditioned media. In agreement with our previous data (Fig. 1b), X11α or X11α ΔN but not X11α ΔPDZ inhibited Aβ42 secretion and suppressed PS-1Δ9-induced elevation of Aβ42 levels (Fig. 6b). Thus, similar results with the X11α deletion constructs were obtained whether utilizing endogenous (Fig. 1b) or exogenous (transfected) (Fig. 6b) γ-secretase activity.

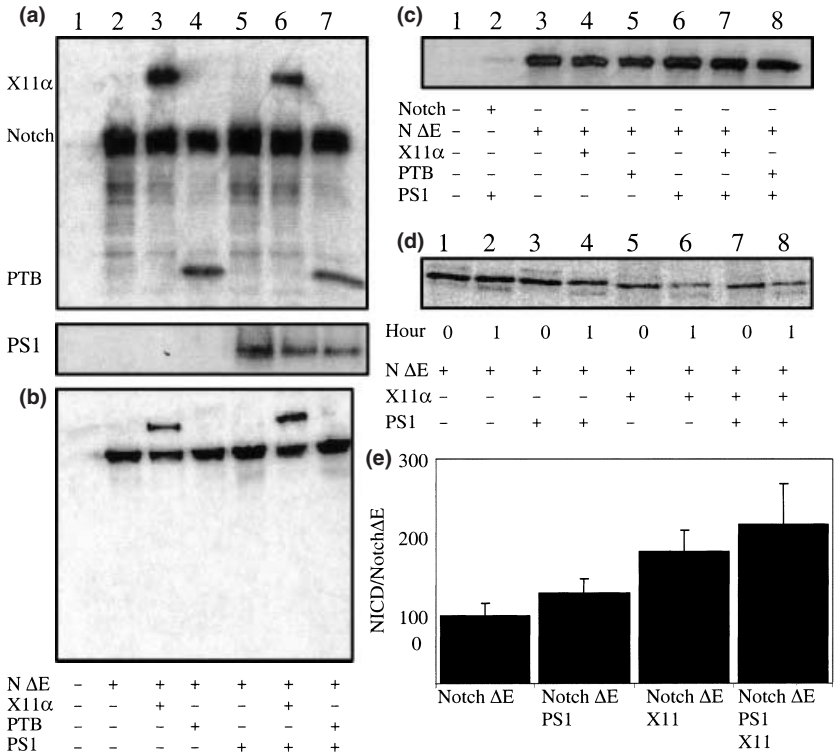

Fig. 5 X11α did not inhibit NICD generation from NotchΔE. (a) Representative immunoblot (anti-myc) of proteins extracted from cell lysates of HEK 293 cells demonstrating equivalent expression of the constructs indicated. NotchΔE, X11α, and X11α PTB were all myc-epitope tagged and detected on a single immunoblot (upper panel). PS-1 was detected by immunoblot separately (lower panel). (b) Representative anti-myc immunoblot after immunoprecipitation of Notch1 revealed interaction of X11α–Notch1 but not PTB–Notch1. (c) Representative immunoblot using an antibody to NICD specific to the neoepitope of cleaved NotchΔE. (d) Representative autoradiograph of proteins after metabolic radiolabeling with [^{35}S]methionine. Cells were pulsed for 20 min followed by a 1-h chase to detect NICD generation from NotchΔE. Data represent mean ± SEM, $n = 8$. (e) Quantitative analysis of autoradiographs plotted as amount of NICD generated relative to NotchΔE expressed and normalized to NotchΔE (100%). Data represent mean ± SEM, $n = 5$.

We next tested the hypothesis that X11α may impair trafficking of APP to cellular compartments harboring the active γ-secretase complex. HEK 293 cells were transfected with C99 with or without X11α and cell-free microsomal membranes prepared. C-terminal fragment levels before and after a 1-h incubation at 37°C (as a percentage of time 0) revealed that X11α coexpression had no effect on C99 degradation (Fig. 6c,d) or C83 degradation (Fig. 6c). Aβ levels in these membrane preparations were undetectable. These data are consistent with the notion that X11α impairs γ-cleavage of APP by impairing its trafficking to subcellular sites containing active γ-secretase complex. In other words, intact cells are essential to observe the inhibitory effect of X11α on γ-cleavage of APP.

Discussion

X11α consistently inhibits APP and APPswe catabolism and Aβ secretion *in vitro* (Borg *et al*. 1998a; Sastre *et al*. 1998; Mueller *et al*. 2000; King *et al*. 2003) by impairing γ- but not β-cleavage and presumably by impairing trafficking of substrate to subcellular compartments harboring active γ-secretase complexes. We probed the effect of X11α on β- versus γ-cleavage of APP, APPswe, and C99 using both endogenous and overexpressed (transfected) levels of secretases. While the PS-1Δ9-mediated increase in Aβ42 was inhibited by X11α, APPsβswe secretion and BACE-1 mediated generation of C99 from APPswe and APP were unaffected. These data point to a relatively specific inhibition of γ- but not β-cleavage of APP and APPswe by X11α. BACE1 expression paradoxically diminished Aβ levels in conditioned media presumably due to additional cleavage sites within the Aβ sequence (Liu *et al*. 2002; Fluhrer *et al*. 2003). We also noted differences in BACE1 cleavage of wild type APP versus APPswe. C89 in cell lysates was apparent with APP but not APPswe coexpression suggesting that the Swedish mutation strongly promotes β-cleavage at the beginning of the Aβ sequence at the expense of β′-cleavage at Glu11.

To probe the specificity of X11α effects, we examined another substrate of γ-cleavage (or RIP, regulated intramembranous proteolysis), namely NotchΔE, and found that X11α coexpression, and interaction, did not inhibit γ-cleavage of

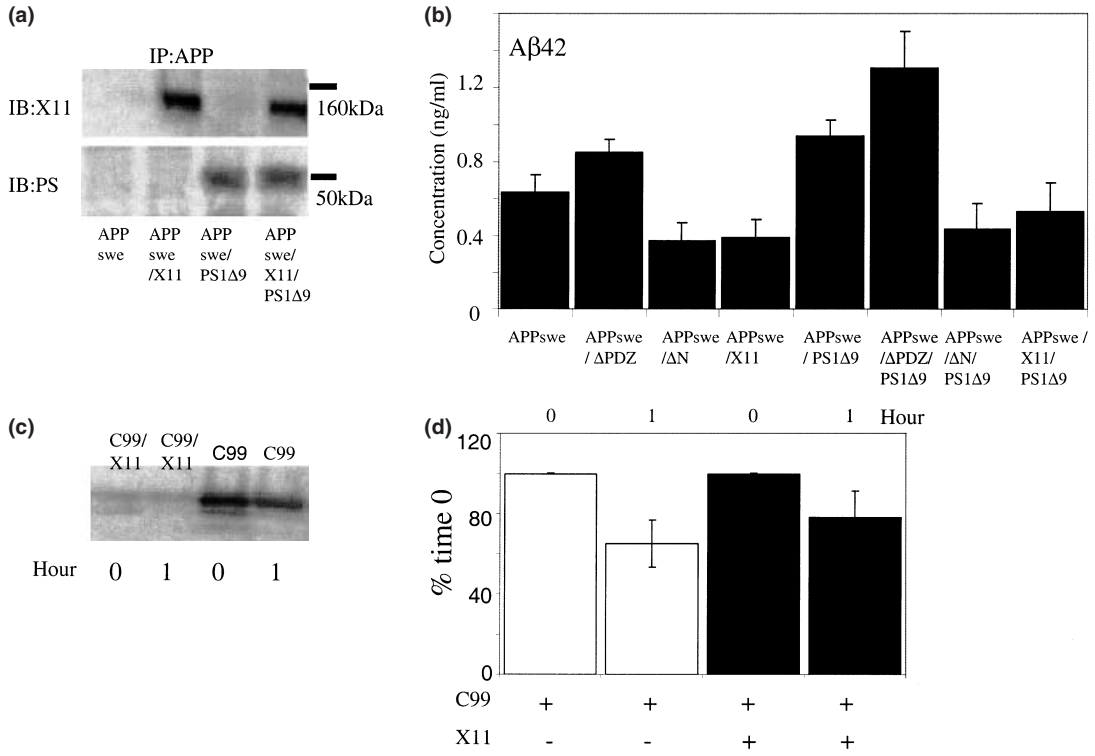

Fig. 6 X11α impairment of γ-cleavage may require altered APP trafficking. (a) Immunoprecipitation of APPswe from cell lysates followed by separation of proteins by SDS–PAGE and detection by immunoblot revealed interaction of APPswe with both X11α (upper panel) and PS-1Δ9 (lower panel) to form a heterotrimeric complex. (b) Aβ42 measured by ELISA in conditioned media from HEK 293 cells transfected as indicated. Data represent mean ± SEM, $n = 6$. (c) Representative immunoblot of C-terminal fragments of APP (C99 and C83) in microsomal membrane preparations from cells transfected as indicated. C99 was expressed with and without X11α and C-terminal fragments were detected in microsomal membranes at baseline (0) and after 1 h of incubation at 37°C. (d) Semiquantitative phosphorimager analysis of multiple immunoblots revealed no inhibitory effect of X11α on C99 catabolism. Data represent mean ± SEM, $n = 6$.

NotchΔE to NICD (Fig. 5). Collectively these data indicate for the first time that X11α may selectively inhibit γ-cleavage of APP and APPswe, spare their β-cleavage, and spare γ-cleavage of other substrates of RIP such as Notch1. In contrast, γ-secretase inhibitors that target enzyme (the presenilin/γ-secretase complex) may be non-specific or less specific to APP. These data are also consistent with the notion that the X11α PDZ–PS-1 interaction (Lau *et al*. 2000) may be irrelevant to γ-cleavage activity. Instead, data obtained with a cell-free system suggest that X11α impairs trafficking of APP, APPswe, or C99 to subcellular sites harboring active γ-secretase complexes.

Although the PTB domain usually mimicked results obtained with intact X11α, these data revealed differences that invoke potential modulatory effects of other X11α protein interaction domains. X11α, but not the PTB domain, inhibited Aβ secretion when coexpressed with C99 (Fig. 4), suggesting that X11α may be a more potent inhibitor of γ-cleavage due to its additional PDZ domains. The most immediate or best substrate for γ-cleavage, C99, required the most potent inhibitors, X11α with PDZ domains, to diminish Aβ secretion. In contrast, the PTB domain, but not X11α, impaired BACE-1-mediated C99 generation from APP but not APPswe (Fig. 3) perhaps due to a pro-catabolic effect of XIIαN-terminal domains. APPswe is a superior substrate for BACE1 compared to wild type APP. This may explain why BACE1-mediated cleavage of APP, but not APPswe, was inhibited by the PTB domain. These data are summarized in Table 1. These results are consistent with a model in which C terminal domains of X11α are inhibitory to APP catabolism, but stimulatory effects of N-terminal domains become apparent only in the absence of dominant PDZ domains (Mueller *et al*. 2000).

X11α may regulate the generation of γCTF (the γ-cleaved C-terminal fragment, or AICD (APP intracellular domain)). Analogous to Notch and NICD generation, the complex γCTF–Fe65, with or without Tip60, may translocate to the nucleus to regulate gene transcription (Kimberly *et al*. 2001). Immunolocalization of X11α demonstrates a perinuclear and vesicular pattern consistent with ER/Golgi localization but no nuclear staining (Rongo *et al*. 1998; Biederer *et al*. 2002). Competitive interactions between the X11, Fe65, and other adaptor protein families may regulate γCTF nuclear translocation and by extension, gene transcription. Interest-

Table 1 Summary of the inhibitory effects of X11α and its deletion constructs on α-, β, and γ-cleavage of APPswe, APP and C99. A schematic diagram of X11α and its deletion constructs is shown in Fig. 1. Inhibitory effects may be negated by the use of a weaker inhibitor or a superior substrate. For example, X11α inhibits γ-cleavage of APPswe, APP, and C99, but the presumably weaker inhibitor PTB inhibits γ-cleavage of APPswe, APP but not C99. In contrast, the PTB domain inhibits β-cleavage of APP but not the superior BACE substrate APPswe, while X11α has no effect on β-cleavage of either

	α	β	γ
APPswe			
X11 α	↓	No	↓
X11 α PTB	↓	No	↓
X11 α ΔPDZ	–	No	No
X11 α ΔN	–	No	↓
APP			
X11 α	↓	No	↓
X11 α PTB	↓	↓	↓
X11 α ΔPDZ	–	–	–
X11 α ΔN	–	–	–
C99			
X11 α	–	–	↓
X11 α PTB	–	–	No
X11 α ΔPDZ	–	–	No
X11 α ΔN	–	–	↓

No, no effect; –, unknown.

ingly, Fe65L1 promotes Aβ40 secretion and AICD generation by facilitating access of APP C-terminal fragments to γ-secretase cleavage, and not by a direct effect of Fe65L1on γ-secretase activity, as NICD generation is unaffected (Chang *et al*. 2003).

Potential drugs for the treatment or prevention of AD based on the amyloid hypothesis include inhibitors of β- or γ-secretases. However, neither protease is specific to APP; thus, inhibition of these proteases may engender intolerable side-effects and toxicity. For example, the γ-secretase complex cleaves multiple substrates including APP and Notch1. Similar to PS-1 knockout (–/–) mice, Notch1 knockout mice are lethal *in utero* (Swiatek *et al*. 1994; Conlon *et al*. 1995) and Notch1 processing also plays important roles in the adult. To date, small-molecule inhibitors of β-secretase are unknown, perhaps due to its large active site (Gruninger-Leitch *et al*. 2002). In order to penetrate the blood–brain barrier, a small molecule may be prerequisite to serve as an effective drug for AD. However, a more specific approach to secretase inhibition may be required to realize safe and effective drugs for AD.

Modulation of APP catabolism by adaptor protein interactions provides novel drug discovery targets that may specifically inhibit Aβ generation from APP. This will require identification of small compounds that promote or inhibit APP–adaptor protein interactions. There are precedents for drugs (in use and in clinical trials) and toxins that promote or impair protein interactions to achieve their biologic effects, and this is a rapidly developing strategy for drug discovery. Alternatively, a gene therapy approach may become feasible despite enormous technical barriers including CNS delivery, duration of expression, and adverse effects. An X11α minigene (the PTB domain) may be preferable to intact X11α for gene therapy because: (i) while intact X11α impaired α- and γ-cleavage of APP, the PTB domain inhibited β- and γ-cleavage; (ii) N-terminal X11α domains may promote APP catabolism; and (iii) PDZ domains are relatively promiscuous in their interactions and may introduce toxicity.

We recognize the limitations of these data obtained with transient transfection in non-neuronal cells. Potential effects of X11α on APP in neurons in brain (*in vivo*) remain speculative, despite their colocalization in neurons in culture and brain sections, and their coimmunoprecipitation from mammalian brain. A recent human genome-wide study revealed significant linkage of sporadic AD to polymorphisms on chromosome 9 (perhaps including X11α) (Menon *et al*. 2003). These data suggest that the X11α–APP interaction may have physiologic and pathologic (*in vivo*) relevance, and that X11α may be a normal mediator of APP function, trafficking, targeting, tethering, processing, and risk of sporadic AD. Ongoing studies with hX11α transgenic and X11α knockout mice will shed light on the *in vivo* significance of the APP–X11α interaction in CNS neurons. For example, hX11α and hAPPswe double transgenic mice exhibit lower Aβ levels in brain homogenates and no amyloid pathology in brain sections compared to age-matched hAPPswe transgenic control mice (Lee *et al*. 2003).

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References

Ando K., Iijima K., Elliott J. I., Kirino Y. and Suzuki T. (2001) Phosphorylation-dependent regulation of the interaction of amyloid precursor protein with Fe65 affects the production of beta-amyloid. *J. Biol. Chem.* **276**, 40353–40361.

Biederer T., Cao X. W., Sudhof T. C. and Liu X. R. (2002) Regulation of APP-dependent transcription complexes by Mint/X11s: Differential functions of mint isoforms. *J. Neurosci.* **22**, 7340–7351.

Borg J. P., Ooi J., Levy E. and Margolis B. (1996) The phosphotyrosine interaction domains of X11 and FE65 bind to distinct sites on the YENPTY motif of amyloid precursor protein. *Mol. Cell Biol.* **16**, 6229–6241.

Borg J. P., Yang Y., De Taddeo-Borg M., Margolis B. and Turner R. S. (1998a) The X11 alpha protein slows cellular amyloid precursor protein processing and reduces A beta 40 and A beta 42 secretion. *J. Biol. Chem.* **273**, 14761–14766.

Borg J. P., Straight S. W., Kaech S. M., de Taddeo-Borg M., Kroon D. E., Karnak D., Turner R. S., Kim S. K. and Margolis B. (1998b) Identification of an evolutionarily conserved heterotrimeric protein complex involved in protein targeting. *J. Biol. Chem.* **273**, 31633–31636.

Chang Y., Tesco G., Jeong W. J., Lindsley L., Eckman E. A., Eckman C. G., Tanzi R. E. and Guenette S. Y. (2003) Generation of beta-amyloid peptide and the amyloid precursor protein C-terminal fragment gamma are potentiated by Fe65L1. *J. Biol. Chem. Papers* in press.

Chen F., Gu Y. J., Hasegawa H., Ruan X., Arawaka S., Fraser P., Westaway D., Mount H. and St George-Hyslop P. (2002) Presenilin 1 mutations activate gamma42-secretase but reciprocally inhibit epsilon-secretase cleavage of amyloid precursor protein (APP) and S3-cleavage of Notch. *J. Biol. Chem.* **277**, 36521–36526.

Chow N., Korenberg J. R., Chen X.-N. and Neve R. L. (1996) APP-BP1, a novel protein that binds to the carboxy-terminal region of the amyloid precursor protein. *J. Biol. Chem* **271**, 11339–11346.

Citron M., Oltersdorf T., Haass C., McConlogue L., Hung A. Y., Seubert P., Vigopelfrey C., Lieberburg I. and Selkoe D. J. (1992) Mutation of the beta-Amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. *Nature* **360**, 672–674.

Conlon R. A., Reaume A. G. and Rossant J. (1995) Notch-1 is required for the coordinate segmentation of somites. *Development* **121**, 1533–1545.

De Strooper B., Annaert W., Cupers P., Saftig P., Craessaerts K., Mumm J. S., Schroeter E. H., Schrijvers V., Wolfe M. S., Ray W. J. *et al.* (1999) A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. *Nature* **398**, 518–522.

Felsenstein K. M., Hunihan L. W. and Roberts S. B. (1994) Altered cleavage and secretion of a recombinant beta-APP bearing the Swedish familial Alzheimers-disease mutation. *Nature Genet.* **6**, 251–256.

Fluhrer R., Multhaup G., Schlicksupp A., Okochi M., Takeda M., Lammich S., Willem M., Westmeyer G., Bode W., Walter J. *et al.* (2003) Identification of a beta-secretase activity, which truncates amyloid beta-peptide after its presenilin-dependent generation. *J. Biol. Chem.* **278**, 5531–5538.

Forman M. S., Cook D. G., Leight S., Doms R. W. and Lee V. M. Y. (1998) Differential effects of the Swedish mutant amyloid precursor protein on beta-amyloid accumulation and secretion in neurons and non-neuronal cells. *J. Neuropathol. Exp. Neurol.* **57**, 175.

Gruninger-Leitch F., Schlatter D., Kung E., Nelbock P. and Dobeli H. (2002) Substrate and inhibitor profile of BACE (beta-secretase) and comparison with other mammalian aspartic proteases. *J. Biol. Chem.* **277**, 4687–4693.

Guenette S. Y., Chen J., Ferland A., Haass C., Capell A. and Tanzi R. E. (1999) hFE65L influences amyloid precursor protein maturation and secretion. *J. Neurochem.* **73**, 985–993.

Ho C. S., Marinescu V., Steinhilb M. L., Gaut J. R., Turner R. S. and Stuenkel E. L. (2002) Synergistic effects of Munc18a and X11 proteins on amyloid precursor protein metabolism. *J. Biol. Chem.* **277**, 27021–27028.

Howell B. W., Lanier L. M., Frank R., Gertler F. B. and Cooper J. A. (1999) The disabled 1 phosphotyrosine-binding domain binds to the internalization signals of transmembrane glycoproteins and to phospholipids. *Mol. Cell Biol.* **19**, 5179–5188.

Huse J. T., Liu K., Pijak D. S., Carlin D. and Lee V. M. Y. (2002) Beta secretase processing in the trans golgi network preferentially generates truncated amyloid species that accumulate in Alzheimer's disease brain. *J. Biol. Chem.* **277**, 16278–16284.

Kamal A., Stokin G. B., Yang Z. H., Xia C. H. and Goldstein L. S. B. (2000) Axonal transport of amyloid precursor protein is mediated by direct binding to the kinesin light chain subunit of kinesin-I. *Neuron* **28**, 449–459.

Kimberly W. T., Zheng J. B., Guenette S. Y. and Selkoe D. J. (2001) The intracellular domain of the beta-amyloid precursor protein is stabilized by Fe65 and translocates to the nucleus in a Notch-like manner. *J. Biol. Chem.* **276**, 40288–40292.

King G. D., Perez R. G., Steinhilb M. L., Gaut J. R. and Turner R. S. (2003) X11alpha modulates secretory and endocytic trafficking and metabolism of amyloid precursor protein: mutational analysis of the YENPTY sequence. *Neuroscience* **120**, 143–154.

Lai A., Sisodia S. S. and Trowbridge I. S. (1995) Characterization of sorting signals in the beta-amyloid precursor protein cytoplasmic domain. *J. Biol. Chem.* **270**, 3565–3573.

Lau K. F., McLoughlin D. M., Standen C. and Miller C. C. J. (2000) X11alpha and X11beta interact with presenilin-1 via their PDZ domains. *Mol. Cell Neurosci.* **16**, 557–565.

Lee S.-F., Shah S., Li H., Yu C., Han W. and Yu G. (2002) Mammalian APH-1 interacts with presenilin and nicastrin and is required for intramambrane proteolysis of amyloid-beta precursor protein and Notch. *J. Biol. Chem.* **277**, 45013–45019.

Lee J.-H., Lau K. F., Perkinton M. S., Standen C. L., Shemilt S. J. A., Mercken L., Cooper J. D., McLoughlin D. M. and Miller C. J. (2003) The neuronal adaptor protein X11alpha reduces Abeta levels in the brains of Alzheimer's APPswe Tg2576 transgenic mice. *J. Biol. Chem.* **278**, 47025–47029.

Li Y. M., Lai M. T., Xu M., Huang Q., DiMuzio-Mower J., Sardana M. K., Shi X. P., Yin K. C., Shafer J. A. and Gardell S. J. (2000a) Presenilin 1 is linked with gamma-secretase activity in the detergent solubilized state. *Proc. Natl Acad. Sci. USA* **97**, 6138–6143.

Li Y. M., Xu M., Lai M. T., Huang Q., Castro J. L., DiMuzio-Mower J., Harrison T., Lellis C., Nadin J. L., Neduvelil J. G. *et al.* (2000b) Photoactivated gamma-secretase inhibitors directed to the active site covalently label presenilin 1. *Nature* **405**, 689–694.

Lichtenthaler S. F., Multhaup G., Masters C. L. and Beyreuther K. (1999) A novel substrate for analyzing Alzheimer's disease gamma-secretase. *FEBS Lett.* **453**, 288–292.

Liu K., Doms R. W. and Lee V. M. Y. (2002) Glu11 site cleavage and N terminally truncated Abeta production upon BACE overexpression. *Biochemistry* **41**, 3128–3136.

Matsuda S., Yasukawa T., Homma Y., Ito Y., Niikura T., Hiraki T., Hirai S., Ohno S., Kita Y., Kawasumi M. *et al.* (2001) c-Jun N-terminal kinase (JNK)-interacting protein-1b/islet-brain-1 scaffolds Alzheimer's amyloid precursor protein with JNK. *J. Neurosci.* **21**, 6597–6607.

Mehta N. D., Refolo L. M., Eckman C., Sanders S., Yager D., Perez-Tur J., Younkin S., Duff K., Hardy J. and Hutton M. (1998) Increased A beta 42 (43) from cell lines expressing presenilin 1 mutations. *Ann. Neurol.* **43**, 256–258.

Menon R., Bertram L., Mullin K., Parkinson M., Bradley M. L., Becker K. D., Velicelebi G., Moscarillo T., Saunders A. J., Blacker D. *et al.* (2003) Search for novel Alzheimer's disease genes on chromosome 9. Soc. Neurosci. Abstract 202.7.

Mueller H. T., Borg J. P., Margolis B. and Turner R. S. (2000) Modulation of amyloid precursor protein metabolism by X11 alpha/Mint-1: a deletion analysis of protein–protein interaction domains. *J. Biol. Chem.* **275**, 39302–39306.

Nakajima M., Shimizu T. and Shirasawa T. (2000) Notch-1 activation by familial Alzheimer's disease (FAD)-linked mutant forms of presenilin-1. *J. Neurosci. Res.* **62**, 311–317.

Nishimoto I., Okamoto T., Matsuura Y., Takahashi S., Murayama Y. and Ogata E. (1993) Alzheimer amyloid protein-precursor complexes with brain GTP- binding protein-G(o). *Nature* **362**, 75–79.

Perez R. G., Soriano S., Hayes J. D., Ostaszewski B., Xia W. M., Selkoe D. J., Chen X. H., Stokin G. B. and Koo E. H. (1999) Mutagenesis identifies new signals for beta-amyloid precursor protein endocytosis, turnover, and the generation of secreted fragments, including A beta 42. *J. Biol. Chem.* **274**, 18851–18856.

Pheil C. J., Wilson C. A., Lee V. M. Y. and Klein P. S. (2003) GSK-3alpha regulates production of Alzheimer's disease amyloid-beta peptides. *Nature* **423**, 435–439.

Rongo C., Whitfield C. W., Rodal A., Kim S. K. and Kaplan J. M. (1998) LIN-10 is a shared component of the polarized protein localization pathways in neurons and epithelia. *Cell* **94**, 751–759.

Russo C., Dolcini V., Salis S., Venezia V., Zambrano N., Russo T. and Schettini G. (2002) Signal transduction through tyrosine-phosphorylated C-terminal fragments of amyloid precursor protein via an enhanced interaction with Shc/Grb2 adaptor proteins in reactive astrocytes of Alzheimer's Disease brain. *J. Biol. Chem.* **277**, 35282–35288.

Sabo S. L., Lanier L. M., Ikin A. F., Khorkova O., Sahasrabudhe S., Greengard P. and Buxbaum J. D. (1999) Regulation of beta-amyloid secretion by FE65, an amyloid protein precursor-binding protein. *J. Biol. Chem.* **274**, 7952–7957.

Sastre M., Turner R. S. and Levy E. (1998) X11 interaction with beta-amyloid precursor protein modulates its cellular stabilization and reduces amyloid beta-protein secretion. *J. Biol. Chem.* **273**, 22351–22357.

Scheinfeld M. H., Roncarati R., Vito P., Lopez P. A., Abdallah M. and D'Adamio L. (2002) Jun NH2-terminal kinase (JNK) interacting protein 1 (JIP1) binds the cytoplasmic domain of the Alzheimer's beta-amyloid precursor protein (APP). *J. Biol. Chem.* **277**, 3767–3775.

Schroeter E. H., Kisslinger J. A. and Kopan R. (1998) Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* **393**, 382–386.

Selkoe D. J. (2001) Alzheimer's disease: genes, proteins, and therapy. *Physiol. Rev.* **81**, 741–766.

Song W. H., Nadeau P., Yuan M. L., Yang X. D., Shen J. and Yankner B. A. (1999) Proteolytic release and nuclear translocation of Notch-1 are induced by presenilin-1 and impaired by pathogenic presenilin-1 mutations. *Proc. Natl Acad. Sci. USA* **96**, 6959–6963.

Steiner H., Romig H., Grim M. G., Philipp U., Pesold B., Citron M., Baumeister R. and Haass C. (1999) The biological and pathological function of the presenilin-1 Delta exon 9 mutation is independent of its defect to undergo proteolytic processing. *J. Biol. Chem.* **274**, 7615–7618.

Steiner H., Winkler E., Edbauer D., Prokop S., Basset G., Yamasaki A., Kostka M. and Haass C. (2002) PEN-2 is an integral component of the gamma-secretase complex required for the coordinated expression of presenilin and nicastrin. *J. Biol. Chem.* **277**, 39062–39065.

Steinhilb M. L., Turner R. S. and Gaut J. R. (2001) The protease inhibitor, MG132, blocks maturation of the amyloid precursor protein Swedish mutant preventing cleavage by beta-secretase. *J. Biol. Chem.* **276**, 4476–4484.

Steinhilb M. L., Turner R. S. and Gaut J. R. (2002) ELISA analysis of beta-secretase cleavage of the Swedish amyloid precursor protein in the secretory and endocytic pathways. *J. Neurochem.* **80**, 1019–1028.

Suzuki N., Cheung T. T., Cai X. D., Odaka A., Otvos L., Eckman C., Golde T. E. and Younkin S. G. (1994) An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta-APP 717) mutants. *Science* **264**, 1336–1340.

Swiatek P. J., Lindsell C. E., Amo F. F., Weinmaster G. and Gridley T. (1994) Notch 1 is essential for postimplantation development in mice. *Genes Dev.* **8**, 707–719.

Tarr P. E., Roncarati R., Pelicci G., Pelicci P. G. and D'Adamio L. (2002) Tyrosine phosphorylation of the beta-amyloid precursor protein cytoplasmic tail promotes interaction with Shc. *J. Biol. Chem.* **277**, 16798–16804.

Taru H., Kirino Y. and Suzuki T. (2002a) Differential roles of JIP scaffold proteins in the modulation of amyloid precursor protein metabolism. *J. Biol. Chem.* **277**, 27567–27574.

Taru H., Iijima K., Hase M., Kirino Y., Yagi Y. and Suzuki T. (2002b) Interaction of Alzheimer's beta-amyloid precursor family proteins with scaffold proteins of the JNK signaling cascade. *J. Biol. Chem.* **277**, 20070–20078.

Turner R. S., Suzuki N., Chyung A. S. C., Younkin S. G. and Lee V. M.- Y. (1996) Amyloids beta(40) and beta(42) are generated intracellularly in cultured human neurons and their secretion increases with maturation. *J. Biol. Chem.* **271**, 8966–8970.

Vassar R., Bennett B. D., Babu-Khan S., Kahn S., Mendiaz E. A., Denis P., Teplow D. B., Ross S., Amarante P., Loeloff R. *et al.* (1999) beta-Secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* **286**, 735–741.

Verdile G., Martins R. N., Duthi M., Holmes E., St George-Hyslop P. and Fraser P. (2000) Inhibiting amyloid precursor protein C-terminal cleavage promotes an interaction with presenilin 1. *J. Biol. Chem.* **275**, 20794–20798.

Wolfe M. S., Xia W. M., Ostaszewski B. L., Diehl T. S., Kimberly W. T. and Selkoe D. J. (1999) Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. *Nature* **398**, 513–517.

Xia W. M., Zhang J., Perez R. G., Koo E. H. and Selkoe D. J. (1997) Interaction between amyloid precursor protein and presenilins in mammalian cells: Implications for the pathogenesis of Alzheimer disease. *Proc. Natl Acad. Sci. USA* **94**, 8208–8213.

Xia W. M., Ray W. J., Ostaszewski B. L., Rahmati T., Kimberly W. T., Wolfe M. S., Zhang J. M., Goate A. M. and Selkoe D. J. (2000) Presenilin complexes with the C-terminal fragments of amyloid precursor protein at the sites of amyloid beta-protein generation. *Proc. Natl Acad. Sci. USA* **97**, 9299–9304.

Yu G., Nishimura H., Arawaka S., Levitan D., Zhang L., Tandon A., Song Y. Q., Rogaeva E., Chen F., Kawarai T. *et al.* (2000) Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and betaAPP processing. *Nature* **407**, 48–54.

Zambrano N., Bruni P., Minopoli G., Mosca R., Molino D., Russo C., Schettini G., Sudol M. and Russo T. (2001) The beta-amyloid precursor protein APP is tyrosine-phosphorylated in cells expressing a constitutively active form of the Abl protoncogene. *J. Biol. Chem.* **276**, 19787–19792.

Zhang Z. T., Lee C. H., Mandiyan V., Borg J. P., Margolis B., Schlessinger J. and Kuriyan J. (1997) Sequence-specific recognition of the internalization motif of the Alzheimer's amyloid precursor protein by the X11 PTB domain. *EMBO J.* **16**, 6141–6150.

Zheng P. Z., Eastman J., Vande Pol S. and Pimplikar S. W. (1998) PAT1, a microtubule-interacting protein, recognizes the basolateral sorting signal of amyloid precursor protein. *Proc. Natl Acad. Sci. USA* **95**, 14745–14750.