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X11 Interaction with β-Amyloid Precursor Protein Modulates Its Cellular Stabilization and Reduces Amyloid β-Protein Secretion*

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The protein interaction domain of the neuronal protein X11 binds to the YENPTY motif within the cytoplasmic domain of β-amyloid precursor protein (βAPP). Amyloid-β protein (Aβ), the major constituent of the amyloid deposited in brain of Alzheimer's disease patients, is generated by proteolytic processing of βAPP, which occurs in part following βAPP internalization. Because the YENPTY motif has a role in the internalization of βAPP, the effect of X11 binding on βAPP processing was studied in mouse neuroblastoma N2a, human embryonic kidney 293, monkey kidney COS-1, and human glial U251 cell lines transfected with wild type or mutated βAPP cDNAs. Secretion of soluble βAPP via α-secretase activity increased significantly in cells transfected with βAPP variants containing mutations that impair interaction with X11 when compared with cells transfected with wild type cDNA. Cotransfection of βAPP and X11 caused retention of cellular βAPP, decreased secretion of sβAPPα, and decreased Aβ secretion. Thus, βAPP interaction with the protein interaction domain of X11 stabilizes cellular βAPP and thereby participates in the regulation of βAPP processing pathways.

Amyloid-β protein (Aβ),[1] deposited in the brain of patients with Alzheimer's disease (AD), Down's syndrome, and sporadic and hereditary cerebral amyloid angiopathy and in the brain of elderly individuals is a proteolytic peptide of a larger β-amyloid precursor protein (βAPP). Aβ has been reported to have heterogeneous carboxyl termini, and $Aβ_{1–40}$ and $Aβ_{1–42}$ appear to be the major species in the parenchymal deposition. Aβ is found in normal cerebrospinal fluid and in conditioned media from various tissue culture cell lines (1–3), suggesting that it is produced and secreted constitutively. Alternative processing pathways of βAPP have been described. Cleavage at position 597 of $βAPP_{695}$ by a β-secretase results in the generation of Aβ. Alternatively, processing by an α-secretase between positions 612 and 613 of $βAPP_{695}$ (positions 16 and 17 of Aβ) precludes the release of intact Aβ (4, 5). Both pathways result in the release of amino-terminal soluble βAPP, sβAPPβ and sβAPPα, into the extracellular compartment. The carboxyl-terminal processing products may undergo an additional cleavage by a protease displaying a γ-secretase activity leading to the formation of Aβ or P3.

α-Secretase processing of βAPP occurs in a late compartment of the constitutive secretory pathway (6, 7), probably in a late trans-Golgi compartment (8, 9). However, βAPP can elude the intracellular cleavage and reach the cell surface as a full-length mature product, and α-secretase activity may occur also at the plasma membrane in several cell systems (10, 11). Immunolabeling of cell surface βAPP in living cells demonstrated that cell surface βAPP is either rapidly released or internalized via clathrin-coated vesicles, such that the duration at the cell surface is very short (10, 12, 13). Whereas most Aβ is produced by a β-secretase at the cell surface (14, 15), via the endosomal/lysosomal pathway, a small fraction of the normally produced $Aβ_{1–42}$ is generated in the endoplasmic reticulum/intermediate compartment, and the trans-Golgi network is a site for $Aβ_{1–40}$ generation (14, 16, 17). γ-Secretase activity occurs during recycling of endosomes to the cell surface (1, 11).

The βAPP cytoplasmic domain has a sequence motif, YENPTY, that is involved in protein internalization (11, 18, 19). Deletion of either the cytoplasmic domain or the YENPTY sequence resulted in reduced βAPP internalization, increased secretion of sβAPPα and P3, and significantly diminished Aβ release (6, 11, 20). No clear effect was observed on the level of intracellular Aβ (21).

βAPP is a cell surface protein with a large extracellular amino-terminal domain, a single transmembrane segment, and a short cytoplasmic tail (22). Its location and structural features are characteristic of a receptor for signal transduction. Screening for potential proteins capable of interacting with its cytoplasmic domain led to the identification of proteins containing phosphotyrosine interaction/phosphotyrosine binding (PI/PTB) domains: Fe65, X11, and their homologues (23–28). The PI/PTB domain was first identified as the component of the adaptor protein Shc (Src homology 2/collagen homology) that binds to activated and tyrosine-phosphorylated receptors (29, 30). This domain was further found in several unrelated regulatory proteins (31), suggesting a general role for this domain in protein-protein interactions and signal transduction. Fe65 was originally proposed as a transcriptional activator (32). However, the presence of a WW domain (a variant of Src homology 3 domains) (33), as well as two PI/PTBs (31), suggests that it is likely to be involved in signal transduction. The expression of Fe65 appears to be enriched in brain in human, rat, and mouse (25, 32, 34). The *X11* gene was originally isolated as a candidate gene for Friedreich ataxia, an autosomal recessive degenerative disorder that affects the cerebellum, spinal cord, and peripheral nerves (35). The X11 protein is a

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[1] The abbreviations used are: Aβ, amyloid-β protein; AD, Alzheimer's disease; βAPP, β-amyloid precursor protein; sβAPPα, soluble βAPP cleaved by α-secretase; sβAPPβ, soluble βAPP cleaved by β-secretase; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; PDZ, postsynaptic density protein, disc-large, zo-1; PI/PTB, phosphotyrosine interaction/phosphotyrosine binding; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

neuron-specific protein of unknown function that contains two postsynaptic density protein, disc-large, zo-1 (PDZ), domains at its carboxyl terminus in addition to a PI/PTB domain (31).

Biochemical characterization of the interaction of X11 and Fe65 with βAPP indicated that the YENPTY motif located at the cytoplasmic carboxyl terminus of βAPP is essential for its association with the PI/PTB domains (23, 24). The interaction of βAPP and the PI/PTB domains of X11 and Fe65 is phosphorylation-independent (23, 24, 27, 28), suggesting that, in contrast to the SH2 domains, the PI/PTB domains are primarily peptide binding domains that have in some cases acquired specificity for phosphorylated tyrosines (36). The binding site of the Fe65 PI/PTB domain appears to be different from that of X11 as mutations within the YENPTY motif differentially affect the binding of X11 and Fe65 (24). The crystal structures of the X11 PI/PTB domain bound to the βAPP peptide (QNGEYENPTYKFFEQ) revealed that the sequence-specific recognition extends to peptide residues that are carboxyl-terminal to the YENPTY motif (36).

Association of PI/PTB domain-containing proteins with the coated pit-mediated internalization signal of βAPP may affect the patterns of βAPP trafficking, generation of Aβ as well as of soluble βAPP, and normal physiologic function. The effect of X11 binding on βAPP processing was studied in different cell lines transiently or permanently transfected with the wild type or mutated βAPP cDNAs. We demonstrate that mutations in βAPP that impair interactions with X11 result in increased proteolysis by an α-secretase. Cotransfection of βAPP with X11 produces the opposite effect. Furthermore, overexpression of both X11 and βAPP results in accumulation of cell-associated βAPP and decreased secretion of Aβ.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse neuroblastoma N2a, human embryonic kidney 293 and African green monkey kidney COS-1 cells were cultured in Dulbecco's modified Eagle's medium, and human glial U251 cells were cultured in RPMI medium at 37 °C in a 5% CO_2 atmosphere. The media were supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml streptomycin sulfate.

Transfection of Cell Lines—The wild type or mutated βAPP_{695} cDNAs and the X11 cDNA were cloned into pRK5 vector as described previously (24). The cDNAs were transiently transfected into N2a, 293, and COS-1 culture cell lines using calcium phosphate (37). U251 cells were stably transfected using LipofectAMINE transfection reagent (Life Technologies, Inc.) and selected with Geneticin (G418, Life Technologies, Inc.). N2a cells stably transfected with wild type βAPP_{695} cDNA (kindly provided by Dr. S. S. Sisodia) and 293 cells stably transfected with human wild type cystatin C gene (38) were transiently transfected with X11 or pRK5 vector cDNAs. Overexpression and secretion of βAPP were confirmed by immunoblot analysis of cell lysates and medium proteins with anti-βAPP_{66-81} antibody (22C11) (Boehringer Mannheim) and with anti-$A\beta_{1-17}$ antibody (6E10) (Senetek). Overexpression of Myc-tagged X11 was confirmed by immunoblot analysis of cell lysates proteins with anti-myc antibody (9E10) (Santa Cruz Biotechnology).

Pulse-Chase Labeling of βAPP—Transiently or stably transfected culture cells were labeled 24 h after transfection. Cells were incubated with 0.3 mCi/ml [^{35}S]methionine/cysteine EXPRE^{35}S^{35}S (DuPont) in methionine/cysteine-free medium with 5% dialyzed serum for a pulse of 20 min at 37 °C. Following a wash with phosphate-buffered saline, pH 7.3, the cells were chased in complete medium with 150 μg/ml methionine at 37 °C for different periods. The media were collected and spun at 4500 × *g* for 10 min at 4 °C. The cells were harvested in phosphate-buffered saline, pH 7.3, and lysed in lysis buffer (1% Triton, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.2). Equal amounts of total proteins from cell lysates or equal volumes of media based upon the relative concentration of total proteins in cell lysates were immunoprecipitated with anti-$A\beta_{1-17}$ antibody (6E10) or anti-βAPP_{66-81} antibody (22C11) overnight at 4 °C and with γ-Bind Plus Sepharose beads (Amersham Pharmacia Biotech) for 2 h at 4 °C. In control experiments, cell lysates and medium proteins were immunoprecipitated with anti-cystatin C antibody (Axell) and protein A-Sepharose (Amersham Pharmacia Biotech) for 4 h at 4 °C. The immunoprecipitated proteins were washed with lysis buffer and phosphate-buffered saline, pH 7.3, boiled in sample buffer (1% SDS, 3% glycerol, 1.5% β-mercaptoethanol, and 20 mM Tris-HCl, pH 6.8), and separated by 8% SDS-PAGE. The gels were enhanced with Amplify (Amersham Pharmacia Biotech) and exposed to x-ray films. The protein bands were scanned using Adobe PhotoShop and quantitated using the NIH Image program. Relative intensity of the bands was calculated as a percentage of the intensity of the protein band in cell lysates at time 0 of the chase. Results are expressed as means ± S.E. Data were compared between experimental groups using one-way analysis of variance combined with Fisher's test. In all cases, p values <0.05 were considered to be statistically significant. Direct quantitation of incorporated radioactivity was performed in vials containing liquid scintillation mixture (Beckman) by liquid scintillation spectrometry (Beckman model LS6000IC). Significant positive correlation between quantification done by densitometry and direct measurements of incorporated radioactivity were obtained ($r = 0.952 \pm 0.008$; $p < 0.0002$). The half-life of intracellular βAPP was calculated from pulse-chase experiments performed for chase periods of up to 8 h.

Metabolic Labeling of Aβ—N2a cells, stably transfected with βAPP cDNA, were labeled 24 h after transient transfection with vector or X11 cDNAs. Cells were incubated with 0.5 mCi/ml [^{35}S]methionine/cysteine EXPRE^{35}S^{35}S (DuPont) in methionine/cysteine-free medium with 5% dialyzed serum for 4 h at 37 °C. The media were collected and spun at 4500 × *g* for 10 min at 4 °C. Equal volumes of media, based upon the relative concentration of total proteins in cell lysates, were immunoprecipitated with anti-$A\beta_{1-17}$ antibody (6E10) and anti-$A\beta_{17-24}$ antibody (4G8) (Senetek) overnight at 4 °C and with protein A-Sepharose beads (Amersham Pharmacia Biotech) for 5 h at 4 °C. The immunoprecipitated proteins were washed with lysis buffer and phosphate-buffered saline, pH 7.3, boiled in sample buffer, and separated by 16.5% Tris-Tricine PAGE. The gels were enhanced with Amplify (Amersham Pharmacia Biotech) and exposed to x-ray films.

Sandwich ELISA—N2a cells stably transfected with βAPP cDNA were transiently transfected with vector or X11 cDNA. 24 h after transfection, the cells were transferred to serum-reduced medium consisting of 50% Dulbecco's modified Eagle's medium, 50% Opti-MEM, and 0.2% fetal bovine serum and incubated at 37 °C and 5% CO_2 for 24 h. The media were collected, spun at 4500 × *g* for 10 min at 4 °C and supplemented with protease inhibitors (82 μM antipain, 0.1 mM bestatin, 2 mM EDTA-Na_2, 10 μM leupeptin, 1 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM 1-chloro-3-tosylamido-7-amino-2-heptanone, 0.2 mM L-1-tosylamido-2-phenylethyl chloromethyl ketone, 0.01% NaN_3). Immunodetection of Aβ in the media was performed as described previously (17, 39). In brief, Aβ in samples were captured with BAN-50, a monoclonal antibody specific for $A\beta_{1-10}$. Peptides terminating at amino acid 40 were specifically detected by horseradish peroxidase-conjugated BA-27 monoclonal antibody, and peptides terminating at amino acid 42 were detected by horseradish peroxidase-conjugated BC-05 monoclonal antibody. This sandwich ELISA has a detection limit of <1 fmol/well.

RESULTS

Mutations That Inhibit Binding of X11 to the YENPTY Motif of βAPP Affect the Secretion of Soluble βAPP—We have previously analyzed the binding sites on βAPP for the X11 PI/PTB domain, using site-directed mutagenesis of the cytoplasmic domain of βAPP (24). The different mutations were placed into full-length βAPP_{695} (see Fig. 1 for the sequence of the carboxyl-terminal domain of βAPP). We found that deletion of the last 18 amino acids severely inhibited the ability of the X11 PI/PTB domain to bind βAPP. Similarly, two mutations within the YENPTY motif, Y682G and N684A, impaired the binding of the protein interaction domain to βAPP (24).

Pulse-chase experiments were performed to study the effect of these mutations on βAPP processing. Mouse neuroblastoma N2a, human embryonic kidney (293), and monkey kidney (COS-1) cells that were transiently transfected and human glial (U251) cells that were stably transfected with wild type or mutated βAPP cDNAs were labeled with [^{35}S]methionine/cysteine for 20 min and chased for different time periods. Results of the SDS-PAGE temporal profile of βAPP expression and secretion are shown in Fig. 2. Immunoprecipitation of cell lysates proteins revealed that mutations within the X11 inter-

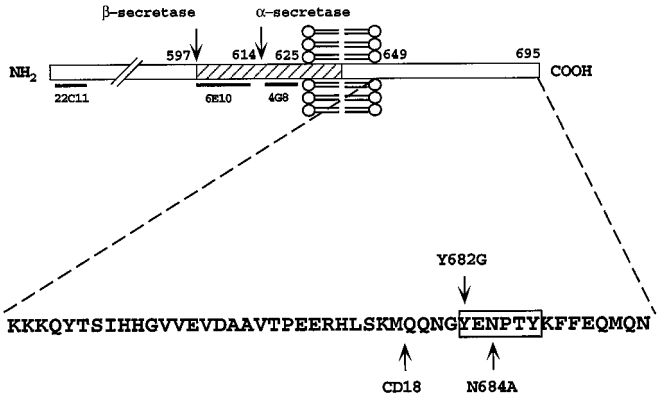

FIG. 1. **Residues within the YENPTY motif of βAPP that are involved in X11 binding.** Locations of Aβ (▨), the transmembrane domain (*circles with double lines*) within βAPP, and residues within the carboxyl-terminal cytoplasmic domain subjected to site-directed mutagenesis with the corresponding mutations (Y682G and N684A) are indicated. The CD18 mutant corresponds to a truncated βAPP protein with deletion of the 18 carboxyl-terminal amino acids. *Horizontal bars* indicate the approximate epitopes of antibodies 22C11, 6E10, and 4G8.

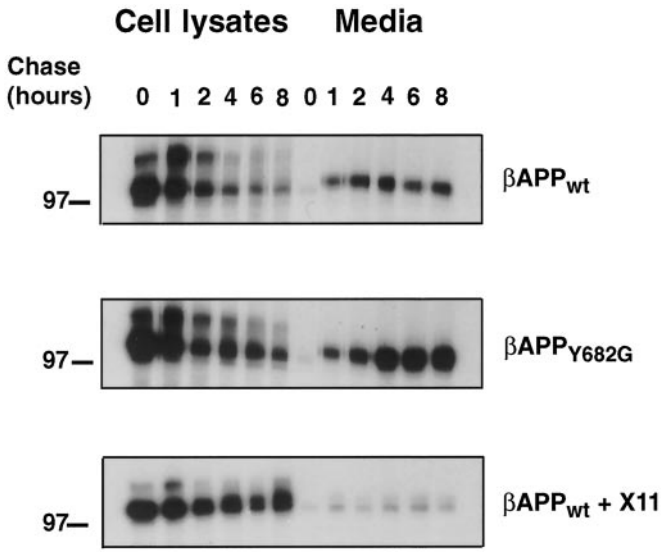

FIG. 2. **Temporal profile of βAPP expression and secretion by cells transiently transfected with wild type or Y682G-mutated βAPP or cotransfected with wild type βAPP and X11 cDNAs.** Transiently transfected 293 cells were labeled with [^{35}S]methionine/cysteine for 20 min and chased for different time periods. Cell lysates and medium proteins were immunoprecipitated with anti $A\beta_{1-17}$ antibody (6E10), separated by 8% SDS-PAGE, and exposed to x-ray film.

active domain of βAPP do not significantly affect the decrease of intracellular βAPP (Fig. 3, *A, C, E,* and *G*). Immunoprecipitation of medium proteins with anti-$A\beta_{1-17}$ antibody (6E10) revealed that the secretion of soluble βAPP via the α-secretase pathway (sβAPPα) was increased in all of the cell lines transfected with βAPP lacking the 18 carboxyl-terminal amino acids, which include the YENPTY motif, when compared with cells transfected with wild type cDNA (data not shown). These results are compatible with previous reports of the effect of deletion of either the cytoplasmic domain or the YENPTY sequence of βAPP on its processing (6, 11, 20). We demonstrate that mutation of the amino-terminal tyrosine (Y682G) or asparagine (N684A) within the YENPTY motif also caused increased secretion of sβAPPα (Fig. 3, *B, D, F,* and *H*).

Effect of Overexpression of X11 Together with βAPP on Cell-associated βAPP and on Soluble βAPP Secretion—Conversely, pulse-chase experiments were performed with N2a, 293, and COS-1 cells transiently cotransfected with wild type βAPP and either vector or X11 cDNAs. Immunoprecipitation of cell lysate proteins with anti-$A\beta_{1-17}$ antibody (6E10) revealed that cotransfection of βAPP and X11 delayed intracellular βAPP depletion (Fig. 4). The half-life of intracellular βAPP in cells cotransfected with βAPP and X11 was prolonged in comparison to cells transfected with βAPP alone (Fig. 5). Half-life in this system refers to turnover rate, dependent on both degradation and secretion. Although X11 overexpression attenuated the depletion of intracellular βAPP in all cell lines tested, the magnitude of the effect was different, with the greatest effect observed in 293 cells and the least in COS-1 cells. Delayed intracellular βAPP depletion was also observed following immunoprecipitation of N2a cell lysates proteins with anti-βAPP_{66-81} antibody (22C11) (Fig. 6).

Furthermore, immunoprecipitation of conditioned medium proteins with anti-$A\beta_{1-17}$ antibody (6E10) (Fig. 4) revealed decreased secretion of sβAPPα. In order to confirm the results obtained with anti-$A\beta_{1-17}$ antibody (6E10), cell lysates and medium proteins were immunoprecipitated with an antibody raised against the amino-terminal end of βAPP, anti-βAPP_{66-81} antibody (22C11). This antibody immunoprecipitates full-length βAPP as well as its amino-terminal fragments, sβAPPα and sβAPPβ. Decreased secretion of sβAPP was also observed in N2a cells cotransfected with βAPP and X11 cDNAs, compared with cells cotransfected with βAPP and vector, following immunoprecipitation with anti-βAPP_{66-81} antibody (22C11) (Fig. 6). The decrease in sβAPP immunoprecipitated with 22C11 antibody may be due to decreased α-secretase activity or may indicate a decrease also in β-secretase activity. The same results were obtained with N2a cells stably transfected with wild type βAPP and transiently transfected with X11 (data not shown).

Coexpression of X11 with mutated βAPP (Y682G or N684A) resulted in a decrease in sβAPP retrieved from the culture media, compared with sβAPP secreted by cells cotransfected with the respective mutated βAPP and vector alone. Pulse-chase experiment with COS-1 cells transiently transfected with wild type βAPP or βAPP N684A cDNAs and either vector or X11 cDNAs is presented in Fig. 7. However, cotransfection of X11 with mutated βAPP had a lesser effect than its effect on wild type sβAPP secretion. These results are compatible with the finding that both mutant βAPPs retain 5–10% of X11 binding activity (24).

To rule out the possibility that X11 affects protein secretion in a more generalized manner, pulse-chase experiments were performed with 293 cells stably overexpressing the human cystatin C gene, transiently transfected with X11 or vector cDNAs. X11 had no effect on the expression or secretion of cystatin C, a cysteine proteinase inhibitor, produced and secreted through the constitutive secretory pathway (Fig. 8).

These results suggest that βAPP binding to X11 delays βAPP turnover, resulting in retention of intracellular βAPP and decreased βAPP secretion.

Effect of Overexpression of X11 and βAPP on Aβ Secretion—In order to test the effect of X11-βAPP binding on Aβ secretion, metabolic labeling experiments were performed. N2a cells permanently transfected with wild type βAPP were transiently transfected with vector or with X11 cDNAs and labeled with [^{35}S]methionine/cysteine, and the conditioned medium proteins were immunoprecipitated with anti-$A\beta_{1-17}$ and anti-$A\beta_{17-27}$ antibodies (6E10 and 4G8) (Fig. 9*A*). X11 binding to wild type βAPP diminished Aβ secretion. A decrease in Aβ secretion was also observed in media conditioned by COS-1 cells transiently cotransfected with X11 and wild type βAPP, compared with cells transiently transfected with βAPP cDNA alone (data not shown).

In order to confirm the finding that X11 binding to βAPP

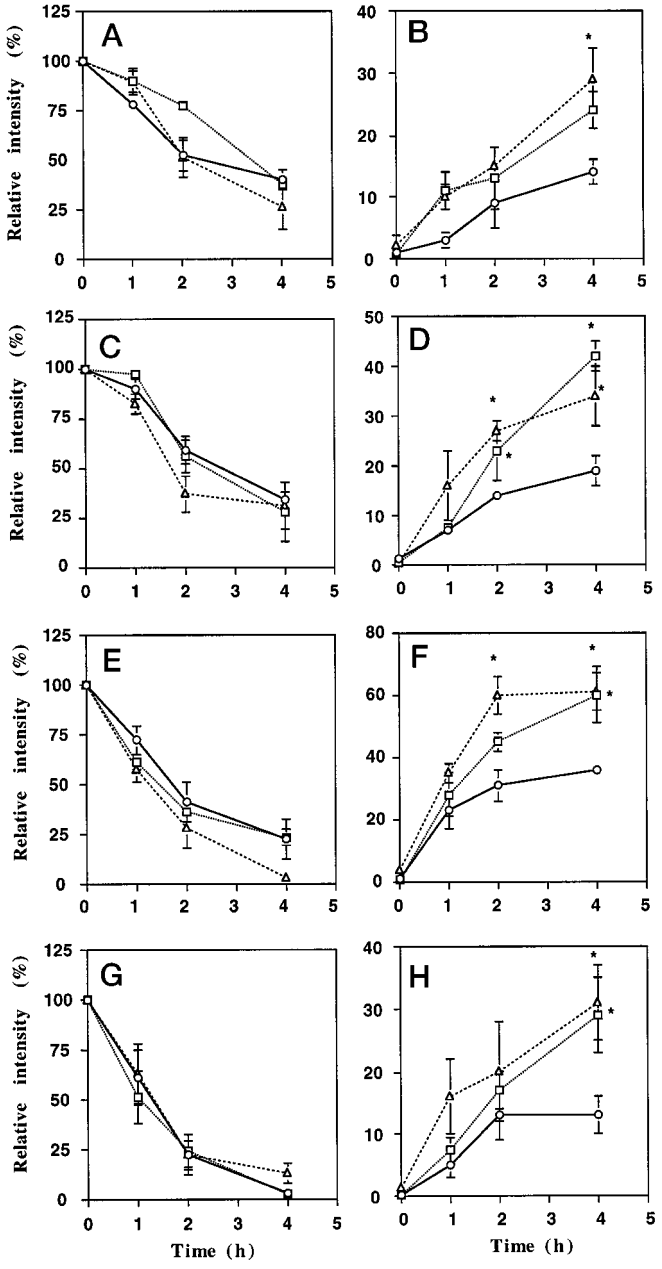

FIG. 3. **Inhibition of X11 binding to the YENPTY motif of βAPP caused increased secretion of soluble βAPP via the α-secretase pathway.** N2a (*A* and *B*), 293 (*C* and *D*) and COS-1 cells (*E* and *F*) that were transiently transfected and U251 cells (*G* and *H*) that were stably transfected with wild type or mutated βAPP cDNAs were labeled with [^{35}S]methionine/cysteine for 20 min and chased for different time periods. Cell lysates (*A, C, E,* and *G*) and medium proteins (*B, D, F,* and *H*) were immunoprecipitated with anti-Aβ_{1-17} antibody 6E10, separated by SDS-PAGE, and exposed to x-ray film. The protein bands were scanned and quantitated. Relative intensity of the bands was calculated as percentage of the intensity of the protein band of cell lysates at the beginning of the chase (time 0). Mean and S.D. from 3–5 different experiments are presented. p values <0.05 for the comparison of the mutated βAPP with wild type βAPP were considered to be statistically significant (*). Symbols represent wild type βAPP (○) and different mutations in βAPP cytoplasmic domain (βAPP$_{N684A}$ (□) and βAPP$_{Y682G}$ (△).

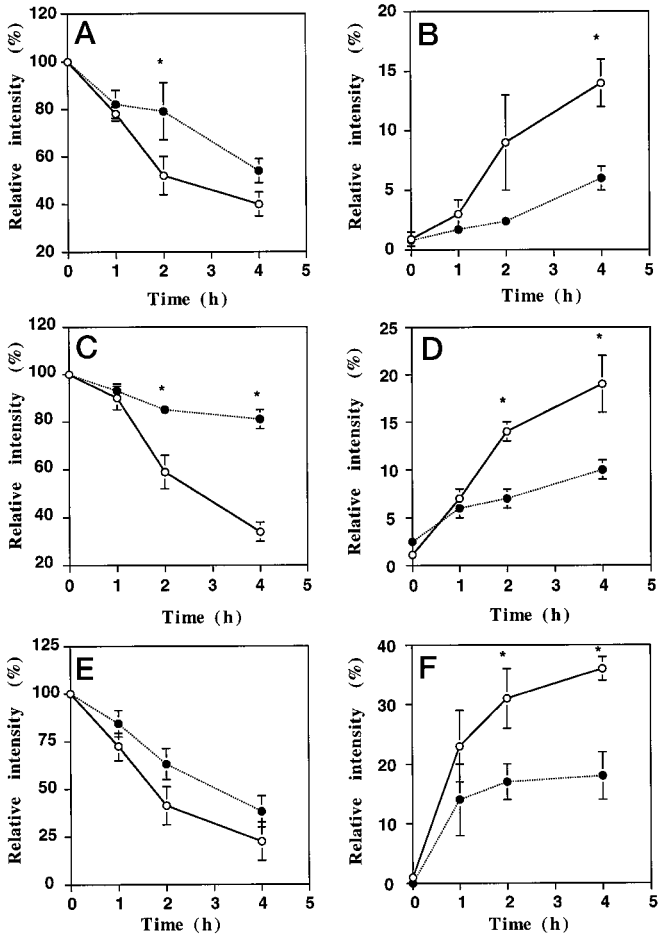

FIG. 4. **Cotransfection of X11 and βAPP resulted in accumulation of cellular βAPP and decreased secretion of sβAPPα.** N2a (*A* and *B*), 293 (*C* and *D*) and COS-1 cells (*E* and *F*) that were transiently transfected with wild type βAPP cDNAs and either vector or X11 cDNAs were labeled with [^{35}S]methionine/cysteine for 20 min and chased for different time periods. Cell lysates (*A, C,* and *E*) and medium proteins (*B, D,* and *F*) were immunoprecipitated with anti-Aβ_{1-17} antibody (6E10), separated by 8% SDS-PAGE, and exposed to x-ray film. The protein bands were scanned and quantitated. Relative intensity of the bands was calculated as the percentage of the intensity of the protein band of cell lysates at the beginning of the chase (time 0). Mean and S.D. from 3–5 different experiments are presented. p values <0.05 for the difference between the experimental conditions were considered to be statistically significant (*). Symbols represent transfection with wild type βAPP with vector (○) or wild type βAPP together with X11 (●).

causes decreased secretion of Aβ peptides, the levels of Aβ secreted into conditioned media were studied by ELISA. The levels of Aβ peptides secreted by N2a cells stably transfected with wild type βAPP cDNA and transiently transfected with X11 cDNA were compared with cells transfected with the βAPP cDNAs alone. The secretion of both Aβ_{1-40} and Aβ_{1-42} was lower in N2a cells transfected with X11 and wild type βAPP as compared with cells transfected with βAPP alone (Fig. 9*B*). These results demonstrate a decrease of 53 and 34% in the levels of Aβ_{1-40} and Aβ_{1-42}, respectively, secreted by cells transfected with wild type βAPP and X11 in comparison to cells transfected only with wild type βAPP.

These data suggest that X11 interaction with βAPP participates in the regulation of βAPP processing, affecting both sβAPP and Aβ secretion in neuronal and nonneuronal cells.

DISCUSSION

Our studies demonstrate that association of βAPP with X11 in cultured cells affects βAPP turnover and processing, which in turn shifts the balance toward decreased production of specific proteolytic peptides. The βAPP binding site for X11 lies within the YENPTY motif present at the carboxyl terminus of βAPP (23, 24). Specific mutations within the binding motif inhibit interaction with X11 and result in increased secretion of soluble βAPP via the α-secretase pathway. Cotransfection of

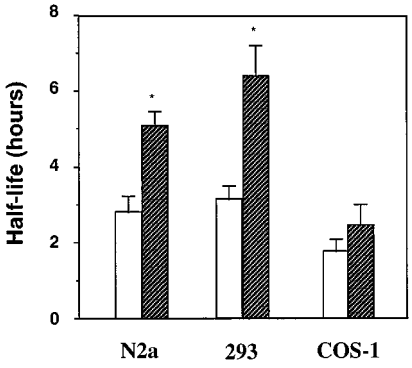

FIG. 5. **Cotransfection of X11 and βAPP resulted in prolongation of the half-life of cellular βAPP.** N2a, 293, and COS-1 cells transiently cotransfected with wild type βAPP and either vector (□) or X11 (▨) cDNAs were labeled with [^{35}S]methionine/cysteine for 20 min and chased for different time periods. Cell lysates proteins were immunoprecipitated with anti-Aβ_{1-17} antibody (6E10), separated by 8% SDS-PAGE, and exposed to x-ray film. The protein bands were scanned and quantitated. Relative intensity of the bands was calculated as percentage of the intensity of the protein band of cell lysates at the beginning of the chase (time 0). Mean and S.D. of the half-life of cell lysates βAPP calculated for individual experiments are presented. p values <0.05 for the difference between the experimental conditions were considered to be statistically significant (*).

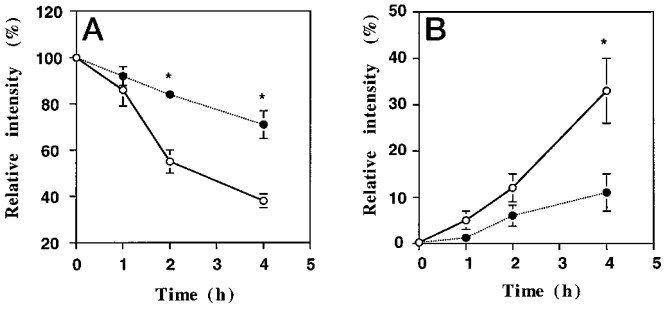

FIG. 6. **Cotransfection of X11 and βAPP resulted in accumulation of cellular βAPP and decreased secretion of sβAPP.** N2a cells transiently transfected with wild type βAPP cDNAs and either vector or X11 cDNAs were labeled with [^{35}S]methionine/cysteine for 20 min and chased for different time periods. Cell lysates (*A*) and medium proteins (*B*) were immunoprecipitated with anti-βAPP$_{66-81}$ antibody (22C11), separated by 8% SDS-PAGE, and exposed to x-ray film. The protein bands were scanned and quantitated. Relative intensity of the bands was calculated as a percentage of the intensity of the protein band of cell lysates at the beginning of the chase (time 0). Mean and S.D. from six different experiments are presented. p values <0.05 for the difference between the experimental conditions were considered to be statistically significant (*). Symbols represent transfection with wild type βAPP and vector (○) or wild type βAPP together with X11 (●).

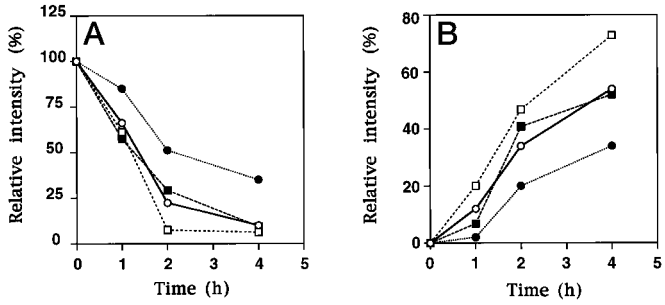

FIG. 7. **The effect of coexpression of X11 with mutated βAPP$_{N684A}$ on βAPP expression and secretion.** COS-1 cells transiently transfected with wild type or mutated βAPP cDNAs and either vector or X11 cDNAs were labeled with [^{35}S]methionine/cysteine for 20 min and chased for different time periods. Cell lysates (*A*) and medium proteins (*B*) were immunoprecipitated with anti-Aβ_{1-17} antibody (6E10), separated by 8% SDS-PAGE, and exposed to x-ray film. The protein bands were scanned and quantitated. Relative intensity of the bands was calculated as a percentage of the intensity of the protein band of cell lysates at the beginning of the chase (time 0). The results from a single representative experiment are presented. Less than 10% variation was observed between experiments. Symbols represent transfection with wild type βAPP with vector (○) or with X11 (●) and transfection with βAPP$_{N684A}$ with vector (□) or with X11 (■).

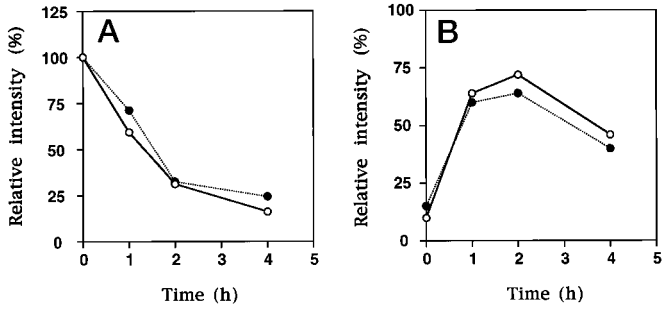

FIG. 8. **Coexpression of X11 with cystatin C does not affect its expression and secretion.** 293 cells stably overexpressing the human cystatin C gene that were transiently transfected with X11 or vector cDNAs were labeled with [^{35}S]methionine/cysteine for 20 min and chased for different time periods. Cell lysates (*A*) and medium proteins (*B*) were immunoprecipitated with anti-cystatin C antibody, separated on 10% Tris-Tricine gel, and exposed to x-ray film. The protein bands were scanned and quantitated. Relative intensity of the bands was calculated as percentage of the intensity of the protein band of cell lysates at the beginning of the chase (time 0). The results from a single representative experiment are presented. Less than 10% variation was observed between experiments. Symbols represent transfection with cystatin C with vector (○) or with X11 (●).

wild type βAPP and X11 cDNAs stabilizes cellular full-length βAPP, down-regulates sβAPP secretion, and concomitantly triggers a decrease in Aβ secretion. These data suggest that βAPP-X11 interaction plays a role in the regulation of βAPP processing, differentially affecting sβAPP and Aβ secretion in neuronal and nonneuronal cells.

Numerous studies have established that stimulation of protein kinase C leads to cell type-specific enhancement of sβAPP with a concurrent suppression of Aβ production (40, 41). The secretory processes of sβAPP and Aβ were also differentially affected by interleukin-1β stimulation (42). Thus, in certain cell systems sβAPP secretion and Aβ generation apparently are not mutually exclusive. These observations are the basis for the hypothesis that there is a surplus of full-length βAPP, which gives rise to sβAPP, Aβ, or lysosomal degradation products, depending on the enzymatic activities and regulatory mechanisms present (40). Because stimulation of protein kinase C causes a rapid reduction of cell surface βAPP (43) and increases secretory vesicle formation (44), it was suggested that its effects are due primarily to a reduction in the transport of βAPP to the cell surface. This alteration correlates with an acceleration of intracellular α-secretase βAPP cleavage and of βAPP trafficking in the exocytic pathway. In most cell cultures, displacement of cell surface βAPP reduces the substrate available for endocytic processing with resultant inhibition of Aβ production (43).

The normal function of βAPP within a signal transduction pathway may be elucidated by studying the interaction of βAPP with PI/PTB-containing proteins. NPXY is a conserved protein binding motif that has been implicated in signal transduction (30, 31) and internalization of βAPP via clathrin-mediated endocytosis (11, 18, 19). X11 binds to the YENPTY motif within the cytoplasmic domain of βAPP. Our data demonstrate that X11 modulates βAPP processing via a direct protein/protein interaction. Because X11 has multiple protein interaction domains, other proteins may be complexed to the βAPP-X11 moiety, affecting βAPP processing or the normal physiologic function of βAPP. X11 has two PDZ motifs implicated in the

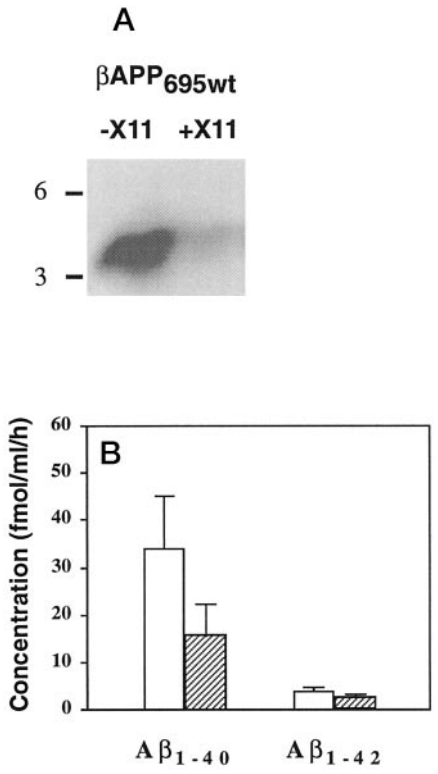

FIG. 9. **Reduced secretion of Aβ in cells overexpressing both X11 and wild type βAPP.** N2a cells stably transfected with wild type βAPP and transiently transfected with vector or X11 cDNAs were labeled with [^{35}S]methionine/cysteine for 4 h. Conditioned medium proteins were immunoprecipitated with anti-Aβ_{1-17} and anti-Aβ_{17-27} antibodies (6E10 and 4G8), separated in 16.5% Tris-Tricine gel, and exposed to x-ray film. The results from a single metabolic labeling representative experiment are presented. Less than 10% variation was observed between experiments (*A*). N2a cells stably transfected with wild type βAPP were transiently transfected with vector (□) or X11 (▨) cDNAs. Three anti-Aβ antibodies were used for ELISA analysis of Aβ medium proteins (*B*): anti-Aβ_{1-10} antibody (BAN-50), anti-Aβ_{x-40} antibody (BA-27), and anti-Aβ_{x-42} antibody (BC-05). The concentrations of Aβ_{1-40} and Aβ_{1-42} are presented in fmol/ml/h as the mean ± S.D. of seven different experiments.

binding of sequences found on Fas and *N*-methyl-D-aspartate receptors (45, 46). These domains are also implicated in PDZ dimerization as exemplified in the interaction of PDZ domains on nitric oxide synthase and PSD95 (47). Thus, PDZ dimerization may induce stabilization of the complex βAPP-X11-X11 dimerization associate within cellular membranes. Stabilization of the complex may block the accessibility of the secretase cleavage sites of βAPP, resulting in decreased secretion of both sβAPP and Aβ. However, X11 may directly affect βAPP processing, preventing secretion of its soluble fragments and thereby leading to an accumulation of βAPP in the cell.

Although numerous functions have been attributed to βAPP, the precise physiological role of the full-length protein, as well as that of the proteolytic peptides, remains to be defined. Cell-associated βAPP and secreted βAPP products have been implicated in cell growth, neuronal survival and viability, neurite outgrowth, and neuroprotection (48–51). sβAPPα more effectively protects hippocampal neurons against excitoxicity, Aβ toxicity, and glucose deprivation than does sβAPPβ. sβAPPα has trophic activity on various cell lines that was localized to amino acids 591–612 at the carboxyl terminus (48, 52, 53). Therefore, down-regulation of sβAPPα as a result of βAPP-X11 coupling could result in reduced trophic activity. However, stabilization of the full-length protein within the cell surface may be neuroprotective.

The pathological processes that lead to the deposition of insoluble Aβ in the brain are unknown. Several observations indicate that aberrant expression or processing of the human βAPP gene products may lead to the development of AD. Patients with trisomy 21 (Down's syndrome patients) have overexpression of the protein and tend to develop AD-type pathology early in life (54, 55). Several familial forms of Alzheimer's disease have been described that are linked to mutations in the βAPP gene that result in amino acid substitutions within Aβ or near its amino- or carboxyl-terminal ends. A Swedish kindred develops an AD phenotype that cosegregates with a double mutation, K595N/M596L, amino-terminal to the Aβ region (56). This double mutation leads to overproduction of Aβ and sβAPPβ (57, 58). Moreover, transgenic mice overexpressing human βAPP exhibit age-dependent and brain region-specific extracellular deposits of fibrillar Aβ (59–61). Thus, perhaps even slightly increased amounts of Aβ might be sufficient to cause AD, and inhibition of Aβ production could aid in prevention of AD.

It was hypothesized that Aβ and sβAPP are physiological ligands with reciprocal effects on neurons and that alterations in the Aβ to sβAPP ratio may have beneficial or deleterious effects on neuronal survival and growth (62). Thus, stabilization of cellular βAPP and inhibition of the amyloidogenic processing pathway by stimulating the interaction of βAPP with an adaptor protein, such as X11 or another family member, may provide a novel approach for the pharmacological modulation of βAPP processing in Alzheimer's disease.

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