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X11α MODULATES SECRETORY AND ENDOCYTIC TRAFFICKING AND METABOLISM OF AMYLOID PRECURSOR PROTEIN: MUTATIONAL ANALYSIS OF THE YENPTY SEQUENCE

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Abstract—The neuronal adaptor X11α interacts with the conserved −GYENPTY− sequence in the C-terminus of amyloid precursor protein (APP) or its Swedish mutation (APPswe) to inhibit Aβ40 and Aβ42 secretion. We hypothesized that the −YENP− motif essential for APP endocytosis is also essential for X11α-mediated effects on APP trafficking and metabolism, and that X11α modulates APP metabolism in both secretory and endocytic pathways. X11α failed to interact with the endocytic-defective APPswe mutants Y738A, N740A, or P741A, and thus did not modulate their trafficking or metabolism. However, endocytic-competent APPswe Y743A had unique trafficking and metabolism including a prolonged half-life and increased secretion of catabolites compared with APPswe. In contrast to endocytic-defective mutants, X11α interacted with APPswe Y743A as well as with APPswe. Thus, similar to APPswe, coexpression of X11α with APPswe Y743A retarded its maturation, prolonged its half-life, and inhibited APPs, Aβ40, and Aβ42 secretion. Collectively, these data suggest that by direct interaction with the APPswe −YENP− motif in the cytoplasmic tail, X11α modulated its trafficking and processing in both secretory and endocytic compartments, and may reduce secretion of Aβ generated in either pathway.

Key words: amyloid precursor protein, XIIα, mint-1 , Aβ, Alzheimer, processing.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that is pathologically defined by the density of neurofibrillary tangles and amyloid plaques in brain. The major components of amyloid plaque in AD brain are Aβ peptides, including Aβ40 and Aβ42, that are derived by β- and γ-secretase cleavage of the type I transmembrane glycoprotein amyloid precursor protein (APP). In the β-/γ-secretase pathway of APP catabolism, the secreted N-terminal ectodomain of APP is known as APPsβ. Alternatively, APP may be metabolized by α-secretases that shed the ectodomain APPsα and preclude Aβ generation. Aβ accumulation and deposition in brain may be pathogenic and set into motion a series of events referred to collectively as the amyloid hypothesis of AD (Selkoe, 2001). Major support for this hypothesis comes from the identification and analysis of the effects of gene mutations identified in early-onset autosomal dominant familial AD (FAD). All known FAD mutations increase either Aβ deposition in brain or the generation of Aβ from APP, in particular the more amyloidogenic Aβ42. For example, the APP Swedish mutation (APPswe; K651N/M652L in 751 isoform numbering) enhances APP cleavage by β-secretase, resulting in increased Aβ40 and Aβ42 production (Citron et al., 1992; Perez et al., 1996).

The intracellular cytoplasmic C-terminus of APP is a site for multiple adaptor protein interactions that regulate its trafficking and metabolism. For example, the APP tail contains the AP-2/clathrin-coated pit consensus motif −GYENPTY− essential for internalization and trafficking of certain transmembrane proteins (Lai et al., 1998; Perez et al., 1999). This domain is unique in being absolutely conserved across species and APP family members, thus indicating its pivotal role in normal APP functions (Table 1). Mutation of both tyrosine residues in this sequence reduces APP internalization five-fold (Lai et al., 1998; Tomita et al., 1998). However, the critical motif for internalization of APP is the tetrapeptide −YENP− (Perez et al., 1999). The −GYENPTY− sequence also mediates interaction with phosphotyrosine binding (PTB) domain-containing partners such as the Fe65 and X11 families of adaptor

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Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; APPs, secreted N-terminal ectodomain of APP; APPsα, secreted α-secretase cleavage product of APP; APPsβ, secreted β-secretase cleavage product of APP; APPsαswe, secreted α-secretase cleavage product of APPswe; APPsβswe, secreted β-secretase cleavage product of APPswe; APPswe, amyloid precursor protein Swedish mutation (K651N/M652L); BM, binding medium; DMEM, Dulbecco Modified Eagle medium; ELISA, enzyme-linked immunosorbent assay; FAD, familial Alzheimer's disease; HEK, human embryonic kidney; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PDZ, repeated sequences in *P*ost-synaptic density-95 (PSD-95); *D. melanogaster* septate junction protein *D*isks-large, and epithelial tight junction protein *Z*ona occludens-1; PTB, phosphotyrosine binding; Y738A, N740A, P741A, and Y743A all refer to APPsweY738A, APPswe N740A, APPswe P741A, and APPswe Y743A, respectively.

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Table 1. Multiple sequence alignment of the cytoplasmic domain of the APP family[a]

Species	Gene	Amino acid sequence
H. sapiens	APP	...LVMLKKKQYTSIHHGVVEVDAAVTP**EE**RHLSKM**Q**QN**GYENPTY**KFFEQMQN
M. fascicularis	APP	...LVMLKKKQYTSIHHGVVEVDAAVTP**EE**RHLSKM**Q**QN**GYENPTY**KFFEQMQN
S. sciureus	APP	...LVMLKKKQYTSIHHGVVEVDAAVTP**EE**RHLSKM**Q**QN**GYENPTY**KFFEQMQN
M. musculus	APP	...LVMLKKKQYTSIHHGVVEVDAAVTP**EE**RHLSKM**Q**QN**GYENPTY**KFFEQMQN
R. rattus	APP	...LVMLKKKQYTSIHHGVVEVDAAVTP**EE**RHLSKM**Q**QN**GYENPTY**KFFEQMQN
Cavia sp.	APP	...LVMLKKKQYTSIHHGVVEVDAAVTP**EE**RHLSKM**Q**QN**GYENPTY**KFFEQMQN
S. scrofa	APP	...LVMLKKKQYTSIHHGVVEVDAAVTP**EE**RHLSKM**Q**QN**GYENPTY**KFFEQMQN
G. gallus	APP	...LVMLKKKQYTSIHHGVVEVDAAVTP**EE**RHLSKM**Q**QN**GYENPTY**KFFEQMQN
C. serpentina	APP	...LVMLKKKQYTSIHHGVVEVDAAVTP**EE**RHLSKM**Q**QN**GYENPTY**KFFEQMQN
Xenopus sp.	APP	...LVMLKKKQYTTIHHGVVEVDAAVTP**EE**RHLTKM**Q**QN**GYENPTY**KFFEQMQN
D. rerio	APP	...LVMLRKKQYTSIHHGIIEVDAAVTP**EE**RHLSKM**Q**QN**GYENPTY**KFFEQMHN
T. rubripes	APP	...LVMLRKKQYTSIHHGVIEVDAAVTP**EE**RHLARM**Q**QN**GYENPTY**KFFEQMQN
T. fluviatilis	APP	...LVMLRKKQYTSIHHGIIEVDAAVTP**EE**RHLSKM**Q**QN**GYENPTY**KFFEQMQN
N. japonica	APP	...LVMLKKKQYTSIHHGVVEVDAAVTP**EE**RHLSKM**Q**QN**GYENPTY**KFFEQMQN
A. gambiae	APPL	...WKASRSPHAQGFVEVDQAVGAPVTP**EE**RHVANM**Q**IN**GYENPTY**KYFEIKE
D. melanogaster	APPL	...TSRSPHAQGFIEVDQNVTTHHPIVR**EE**KIVPNM**Q**IN**GYENPTY**KYFEVKE
C. elegans	APL1	...AFAITNARRRRAMRGFIEVD-VYTP**EE**RHVAGM**Q**VN**GYENPTY**SFFDSKA
H. sapiens	APLP1	...LLLRRKKPYGAISHGVVEVDPMLTL**EE**QQLREL**Q**RH**GYENPTY**RFLEERP
M. musculus	APLP1	...LLLRKKKPYGTISHGVVEVDPMLTL**EE**QQLREL**Q**RH**GYENPTY**RFLEERP
H. sapiens	APLP2	...LVMLRKRQYGTISHGIVEVDPMLTP**EE**RHLNKM**Q**NH**GYENPTY**KYLEQMQI
M. musculus	APLP2	...LVMLRKRQYGTISHGIVEVDPMLTP**EE**RHLNKM**Q**NH**GYENPTY**KYLEQMQI
R. norvegicus	APLP2	...LVMLRKRQYGTISHGIVEVHPMLTP**EE**RHLNKM**Q**NH**GYENPTY**KYLEQMQ
Transmembrane region		...LVML
Conserved consensus sequences		**EE** **Q** **GYENPTY**
Required for endocytosis		YXNP
Required for basolateral sorting		YTSI
X11α binding region		QNGYENPTYKFF

[a] Multiple sequence alignment revealed the uniquely conserved –YENPTY– motif in the cytoplasmic domains of the APP family. The –YENPTY– sequence is a consensus motif for AP-2/clathrin-mediated endocytosis and contains the consensus for PTB domain-containing adaptor protein interactions. The –YXNP– motif of APP is required for endocytosis (Perez et al., 1999). The –YTSI– motif localized near the cell membrane is essential for basolateral targeting in polarized epithelial cells (Lai et al., 1998). The –YENPTY– motif also spans the X11α binding region (Zhang et al., 1997). APLP1 and APLP2 are amyloid precursor protein like-1 and -2.

proteins (Borg et al., 1996; Zambrano et al., 1998). Fe65 binding to APP accelerates its transit in the secretory pathway and promotes Aβ secretion (Sabo et al., 1999). In contrast, X11α retards APP catabolism and inhibits Aβ secretion (Borg et al., 1998b; Sastre et al., 1998; Mueller et al., 2000) by unclear mechanisms.

X11α (also known as mint1 and lin-10) is one of three family members (X11α, β, and γ) containing highly conserved C-terminal PTB and repeated sequences in *P*ost-synaptic density-95 (PSD-95), *D. melanogaster* septate junction protein *D*isks-large, and epithelial tight junction protein *Z*ona occludens-1 (PDZ) domains and divergent N-terminal domains as well as divergent tissue distributions (Blanco et al., 1998; Butz et al., 1998; McLoughlin et al., 1998; Okamoto and Sudhof, 1998; Tanahashi and Tabira, 1999a,b). A munc-interaction domain present in X11α and X11β, but not X11γ, binds to munc-18, which is critical to synaptic vesicle exocytosis (Okamoto and Sudhof, 1997). A CASK interaction domain found in X11α, but not X11β or X11γ, binds to CASK to form a heterotrimeric complex with Veli that targets certain transmembrane receptors in polarized cells (epithelia and neurons). For example, X11α is essential for GLR-1 glutamate receptor localization to the postsynaptic membrane in neurons (Rongo et al., 1998). These findings suggest a role for X11α in transmembrane receptor targeting and tethering in polarized cells, and we propose a similar role for X11α on APP trafficking and function in neurons. In mammalian brain the complex X11α/CASK/Veli is enriched at synaptic terminals (Borg et al., 1998a; Butz et al., 1998) but localizes mostly in perinuclear vesicular (membranous) compartments in neurons, which is identical to the localization of APP (Borg et al., 1999).

The specific conformational requirements for the APP:X11α interaction are only partly mapped. Similarly, our knowledge of the key roles of the −YENPTY− motif in cellular APP trafficking and processing is incomplete. With wild type APP, the mutations APP Y738A or N740A inhibit X11α binding and impair APP endocytosis (Borg et al., 1996; Sastre et al., 1998; Perez et al., 1999). APP P741A also impairs endocytosis (Perez et al., 1999) but the effect of this mutation on X11α binding and X11α-mediated effects on APP metabolism are unknown. The APP Y743A mutation does not impair endocytosis or X11α binding but increases APP half-life (Borg et al., 1996; Perez et al., 1999). The effects of X11α on APP Y743A metabolism are also unknown. Although Aβ secretion is consistently inhibited by X11α coexpression with APP, the mechanism and localization of these X11α-mediated effects are unclear. Therefore, we explored the effects of point mutations in the

−YENPTY− motif of APPswe, expressed alone or with X11α, on its trafficking and metabolism and report several novel observations.

EXPERIMENTAL PROCEDURES

Cell culture

Human embryonic kidney (HEK) 293 cells (American Type Culture Collection) were passaged in Dulbecco Modified Eagle medium (DMEM; Invitrogen) containing penicillin (100 U/ml) and streptomycin sulfate (Invitrogen) (100 μg/ml) supplemented with 10% fetal bovine serum (Atlanta Biologicals).

DNA constructs

Myc-tagged X11α was cloned in pRK5 as described (Borg et al., 1996). $APPswe_{751}$ and its C-terminal missense mutations were cloned into pcDNA3 as described (Perez et al., 1999). All constructs were created by a PCR method and sequenced using Sequenase Version 2.0 (U.S. Biochemical). The APP_{751} isoform and Swedish mutation (K651N/M652L; APPswe) in pcDNA3 were used to permit robust detection of both Aβ40 and Aβ42 in conditioned media, and to more closely mimic neuronal APP metabolism in non-neuronal HEK 293 cells (Forman et al., 1998).

Cell transfection and protein extraction

Six centimeter plates were precoated with poly-D-lysine (Sigma; 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 3 μg of DNA for single transfections or 6 μg of DNA for double transfections 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 were removed and centrifuged at $15{,}000\times g$ for 10 min at 4 °C to remove cellular debris. After washing in 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 phenylmethyl-sulfonylfluoride (Sigma) and complete protease inhibitor cocktail (Roche). Cell lysates were centrifuged at $15{,}000\times g$ for 10 min at 4 °C.

Immunoprecipitation

To probe X11α:APP interaction, X11α was immunoprecipitated from cell lysates with anti-myc (9E10, Santa Cruz) followed by immunoblot of recovered proteins with the anti-APP antibody (22C11, Chemicon). APP or APPs were recovered from 1 ml of cell lysate or conditioned media, respectively, by incubation with antibody to APP (Biosource) or APPs (Karen, a polyclonal serum raised to the N-terminus of APP) (Turner et al., 1996) and protein-A sepharose beads (Sigma) for 4 h at 4 °C. Beads were then washed twice with wash buffer (400 mM NaCl, 50 mM Tris, 0.25% DOC, 0.25% NP40, pH 7.5). After boiling with sample buffer containing β-mercaptoethanol, recovered proteins were separated by 8% Tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Invitrogen), and detected by immunoblot.

Immunoblotting

Proteins were transferred from gels to polyvinyldiene difluoride (Roche) membranes presoaked in methanol. Membranes were blocked in 5% milk in TBST. The monoclonal antibody 22C11 (Chemicon) raised to an N-terminal domain of APP (residues 61–88) was used to detect APP. Anti-myc (9E10, Santa Cruz) was used to confirm expression of myc-tagged X11α constructs in cell lysates. ECL Plus (Amersham) detected horseradish peroxidase-conjugated goat anti-mouse (Chemicon) by PhosphorImager analysis. Protein bands were quantitated with a STORM Scanner using ImageQuant software (Molecular Dynamics).

Metabolic radiolabeling

Twenty-four hours after transfection, cells were starved for 15 min in cysteine/methionine-free DMEM (Invitrogen). Subsequently, cells were incubated in DMEM containing [^{35}S]methionine (ICN) for 1 h for pulse-labeling. Cells were then rinsed with PBS, lysed, and proteins immunoprecipitated as described above, using anti-APP antibody (Biosource). For pulse-chase experiments, cells were further incubated with DMEM containing excess unlabeled methionine for 0–8 h. Immunoprecipitated proteins were separated by 8% Tris–glycine SDS-PAGE, and radiolabeled bands detected by autoradiography and quantitated by PhosphorImager analysis using a STORM Scanner with ImageQuant software (Molecular Dynamics).

Uptake assay

Triplicate cultures of cells transfected 24 h before (see above) were grown in 12 well tissue culture plates. We measured internalization of cell surface APP, as previously described (Perez et al., 1999) except with the 22C11 antibody (Chemicon). Antibody was iodinated using Chloramine-T (Perkin-Elmer) to approximately 3–6 μCi/μg as previously described (Perez et al., 1999) and added to binding medium (BM; RPMI 1640 supplemented with 20 mM HEPES, 0.2% BSA; Invitrogen) before being applied to confluent layers of cells and incubated at 37 °C for 30 min. After incubation, cells were rapidly chilled on ice and the reaction was quenched by addition of ice cold BM. After 10 min, chilled cells were washed multiple times with ice cold Dulbecco's Phosphate-buffered saline (Invitrogen) to eliminate unbound antibody. Residual antibody bound to APP (acid-labile fraction) was then detached from cell surfaces by two 5 min washes with ice cold PBS, pH 2.5, and collected into sample tubes. Cells were lysed in 0.2 M NaOH (acid-resistant fraction) and collected into sample tubes. Acid-labile and acid-resistant counts were measured by γ counting. The ratio of acid-resistant to acid-labile counts provided a measure of internalized versus cell-surface pools of APP. Specific binding was calculated after subtraction of the radioactive counts obtained from labeled nontransfected cells.

Secreted β-secretase cleavage product of APPswe (APPsβswe) enzyme-linked immunosorbent assay (ELISA)

APPsβswe in conditioned media was detected by a sandwich ELISA using 931 antiserum as the capture antibody (Steinhilb et al., 2001). This antiserum does not recognize full length APPswe, APPsαswe, APP, APPsα, or APPsβ, and is thus specific for the neoepitope in the C-terminus of APPsβswe. The affinity purified capture antibody was diluted to 1.2 μg/mL in PBS and 100 μl/well added to a 96 well Nunc-Immuno Maxisorp plate (Nalge Nunc International). Twenty-five microliters of conditioned media/well were aliquotted in triplicate and the following day wells were incubated with 100 μl of diluted detector antibody (mouse monoclonal antibody mAbP2-1, Biosource) at 0.25 μg/ml. Lastly, wells were incubated for 1 h with horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:4000; Southern Biotechnology Associates, Inc.). TMB solution (Pierce Chemicals) and 2 M sulfuric acid were used to detect bound antigen in wells by measuring absorbance at 450 nm spectrophotometrically.

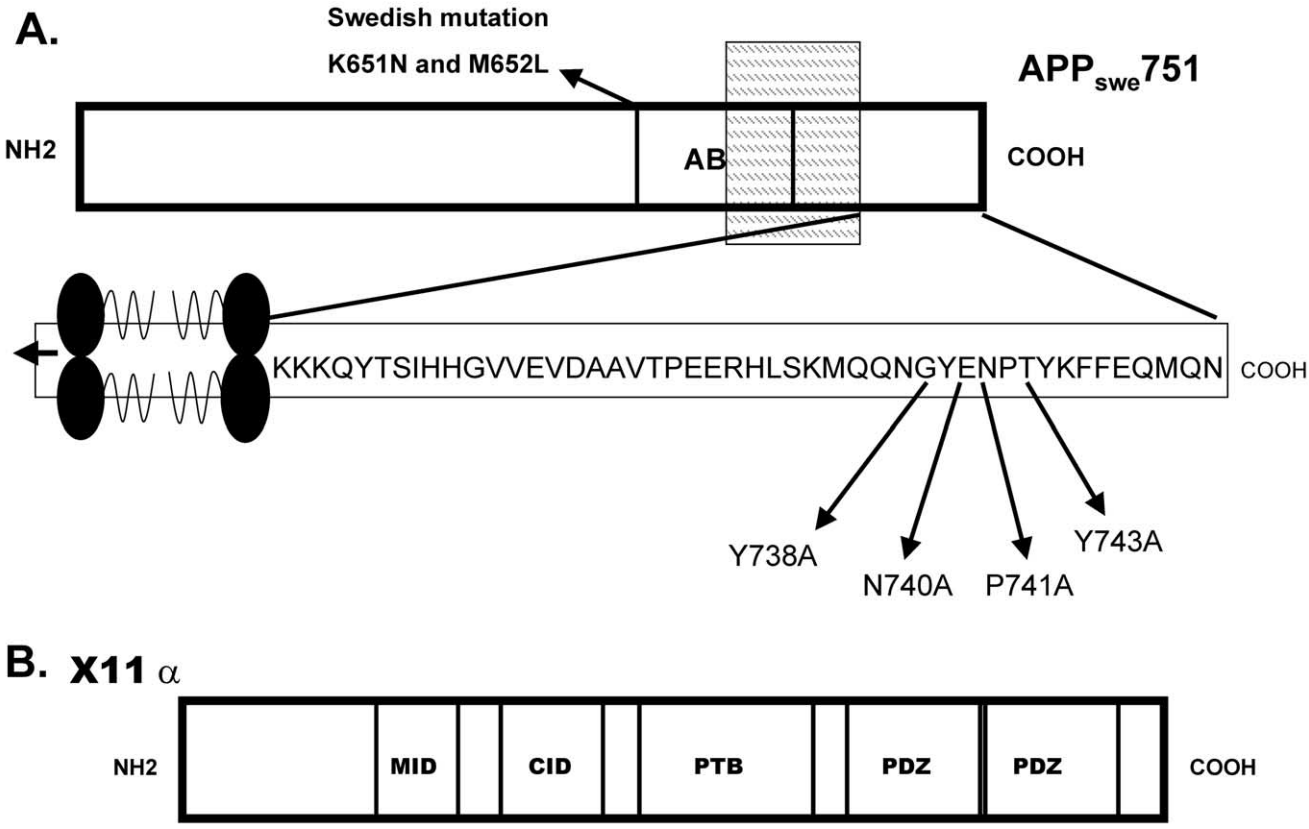

Fig. 1. Schematic representations of APPswe, its C-terminal missense mutations studied herein, and the protein interaction domains of X11α. (A) The Swedish mutation of APP (APPswe; K651N/M652L) was used in these studies. The missense mutations generated within the −YENPTY− motif of APPswe are also indicated. AB indicates the Aβ sequence within APP. (B) X11α contains a Munc18-interacting domain (MID, residues 226–314), a CASK/lin-2 interacting domain (CID, residues 373–436), a PTB/protein interaction domain (residues 457–643) and two PDZ domains (residues 656–742 and 747–822).

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 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. BAN-50 is a monoclonal antibody specific for Aβ1-10. Standard curves were generated for Aβ40 and Aβ42 (Bachem) for quantitation.

Statistical analysis

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

RESULTS

The −YENP− motif of APPswe is essential for X11α interaction

Our previous data (Borg et al., 1998) suggested that the −YENP− internalization motif, not the −NPTY− sequence, was essential for the PTB domain of X11α to bind APP. To further define the specific conformational requirements of the X11α:APP interaction, HEK 293 cells were transfected with APPswe or its C-terminal point mutations (Fig. 1A, B). To probe *in vitro* interaction, X11α was immunoprecipitated from cell lysates and binding to APPswe mutants was determined by immunoblot. X11α interacted with APPswe and APPswe Y743A, much less with APPswe Y738A, but not with APPswe N740A or APPswe P741A (abbreviated as Y738A, N740A, P741A, and Y743A hereafter; Fig. 2B). These data indicated that the −YXNP− tetrapeptide motif was essential for X11α binding, and therefore likely to be essential for X11α-mediated modulation of APP trafficking and metabolism.

X11α retards maturation of APPswe and APPswe Y743A

To probe the metabolic effects of the APPswe YENPTY mutations, with and without X11α, we examined APP and its catabolites in cell lysates and conditioned media. X11α coexpression increases the relative level of APPswe in cell lysates (Borg et al., 1996; Mueller et al., 2000) and also increased the cellular level of Y743A, but had no effect on Y738A, N740A, or P741A (Fig. 3A, C). These results are consistent with the interaction assay (Fig. 2), and also revealed the lack of nonspecific effects of X11α on the catabolism of APPswe mutants that were incapable of binding to X11α. Equivalent levels of X11α expression were confirmed by immunoblot (Fig. 3B). When mature

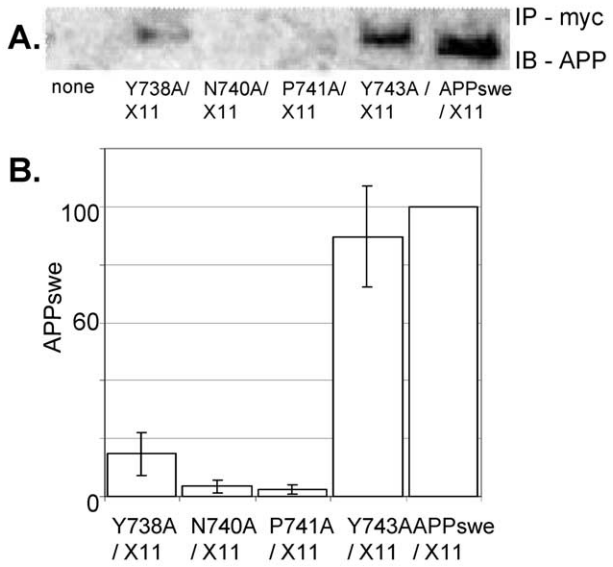

Fig. 2. X11α requires the −YENP− motif of APPswe for binding. (A) Representative immunoblot of APPswe and the APPswe mutations indicated recovered from transiently transfected HEK 293 cells. Proteins in cell lysates were immunoprecipitated with anti-myc (9E10) to recover X11α and bound APPswe or its mutations were subsequently detected by immunoblot (with 22C11). (B) Semi-quantitative analysis of levels of APPswe or mutations as determined with a PhosphorImager. Data were normalized to APPswe (set as 100%), $n=4$.

(post-translationally modified, *N*-glycosylated and *O*-glycosylated APP; Weidemann et al., 1989; Oltersdorf et al., 1990) versus immature (i.e. not fully glycosylated) APPswe were examined separately, X11α inhibited maturation of APPswe and Y743A, but not the endocytic-defective mutants Y738A, N740A, or P741A (Fig. 3A, D). Collectively, these data revealed that point mutations within the −YENP− sequence of APPswe blocked X11α binding to APPswe and consequently the effects of X11α on APPswe metabolism, suggesting that this motif normally contributes to APP/X11α interactions. Like APPswe, Y743A bound to X11α and therefore its maturation and metabolism were affected. Based on quantitative differences in the levels of mature versus immature APPswe, the impairment of APPswe and Y743A maturation suggests that X11α retarded their egress through the secretory compartment, specifically by retarding their transit between the ER and *trans* Golgi. Interestingly, we observed less mature than immature Y743A in cells cotransfected with X11α, suggesting that those cells would have less full length APPswe at the cell surface.

X11α prolongs the half-life of APPswe and APPswe Y743A

To further test the hypothesis that an interaction between X11α and APPswe may alter its catabolism, we performed metabolic radiolabeling experiments to determine the effects of X11α coexpression on the half-life of APPswe, Y743A, or N740A. For these experiments, N740A was selected as representative of the endocytic-defective mutations. X11α coexpression doubled the half-life of APPswe to approximately 1.5 h (Fig. 4A), which is similar to X11α's effect on wild type APP (Borg et al., 1998b; Sastre et al., 1998). The Y743A mutation alone had a unique effect in prolonging half-life (Fig. 4C), and coexpression with X11α further prolonged its half-life to >4 h (Fig. 4C). These data indicated that a point mutation of the −NPTY− tyrosine when coexpressed with X11α exaggerated the effects of Y743A on APP trafficking and metabolism similar to our earlier studies (Perez et al., 1999; Fig. 4C). In contrast, the N740A mutation did not significantly affect half-life, and X11α coexpression with N740A had no effect, as predicted (Fig. 4B). For all APP constructs the results obtained at 8 h of chase (data not shown) were similar to those observed at 4 h. These data are consistent with previous results suggesting that X11α modulated the metabolism of APPswe and Y743A, but not of N740A.

X11α inhibits secreted metabolites from APPswe with an intact −YENP− sequence

We next measured the effects of the APPswe YENPTY mutations, expressed with and without X11α, on secreted metabolites of APPswe (measured from conditioned media of cells transfected to obtain the results in Fig. 3). Compared with APPswe alone, Y743A increased and N740A decreased total APPs secretion (Fig. 5A), while Y738A or P741A had less of an effect. These studies using APPswe are in contrast to our studies using wild type APP in which −YENP− internalization-defective mutants increase APPs secretion (Perez et al., 1999). This difference may reflect the fact that endocytic processing is the predominant catabolic pathway of APP whereas catabolism of APPswe with or without −YENP− mutations occurs predominantly in the secretory pathway. We previously observed that cells overexpressing APPswe produce significantly more Aβ in both the secretory and endocytic pathways (Perez et al., 1996). X11α decreased APPs secretion when coexpressed with APPswe or Y743A, but not with Y738A, N740A or P741A (Fig. 5A) as expected from our observation that these mutants interact very little with X11α. These results further demonstrated that mutations within the −YENP− sequence, but not of the NPTY tyrosine (Y743) abolished X11α-mediated effects on APP catabolism.

In addition, we developed a novel ELISA to specifically detect the neoepitope at the C-terminus of APPsβswe, that does not detect wild type APPsα or APPsβ, or APPsαswe (Steinhilb et al., 2001). This ELISA allowed us to analyze the effect of APP C-terminal mutations, expressed with or without X11α, on β-secretase activity. Similar to the findings with total APPs, Y743A also increased and N740A decreased APPsβswe secretion compared with APPswe alone. Coexpression of X11α with Y743A, but not with APPswe, inhibited secretion of APPsβswe (Fig. 5B), again demonstrating a unique metabolism of Y743A probably associated with its extremely long half-life. X11α inhibits Aβ40 and Aβ42 secretion upon coexpression with APPswe (Borg et al., 1998b; Mueller et al., 2000). X11α also inhibited Aβ40 and Aβ42 secretion when coexpressed with Y743A but not with Y738A, N740A, or P741A (Fig. 5C, D). Collectively, these data suggested specific inhibition of γ-

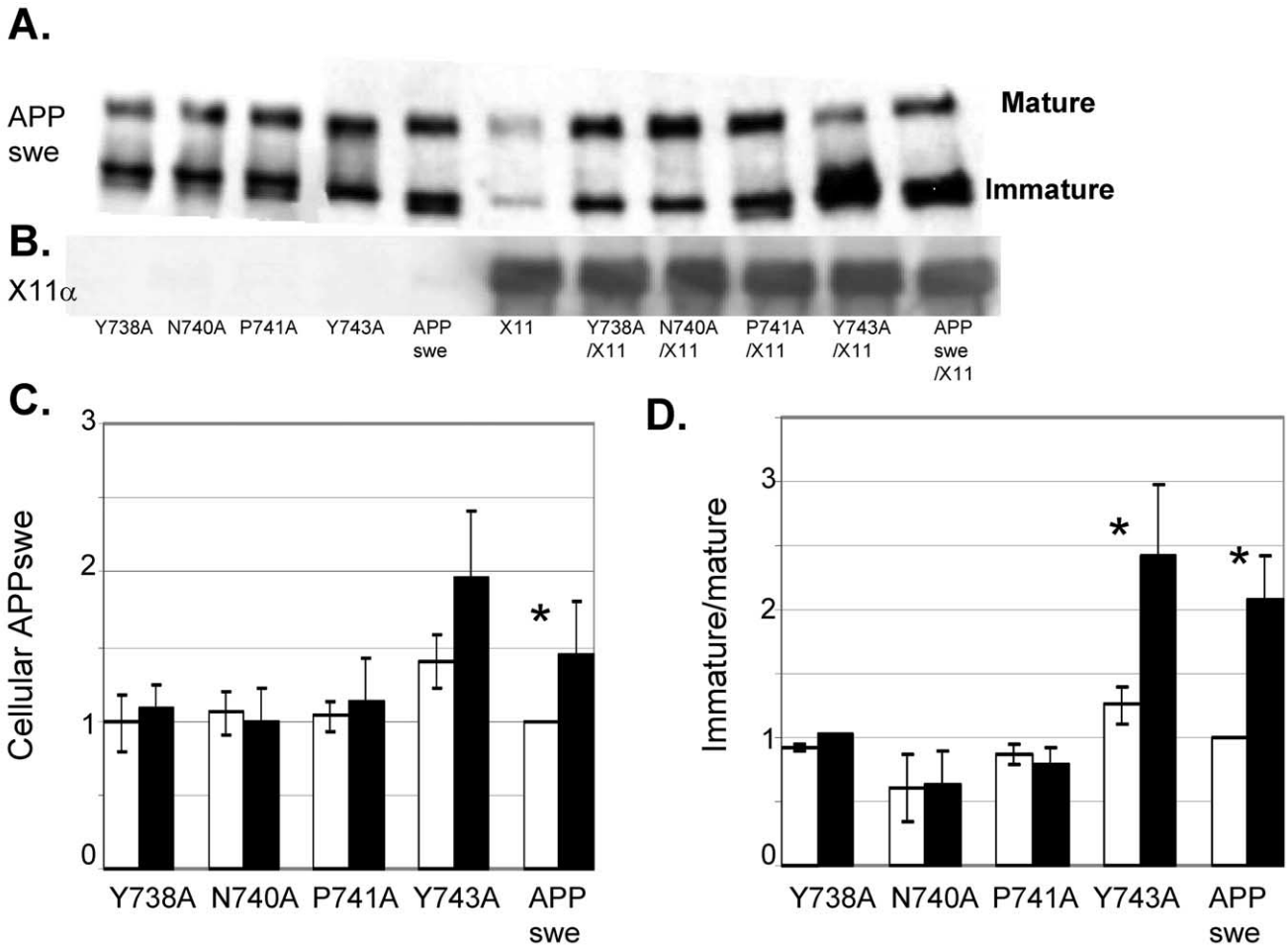

Fig. 3. X11α interaction retards APPswe and Y743A maturation. (A) HEK 293 cells were transiently transfected with the APPswe constructs indicated, with or without X11α. After 24 h of incubation, cells were lysed and proteins separated by 8% Tris–glycine SDS-PAGE. APPswe was detected by immunoblot (with 22C11) of separated proteins. Immature and mature indicate nascent and post-translationally modified (higher Mr) APPswe, respectively. (B) The immunoblot for X11α (with 9E10) confirmed its equivalent expression. (C) Quantitation of total APPswe in cell lysates, as shown in A, relative to APPswe expression alone. (D) Ratio of immature to mature APPswe in cell lysates, relative to APPswe. Protein bands were quantitated with a Molecular Dynamics STORM Scanner. White bars indicate transfection with APPswe or the APPswe mutations alone, and black bars represent their cotransfection with X11α. The data shown are mean±S.E.M; * indicates $P \leq 0.05$; $n=5$.

but not β-cleavage of APPswe by X11α, since Aβ but not APPsβswe secretion was inhibited when X11α was coexpressed (Fig. 5B–D). In contrast to APPswe, however, with Y743A both β- and γ-cleavage of APPswe were inhibited by X11α.

X11α inhibits endocytosis of cell surface APP

X11α normally interacts with the −YENP− internalization motif of APP suggesting a role for X11α in endocytic processing of APP, but such a role has not yet been demonstrated. To test the hypothesis that X11α affects internalization of APP, we utilized a cell surface labeling method (iodinated anti-APP antibody 22C11) in cells transiently transfected with APPswe with and without X11α. YENP mutations were not evaluated since binding to X11α is not significant and we have previously described their role in APP endocytosis (Perez et al., 1999). Likewise the Y743A mutation was not evaluated due to concerns stated above of a very small fraction reaching the cell surface. Compared with APPswe, X11α coexpression significantly diminished uptake of cell-surface APPswe (Fig. 6). Thus, these data are consistent with the notion that X11α binding to APPswe retarded its internalization from the cell surface and therefore endocytic processing.

DISCUSSION

We describe several novel observations on the regulation of APPswe trafficking and metabolism by interaction with X11α: 1) the APPswe Y743A mutation produced unique effects on its metabolism, 2) the −YXNP− tetrapeptide was essential for X11α binding and thus X11α-mediated effects on APPswe processing, 3) X11α coexpression impaired maturation of APPswe and APPswe Y743A, and 4)

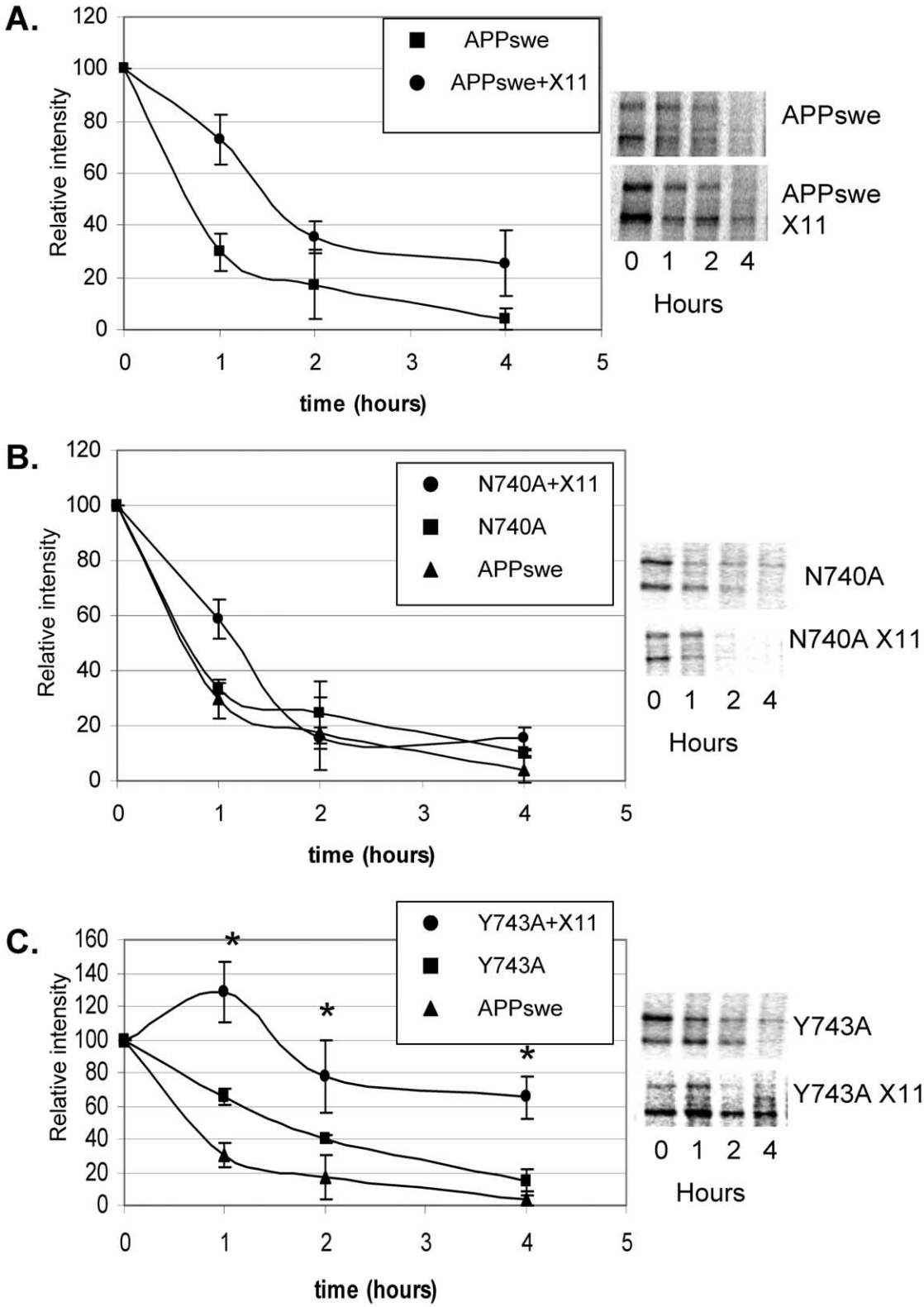

Fig. 4. X11α prolongs APPswe and Y743A half-life. HEK 293 cells were transfected 24 h before metabolic radiolabeling with [^{35}S]methionine for 1 h followed by a 0–4-h chase with excess unlabeled methionine. APPswe in cell lysates was immunoprecipitated (Biosource), separated by 8% Tris–glycine SDS-PAGE, and total APPswe (mature and immature) was quantitated with a Molecular Dynamics STORM Scanner and ImageQuant software. Pulse-chase data of (A) APPswe, (B) APPswe N740A, and (C) APPswe Y743A, with and without X11α, are indicated. Data shown are mean±S.E.M; * indicates $P \leq 0.05$; $n=5$.

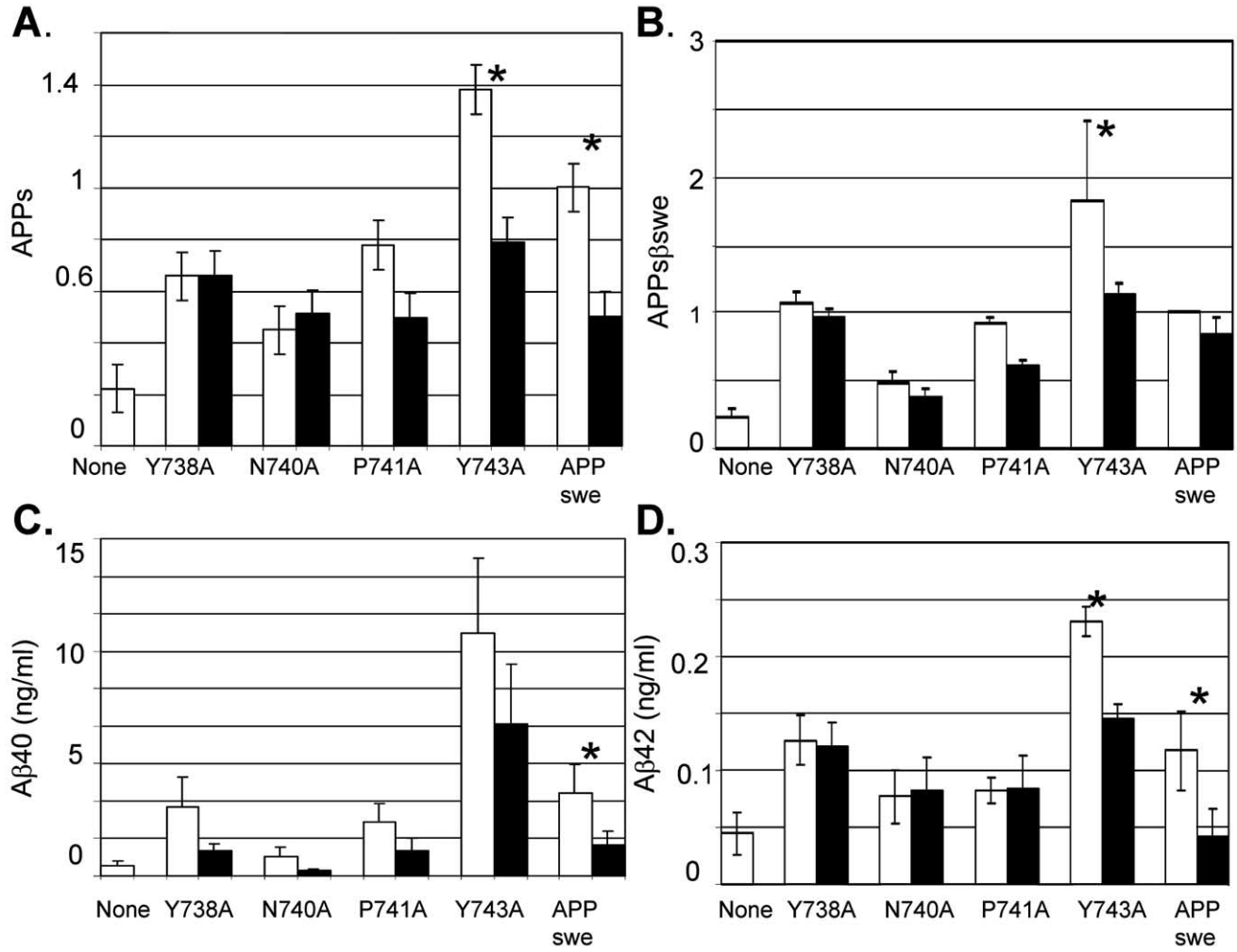

Fig. 5. Effects of X11α on APPs, APPsβswe, Aβ40, and Aβ42 secretion. HEK 293 cells were transiently transfected as indicated and incubated for 48 h. White bars represent APPswe or its mutations transfected alone, and black bars represent their cotransfection with X11α. Samples from conditioned media were subject to specific sandwich ELISA for (B) APPsβswe, (C) Aβ40, and (D) Aβ42. For (C) and (D), absorbance was converted to concentration (ng/ml) using standard curves. For (A), total APPs was immunoprecipitated with Karen, immunoblotted with 22C11, and quantitated by PhosphorImager analysis. The amount of APPs secreted by APPswe-transfected cells was set to 100%. Data shown are mean±S.E.M; * indicates $P \leq 0.05$; $n = 5–6$.

X11α coexpression decreased uptake of cell-surface APPswe. These results suggest that the structural requirements for APP endocytosis and X11α-mediated effects of APPswe metabolism may be identical. In other words, the −YENP− motif may serve as either a retention signal or an endocytic signal for APP depending on differential and competitive adaptor protein interactions. We observed that X11α modulates APPswe trafficking and metabolism in both the secretory and endocytic compartments (as summarized in Fig. 7). Because X11α had little or no effect on internalization-defective mutations of APPswe, we could not dissect the structural requirements for APP endocytosis (Perez et al., 1999) away from the requirements for X11α:APPswe binding and effects on APPswe trafficking and metabolism. X11α retarded both APPswe maturation and internalization, suggesting that X11α may inhibit Aβ40 and Aβ42 generation in either the secretory or endocytic compartments.

The absolutely conserved −YENPTY− motif in the C-termini of the APP gene family suggests a key role for this motif in trafficking, processing, and function. Tyrosine residues, in particular, are pivotal in the regulation of protein trafficking. Mutation of both tyrosine residues in the −GYENPTY− sequence markedly impairs APP endocytosis (Cescato et al., 2000). Mutation of the tyrosine residue within the sequence −YTSI− near the cell membrane abolishes basolateral sorting of APP in polarized cells (Bayer et al., 1999). Mutation of all three tyrosine residues within the −YTSI− and −GYENPTY− motifs impairs lysosomal targeting of APP (Lai et al., 1998) but this targeting appears to be mainly due to the distal tyrosine in the motif as we previously observed for wild type APP (Perez et al., 1999). Mutation of any residue in the −YENP− sequence significantly reduces APP endocytosis and inhibits Aβ secretion in wild type APP (Perez et al., 1999). We now extend these observations by mapping the inter-

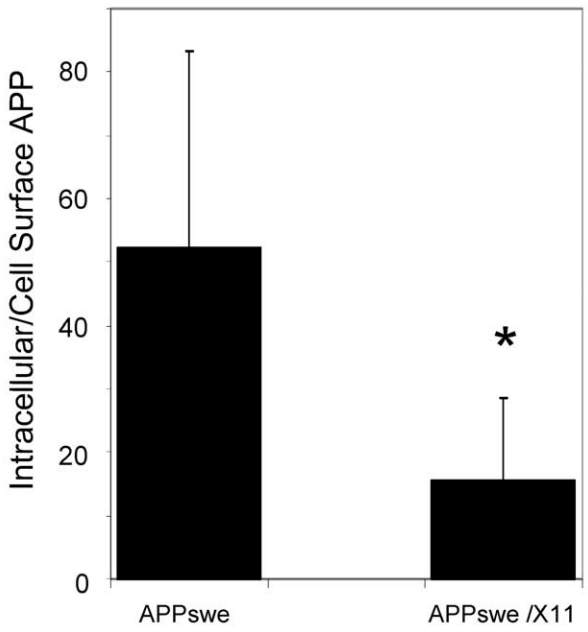

Fig. 6. X11α impairs APPswe internalization. HEK 293 cells were transiently transfected with APPswe alone or with X11α and incubated with [125]I-labeled anti-APP (22C11) for 30 min at 37 °C. Cell surface protein was measured by acid strip of cell surface proteins. Iodinated intracellular protein was measured after subsequent cell lysis. Data shown are mean±S.E.M; * indicates $P \leq 0.05$ as calculated by Mann-Whitney test.

action requirement for X11α to the −YENP− motif, and report for the first time the unique effects of the Y743A mutation on APPswe metabolism.

The PTB-domain binding proteins are recruited to the sequence −NPXpY− when the distal tyrosine residue is phosphorylated, thus mediating receptor tyrosine kinase-signal transduction pathways. When the distal tyrosine, Y743 in the −NPXY− sequence of APP is phosphorylated, the affinity of X11α binding is unaffected (Zhang et al., 1997) whereas the binding of *Dab1* is markedly impaired (Howell et al., 1999). Similar to the requirements for APP endocytosis (Perez et al., 1999) X11α interaction requires the −YXNP− and not the −NPXY− motif. Thus, phosphorylation of Y743 would be unlikely to regulate the APP interaction with X11α *in vivo*. In contrast, phosphorylation of Y738 by the non-receptor tyrosine kinase *abl* generates the −pYXXP− motif recognized by SH2-containing adaptor proteins such as *shc*, *abl*, and *Grb2* (Zambrano et al., 2001; Russo et al., 2002; Tarr et al., 2002). Phosphorylation of Y738 in APP may promote its interaction with SH2-domain containing adaptor proteins and simultaneously preclude its interaction with PTB-containing adaptors such as the X11 and Fe65 families. Thus, tyrosine phosphorylation may modulate APP trafficking, metabolism, and function by regulating the interaction of APP with different adaptor proteins.

There are several known interaction partners of the APP cytoplasmic tail (Okamoto et al., 1995, 1996; Trommsdorff et al., 1998; Zheng et al., 1998; Homayouni et al., 1999; Chen et al., 2000; Kamal et al., 2000; Yu et al., 2000; Gao and Pimplikar, 2001; Gunawardena and Goldstein, 2001; Matsuda et al., 2001; Scheinfeld et al., 2002), and all of these likely regulate APP trafficking and processing. While the X11 family retards APP catabolism and Aβ secretion (Borg et al., 1998b; Sastre et al., 1998; Mueller et al., 2000) the Fe65 family has opposite effects (Guenette et al., 1999; Sabo et al., 1999). Since both families interact with the APP C-terminus via a PTB domain, these opposing effects on APP metabolism may be mediated by their distinct sets of protein interaction domains. For example, the WW domain of Fe65 interacts with mena and the actin cytoskeleton; the non-APP interacting PTB domain of Fe65 binds to the LRP family of lipoprotein receptors (Trommsdorff et al., 1998) or to the transcription factor CP2/LSF/LBP1 (Zambrano et al., 1998). It is unknown whether X11α interacts with LRP. Fe65 enhances the effect of APP in acceleration of cell migration, perhaps by regulation of the amount of cell surface APP (Sabo et al., 2001). Fe65 may also stabilize and translocate the C-terminus of APP remaining after γ-secretase cleavage (the AICD, or APP intracytoplasmic domain) to the nucleus in a Notch-like manner, and thus regulate gene transcription (Cao and Sudhof, 2001; Cupers et al., 2001; Kimberly et al., 2001). Similar to opposing effects on APP catabolism, and perhaps because of this, X11α may have an opposite effect of Fe65 on this putative novel function of APP (Biederer et al., 2002).

Due to its localization and putative interaction partners, roles for X11α in pre- and post-synaptic structure and function are suggested in mammalian brain. The munc18-interaction, CASK-interaction, and PTB domains of X11α are specific with regard to their binding partners. However, the PDZ domains are promiscuous and interact with multiple partners, including presenilin-1 (Lau et al., 2000), a presynaptic voltage-gated calcium channel (Maximov et al., 1999), spinophilin/neurabin II (Ide et al., 1998), the copper chaperone of SOD1 (McLoughlin et al., 2001), the dendritic kinesin KIF-17 (and via X11α/CASK/Veli the *N*-methyl-D-aspartate receptor; Setou et al., 2000) and potentially with itself by dimerization of PDZ domains (Walhout et al., 2000). Many of these interaction partners mediate synaptic functions, again implying a scaffolding/adaptor role for X11α in the pre- and post-synaptic complex. In agreement with this hypothesis, X11α knockout mice exhibit impaired dopaminergic (Hase et al., 2002) and GABAergic neurotransmission (Ho et al., 2003).

We recognize the limitations of our study using transient transfection of APPswe in non-neuronal cells; however, our conclusions may extrapolate to the trafficking and metabolism of APP in neurons *in vivo*. X11α is expressed primarily in CNS neurons, and non-neuronal models of APP often extrapolate to neurons. APP:X11α proteins colocalize in non-neuronal cells and neurons, and this complex occurs in mouse brain homogenates, supporting a role for X11α in APP trafficking, metabolism, and function in neurons. Because of greater β-secretase expression in neurons and more efficient cleavage of APPswe by β-secretase (compared with wild type APP) the metabolism of APP in neuronal cells

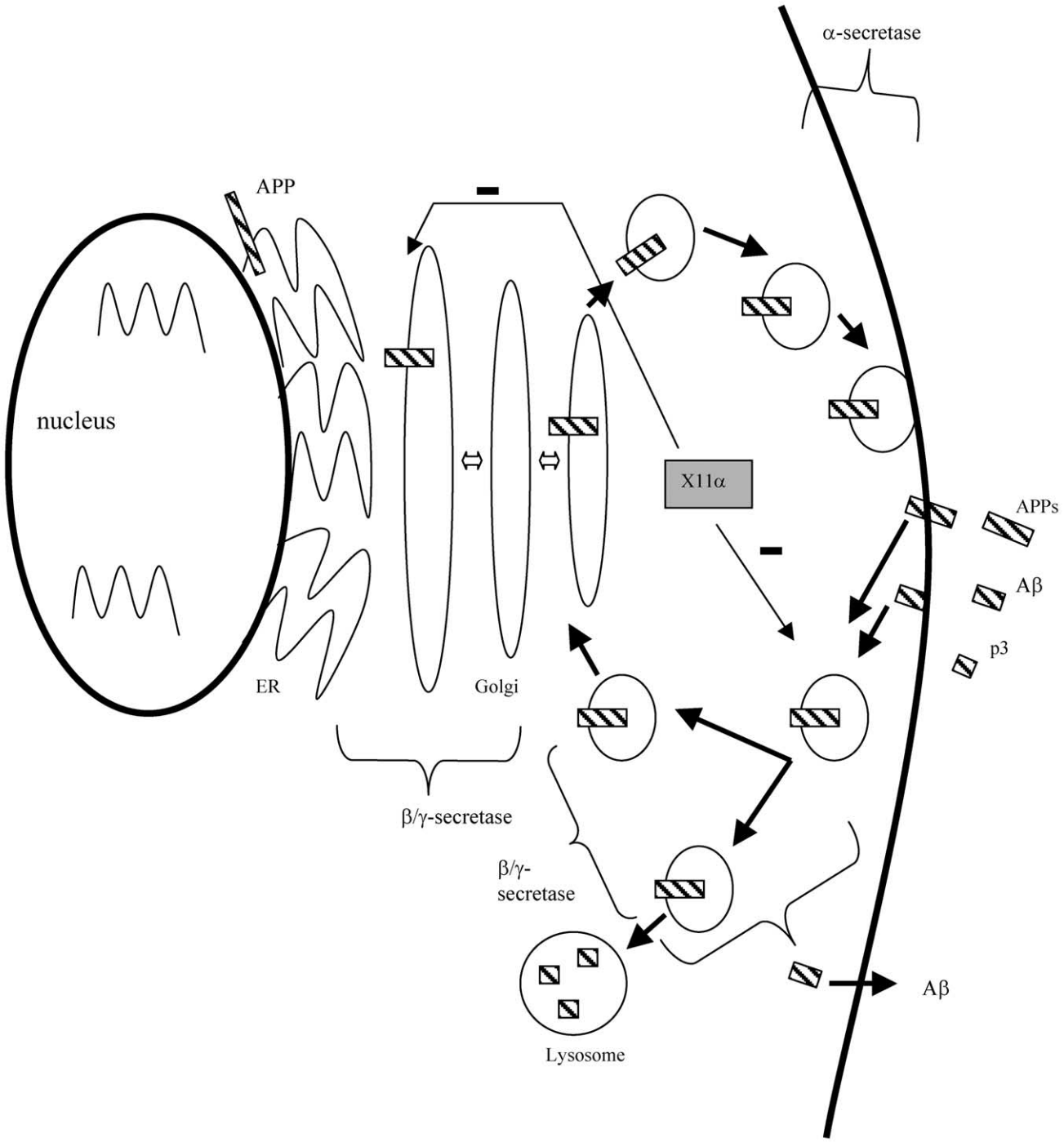

Fig. 7. X11α modulates APP metabolism in secretory and endocytic pathways. APP is cotranslationally translocated into the ER, undergoes post-translational modifications (maturation) including glycosylation in the ER and Golgi, and transits to the cell surface. APP also undergoes endosomal/lysosomal catabolism after internalization from the cell surface, although some APP may be diverted directly from the secretory pathway. While α-secretase cleavage occurs primarily at or near the cell surface in the secretory pathway, β- and γ-secretase cleavage may occur in secretory and endocytic cellular compartments. As shown by delayed maturation, X11α may retard egress of APP through the secretory pathway. X11α may also impair endocytic trafficking and processing of APP, perhaps due to competitive interactions with the −YENP− motif. Thus, X11α may inhibit APPswe metabolism in secretory and endocytic compartments and inhibit secretion of Aβ40 and Aβ42 generated by either pathway.

may be partially mimicked by APPswe in transfectable cell lines such as HEK 293 which possess much less β-secretase activity (Forman et al., 1998). However, proposed modulatory effects of X11α on APP trafficking, metabolism, and function in neurons in brain *in vivo* remain speculative.

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