The Intracellular Domain of the β-Amyloid Precursor Protein Is Stabilized by Fe65 and Translocates to the Nucleus in a Notch-like Manner*

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The β-amyloid precursor protein (APP) is a ubiquitous receptor-like molecule without a known function. However, the recent recognition that APP and Notch undergo highly similar proteolytic processing has suggested a potential signaling function for APP. After ligand binding, Notch is cleaved by the ADAM-17 metalloprotease followed by an intramembrane cleavage mediated by γ-secretase. The γ-secretase cut releases the Notch intracellular domain (NICD), which enters the nucleus and modulates transcription. Because APP is processed similarly by ADAM-17 and γ-secretase, we reasoned that the APP intracellular domain (AICD) has a role analogous to the NICD. We therefore generated a plasmid encoding the AICD sequence and studied the subcellular localization of the expressed protein (C60). Our results demonstrate that the cytoplasmic domain of APP is a highly labile fragment that is stabilized by forming complexes with Fe65 and can then enter the nucleus in neurons and non-neural cells. These findings strongly support the hypothesis that APP signals in the nucleus in a manner analogous to the function of Notch.

* This work was supported by National Institutes of Health Grant AG06173 (to D. J. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: APP, β-amyloid precursor protein; AD, Alzheimer’s disease; AICD, APP intracellular domain; Aβ, amyloid β-protein; NICD, Notch intracellular domain; CSL, CBP/Suppressor of Hairless/Lag-2 protein; PACE, polycyclamid gel electrophoresis; CHO, Chinese hamster ovary; DAPI, 4,6-diamidino-2-phenylindole; CTF, carboxy-terminal fragment; ADAM, protein containing a disintegrin and a metalloprotease; Tricine, N-tris(hydroxymethyl)methylglycine.

The β-amyloid precursor protein (APP) is a type I transmembrane protein that is proteolytically processed by three enzymatic activities (1). The large ectodomain is first cleaved at one of three sites close to the membrane by either α- or β-secretase to liberate a soluble APP extracellular piece (termed α- or β-APP, respectively). In doing so, α-secretase generates an 83-residue carboxy-terminal fragment (CTF) C83, whereas β-secretase cuts at the other two sites to generate an 89- or 99-residue CTF (C99 and C99, respectively) (1, 2). These 3 CTFs are retained in the membrane and become substrates for an unusual intramembrane cleavage mediated by γ-secretase, producing a heterogeneous set of products. The best characterized of these is the amyloid β-protein (Aβ) derived from C99, which accumulates to high abundance in senile plaques and appears to play a central role in the etiology of Alzheimer’s disease (AD) (1). Whereas the several Aβ species have been studied in great detail, the other products generated by γ-secretase have received scant attention. One fragment of particular interest is the APP intracellular domain (AICD), the ~6-kDa extreme C terminus of APP that results from the γ-secretase cleavage of the C83, C89, or C99 fragments.

The potential importance of AICD has recently been emphasized by the recognition of similarities between APP and another type I transmembrane protein, Notch (3). Notch is a cell surface receptor that plays a critical role in many cell fate decisions during embryogenesis and adulthood (4). Following binding to its prototypical ligand Delta, Notch undergoes two cleavages, the first of which is performed by the metalloprotease ADAM-17, also called tumor necrosis factor-α converting enzyme (5, 6). It is subsequently cut by the presenilin-dependent γ-secretase (7), releasing the Notch intracellular domain (NICD), a fragment analogous to AICD. After release from the membrane, the NICD fragment associates with the cellular factor CSL (for CBF1, Suppressor of Hairless and Lag-1) and translocates into the nucleus, where it alters gene transcription (8). APP is the only other known protein that shares similar sequential processing by ADAM-10 (9) or ADAM-17 (10) and γ-secretase (3). The striking similarities between Notch and APP proteolysis therefore raise the intriguing possibility that APP is a cell surface receptor that also signals via release and translocation of AICD into the nucleus.

In the absence of a defined functional assay for APP in vitro or in vivo, we reasoned that an analysis of exogenous AICD expression could provide insights into its potential role in transducing a signal from the cell surface. However, detection of free AICD in intact cells has been elusive, similar to the situation with NICD. Because the binding of the CSL protein to NICD stabilizes the latter (8), we hypothesized that an analogous mechanism might also apply for AICD. We therefore assessed whether a previously described protein, which binds to the APP cytoplasmic tail, Fe65, could stabilize AICD and allow nuclear translocation. Our results show that AICD complexes with and is stabilized by Fe65 in a manner analogous to the NICD and CSL proteins and that the complexes can enter the nucleus in both primary neurons and non-neural cells.

EXPERIMENTAL PROCEDURES

Plasmids, Transfections, and Cells—A cDNA encoding an initiating methionine and the last 59 residues of the APP C terminus was amplified by polymerase chain reaction, inserted into pcDNA5 (Invitrogen), and confirmed by sequencing. Plasmids encoding wild-type human APP695 (11) and human Fe65 with a Myc epitope (12) have been characterized. For transfections, 10 μg of DNA was introduced into COS cells (95% confluent) or mouse primary neurons (18,000 cells/cm²) using LipofectAMINE 2000 (Life Technologies, Inc.) according to the manufacturer’s instructions. Neurons were plated and maintained in neurobasal medium supplemented with B27, GlutaMax, and penicillin/streptomycin.
APP C60 Fragment Translocates to Nucleus

FIG. 1. The APP intracellular domain can be detected in vitro and in transfected cells. a, schematic depiction of APP CTFs generated by sequential secretase cleavages of APP. AICD results from the γ-secretase cleavage of either C83 (α-product) or C89 and C99 (β-product). b, AICD protein is generated from microsomal membranes. Microsomes of CHO cells stably overexpressing human APP695 were incubated at 37 °C for 0 or 4 h, lysed in 1% Nonidet P-40, and subjected to C7 immunoprecipitation followed by 13G8 Western blotting (lanes 1 and 2). The same reaction was performed in the presence of 50 μM compound 11 (18) or its vehicle (dimethyl sulfoxide (DMSO)). c, COS cells transiently expressing empty vector (V), human APP695 (APP), or C60. Cells were first lysed in 1% Nonidet P-40, and the soluble fraction was collected (lanes 1–3). The Nonidet P-40-insoluble pellet was sonicated in 1% SDS and diluted 10-fold with 1% Nonidet P-40 lysis buffer (lanes 4–6). Detection was as in b, d, marker proteins validate the fractionation method. COS cells were fractionated into membrane (M), cytosolic (C), and nuclear (N) fractions (see “Experimental Procedures”). PS1 NTF is exclusively a membrane-bound protein, tubulin is cytosolic, and histone H1 is associated with DNA in the nucleus. e, detection of C60 after transfection and nuclear fractionation of COS cells. A sample similar to lane 6 in c served as a positive size marker (lane 1). Endogenous C83 and C89 are located exclusively in the membrane fraction. Ig, immunoglobulin light chains.

RESULTS AND DISCUSSION

The APP Intracellular Domain Can Be Generated in a Cell-free Microsomal System and by Cellular Overexpression—The APP intracellular domain (AICD) corresponds to the cytoplasmic tail of APP that is released after cleavage of C83, C89, or C99 by γ-secretase (see Fig. 1a). In our search for the AICD fragment, we first attempted to generate it using a cell-free in vitro reaction. We had previously measured Aβ generation in microsome vesicles derived from Chinese hamster ovary (CHO) cells stably overexpressing APP751 (16). We therefore probed for de novo generation of AICD by similarly incubating microsomes at 37 °C. After the incubation, the microsomal membranes were lysed, precipitated with the APP antiserum C7, and resolved by 10–20% Tris/Triton SDS-PAGE. Identical preparations incubated in parallel were analyzed by a sensitive and specific sandwich enzyme-linked immunosorbent assay for Aβ, confirming the de novo production of Aβ (669 + 127 pg/ml (mean ± S.D.) in 4 h; n = 3). In these same reactions, we observed the de novo production of a 6-kDa C-terminal fragment of APP that was present only after the 4-h incubation at 37 °C (Fig. 1b, lane 2). Importantly, production of this fragment was specifically inhibited by a well characterized peptidomimetic inhibitor of γ-secretase, compound 11 (18) (Fig. 1b, lanes 3 and 4). Note that the Tris/Triton gel resolves the three membrane-bound APP CTFs (C83, C89, and C99), which are more readily visible in a shorter exposure (Fig. 1b, lane 5).

We next constructed a plasmid encoding the C-terminal 59 amino acids of APP plus an initiating methionine (C60). The expressed protein corresponds to the APP sequence starting after the principal γ-secretase cleavage site, i.e. after position 40 of Aβ. We transiently expressed this construct in COS cells and sequentially lysed them in 1% Nonidet P-40 followed by 1% SDS. In control COS transfectants expressing full-length APP695, an 8-kDa C-terminal fragment corresponding to C83 was readily seen in the Nonidet P-40-soluble fraction, and only a small amount was found in the Nonidet P-40-insoluble/SDS-soluble fraction (Fig. 1c, lanes 2 and 5). In the COS transfectants expressing C60, a faint ~6-kDa band was visible in the Nonidet P-40-soluble fraction; however, the vast majority of the C60 protein was found in the Nonidet P-40-insoluble pellet (Fig. 1c, compare lanes 3 and 6). Because SDS does not Nonidet P-40 readily dissolves nuclei, we postulated that most of the C60 protein was associated with the nuclei of these cells.

C60 Localizes Primarily to the Nucleus and Is Stabilized by Co-expression of Fe65—To establish the subcellular localization of the expressed C60 protein, we performed a nuclear fractionation technique described previously (17). Following hypotonic swelling, we subjected the transfected COS cells to Dounce homogenization and pelleted their nuclei. After washing the

basal media supplemented with B27 (Life Technologies, Inc.) using standard methods (13).

Antibodies—Polyclonal antibodies C7 and X81 were described previously (Refs. 14 and 15, respectively). Monoclonal antibodies 13G8 and 8E5 were gifts of P. Seubert and D. Schenk and are directed against APP, respectively. Polyclonal antibody C4 is raised against APP222–231 and was a gift of Y. Ibarra. Monoclonal antibodies 9E10 (which recognizes the e-Myc epitope) and histone H1 were purchased from Santa Cruz Biotechnology, and polyclonal tubulin antibody was from Sigma.

In Vitro Generation of AICD and Nuclear Fractionation—De novo generation of Aβ and AICD was performed essentially as described (16). The membrane pellet (microsomes) was resuspended in 50 mm Tris, pH 7.0, 150 mm NaCl, 1 mm EDTA. The resuspended vesicles were incubated for 4 h at 37 °C, and the reaction stopped with Nonidet P-40 at a final concentration of 1%. Experiments were performed in 3 independent replicates. The nuclear fractionation of whole cells was performed as described (17). Following Dounce homogenization, nuclei were pelleted at 800 × g and washed three times in wash buffer containing 0.1% Nonidet P-40. In the postnuclear supernatant, membranes were separated from cytosol with a 100,000 × g spin for 45 min.

Inmunoprecipitation and Western Blotting—Cells were solubilized in 100 μl of an SDS lysis buffer containing 50 mm Tris, pH 7.6, 150 mm NaCl, 2 mm EDTA, 1% SDS, and Complete protease inhibitors (Roche Molecular Biochemicals). Lysates were sonicated at 4 °C for three 30-s pulses and then diluted 1:9 with a detergent buffer identical to 1% SDS lysis buffer except that it contained 1% Nonidet P-40 instead of SDS. Some samples were lysed directly in 1% Nonidet P-40 lysis buffer. Lysates were precipitated with C7 (for APP, 1:200) or 9E10 (for Fe65, 1:100) and 50 μl of protein A-Sepharose (Sigma) or protein G-agarose (Roche) for more than 2 h at 4 °C. Following extensive washing, samples were eluted in 2× sample buffer (20% glycerol, 4% SDS, 10% β-mercaptoethanol) at 100 °C for 5 min, resolved by 10–20% Tris/Tricine SDS-PAGE (Bio-Rad), and transferred to 0.2 μm polyvinylidene difluoride (Bio-Rad). Western blotting using ECL Plus detection was performed according to the manufacturer’s instructions (Amersham Pharmacia Biotech). All experiments were repeated at least three times.

Immunocytochemistry—Cells plated on 18-mm coverglass were fixed in 3% paraformaldehyde and then blocked with 5% goat serum in phosphate-buffered saline containing 0.1% Triton X-100 for 1 h. Primary antibodies (C7, 1:2000; C4, 1:2000; 9E10, 1:1000) were diluted in the blocking buffer and incubated for 2 h. Following three 5-min washes with phosphate-buffered saline, secondary antibodies (goat anti-rabbit Cy3, 1:400; goat anti-mouse FITC, 1:200; Jackson ImmunoResearch) were incubated for 1 h at room temperature. Nuclei were stained with 1 μg/ml DAPI for 30 min and mounted with SlowFade Light anti-fade reagent (Molecular Probes). Images were collected with an Axiovert 100M confocal scanning microscope and analyzed with LSM 510 software (Zeiss).
nuclear pellets with 0.1% Nonidet P-40 to dissolve associated membranes, the nuclei were lysed in 1% SDS. The postnuclear supernatants were spun at 100,000 × g to separate membranes from cytosol. The localization of three protein markers confirmed the validity of the fractionation technique (Fig. 1d). We therefore used this method to analyze the location of C60 in transfected COS cells by immunoprecipitation and Western blotting. First, endogenous APP CTFs, principally corresponding to C89 and C83, exclusively localized to the membrane fraction, as expected (Fig. 1e, lanes 2, 5, 8, and 11). This result confirmed that little or no membrane protein contaminated the nuclear fraction. In cells transfected with C60, a majority of the expressed protein was localized to the nucleus (Fig. 1e, lane 10) with a small amount associating with the membrane fraction (lane 8).

Because the CSL protein is known to stabilize the NICD fragment (8), we reasoned that one of the APP cytoplasmic binding partners might perform a similar function for APP. Although there are several proteins that are reported to bind to APP (19), we focused our efforts on Fe65 because it is a prototypical member of a family of genes that alters APP processing (20–22), localizes to the nucleus (22), and binds to the CP2 transcription factor (23). Indeed, when we co-transfected C60 with Fe65, we observed a marked increase in C60 levels in all three subcellular compartments (Fig. 1e, lanes 11–13). Fe65 was found in the same three subcellular fractions (data not shown), in agreement with previously published observations (22). However, endogenous AICD was not detected when Fe65 was overexpressed alone (Fig. 1e, lanes 5–7). In fact, even the transient co-expression of Fe65 with APP770 did not allow visualization of an AICD fragment (data not shown), suggesting that the latter is degraded with great efficiency and cannot be detected by this technique.

To establish more directly the subcellular localization of C60, we used confocal immunofluorescent microscopy to visualize intact cells. When C60 was transfected alone, we were unable to identify any cells that displayed C7 fluorescence above the background level (Fig. 2, m and n). Samples transfected in parallel and analyzed by immunoprecipitation-Western blotting confirmed successful expression of C60, suggesting that confocal microscopy is less sensitive than Western blotting. Because C60 expression alone yields far lower levels of the protein than does co-expression of C60 and Fe65 (see Fig. 1e), we co-transfected C60 with Fe65. We double-stained cells for the C terminus of APP and Fe65 followed by counterstaining with DAPI to visualize all nuclei. Under these conditions, specific immunoreactivity for C60 was localized primarily to the nucleus but was also present in the cytoplasm (Fig. 2, a–d). Furthermore, Fe65 was readily detectable (Fig. 2h), and when this signal was merged with the C7 fluorescence, the two patterns overlapped to a great extent (Fig. 2c). As further confirmation, this pattern was reproduced identically with a different C-terminal-specific APP antibody, C4 (Fig. 2, e–h).

To control rigorously for the specificity of this staining, we preabsorbed C7 with its peptide immunogen, which eliminated the C7-specific immunofluorescence without affecting Fe65 detection (Fig. 2, i–j). Additionally, when the C7 and Fe65 primary antibodies were omitted, we observed no appreciable fluorescence (Fig. 2, k and l). Finally, we confirmed that Fe65 subcellular localization is independent of C60 because it retained the same expression pattern in the presence or absence of C60 co-transfection (Fig. 2, compare b with a).

Fe65 Binds to the C60 Protein and Markedly Enhances Its Half-life—We next assessed whether Fe65 can bind to the C60 fragment. Previous work demonstrated that Fe65 binds to the C terminus of full-length APP (24). We therefore performed co-immunoprecipitations in COS cells expressing both C60 and Fe65. Immunoprecipitating Fe65 via its Myc tag and then Western blotting for APP with C7 revealed a 6-kDa band only when the two proteins were co-expressed (Fig. 3a, compare lane 4 with lanes 2 and 3). This was confirmed when the order of the antibodies was reversed; immunoprecipitating with an APP-specific antibody and Western blotting with an anti-Myc antibody yielded detectable Fe65 when the two proteins were co-expressed (Fig. 3a, lane 7). Here, we also noticed faint co-immunoprecipitation of Fe65 when it was expressed alone (Fig. 3a, lane 6). Because the precipitating antibody (C7) also recognizes full-length endogenous APP, we interpreted this result to mean that a small amount of Fe65 co-precipitated with endogenous APP. In accord, we found that C7 equally precipitated endogenous APP holoprotein in all three transfection conditions (data not shown). Therefore, these co-immunoprecipitations confirm published evidence that Fe65 binds to APP and demonstrate that this interaction is maintained when just the C60 fragment is expressed.

To address the effect of Fe65 on C60 stability, we performed quantitative pulse-chase analysis. COS cells were incubated with [35S]methionine for 10 min and chased in cold media for various times, followed by immunoprecipitation of C60. To accurately compare the differences in half-life, we used phosphorimaging quantitation followed by graphical analysis. Fig. 3b shows the quantitative results from three independent experiments, depicting an exponential decrease in the level of C60 when expressed alone. A slower pattern of decay was observed with Fe65 co-transfection. We calculated its half-life to be ~10 min when expressed alone but 188 min when Fe65 was also present. Furthermore, the latter curve reveals that a small fraction (~20%) of the C60 protein is more stable (present at chase times >8 h) in the presence of Fe65.

Expression of C60 in Primary Mouse Neurons Is Similar to COS Cells—Although APP is expressed in many tissues in the
AICD generated from full-length APP. A CHO cell line stably expressing CTFs are detected in membranes, not in cytosol or nuclei. Nuclear fractions of double-transfected cells (lanes 2) in membrane, cytosolic, and nuclear fractions (right panel) co-migrates with COS C60 (lanes 2–4), in agreement with the COS results (Fig. 1c, lanes 11–13), although we obtained relatively less nuclear C60 in the neurons.

Having established the importance of Fe65, we next asked whether transient expression of this protein would aid the detection of endogenous AICD derived from full-length APP. To this end, we transiently transfected a CHO cell line that stably overexpresses APP<sub>751</sub> with the V717I mutation (7PA2 cells) (27) with empty vector or Fe65. When vector was transfected, a very faint ~6-kDa band was visible (Fig. 3c, lane 2), which was stabilized by Fe65 transient overexpression (lane 3). Importantly, we obtained similar results when Fe65 was transfected into primary mouse neurons, which express endogenous levels of APP<sub>695</sub> (Fig. 3c, lanes 4 and 5).

In this study, we provide evidence that supports the hypothesis that the free APP intracellular domain (AICD) created by γ-secretase cleavage is markedly stabilized by and reaches the nucleus with the adaptor protein, Fe65. The potential importance of the Fe65-C60 complex that our co-precipitation experiments document in intact cells is highlighted by its striking similarity to CSL and NICD in Notch processing. Both APP and Notch are cleaved precisely 12 amino acids outside of the membrane by an α-secretase activity mediated by ADAM proteases. These scissors release the respective ectodomains, apparently allowing the membrane-retained, C-terminal fragments to be cleaved by γ-secretase. This intramembranous cleavage is highly unusual and is shared by few other proteins (28).

The highly labile NICD fragment then binds to the CSL protein and translocates into the nucleus where it regulates the transcription of downstream genes. The detection of NICD is improved by the co-expression of CSL (8). Our results indicate that C60 and Fe65 have an analogous relationship: C60 is highly labile and binds Fe65, which, like CSL, is present in the cytoplasm and nucleus. Furthermore, Fe65 markedly stabilizes and promotes nuclear translocation of C60 in a manner similar to CSL and NICD. Although our results support an important role for Fe65, other binding partners may also affect AICD production, stability, and/or location. Indeed, certain other binding partners, e.g. X11a, can alter APP metabolism when transiently overexpressed (29). It is possible that a dynamic balance among several proteins capable of binding the cytoplasmic domain of APP may impact on its proteolytic fate and function.

Our findings in this regard are consistent with recent evidence in cell transfection studies that the cytoplasmic domain of chimeric APP molecules can participate in Fe65-containing complexes, which drive transcription of heterologous reporter genes in the nucleus (30). Our demonstration that the free cytoplasmic domain generated by γ-secretase cleavage from full-length APP is detectable in cells after stabilization by Fe65 transfection supports the model proposed by Cao and Sudhof (30) that sequential α- and γ-secretase cleavages serve to release the cytoplasmic domain to signal in the nucleus. Taken together, the emerging data highlight a novel mechanism of APP function that could explain previously identified roles for cell-surface APP in neurite extension and growth (31–33).

In summary, our studies of the APP cytoplasmic domain are

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...adult, it is particularly enriched in neurons (1, 25). Fe65 is also predominantly expressed in the brain (26), suggesting that the physiological location and action of these two proteins resides in neurons. We therefore asked whether C60 protein was detectable when transfected into cultured primary neurons. We transfected the 3- or 4-day-old mouse neurons with C60 (with or without Fe65) and analyzed the lysates by immunoprecipitation and Western blotting (Fig. 3c). The results were qualitatively similar to those seen with COS cells. Although the transfected C60 alone resulted in undetectable C60 expression (Fig. 3c, lane 4), co-transfection with Fe65 resulted in the appearance of a ~6-kDa band (lane 5). The relative difference in the amount of C60 detected in COS cells and neurons is likely due to the relative transfection efficiencies in these two cell types. Therefore, as in COS cells, Fe65 co-expression stabilizes the otherwise rapidly degraded C60 protein. Because the C terminus of APP is known to be phosphorylated in neurons (2), we also detected several endogenous CTF bands, corresponding to the predominant neuronal α- and β-secretase products, C83 and C89, in both their non-phosphorylated and phosphorylated forms (Fig. 3c, lanes 2–5).

To establish the subcellular localization of C60 in neurons, we performed nuclear fractionation on the transfected neurons. As with the COS cells, we found endogenous APP C83 and C89 to be located exclusively in the membrane fraction, confirming the validity of our fractionation technique (Fig. 3d, lane 2). Co-transfection of C60 and Fe65 and subsequent fractionation led to the detection of the ~6-kDa C60 fragment in all three subcellular compartments (Fig. 3d, lanes 2–4), in agreement with the COS results (Fig. 1c, lanes 11–13), although we obtained relatively less nuclear C60 in the neurons.

Importantly, similar results were achieved from endogenous APP in primary mouse neurons (lanes 4 and 5).
consistent with rapidly emerging evidence that a primary function of APP may be as a receptor that signals through cleavage and nuclear translocation of its cytoplasmic tail together with adaptor proteins that include Fe65. Such a mechanism shows striking analogy to that of the Notch receptor. These two proteins, along with a sterol regulatory element-binding protein, appear to define a new class of receptor molecules that may signal following regulated cleavage within their transmembrane domains.

Acknowledgments—We thank Y. Ihara for the kind gift of antibody C4 and D. McLoughlin for the Fe65 clone. We are grateful to S. Vassquez for technical assistance with the mouse neuronal cultures and are indebted to D. Walsh, M. Wolfe, and M. Schlossmacher for invaluable discussions.

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