Communication

Intracellular Accumulation of β-Amyloid in Cells Expressing the Swedish Mutant Amyloid Precursor Protein*

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β-Amyloid (βA) is a normal metabolic product of the amyloid precursor protein (APP) that accumulates in senile plaques in Alzheimer’s disease. Cells that express the Swedish mutant APP (Sw-APP) associated with early onset Alzheimer’s disease overproduce βA. In this report, we show that expression of Sw-APP gives rise to cell-associated βA, which is not detected in cells that express wild-type APP. Cell-associated βA is rapidly generated, is trypsin-resistant, and is not derived from βA uptake, indicating that it is generated from intracellular processing of Sw-APP. Intracellular and secreted βA are produced with different kinetics. The generation of intracellular βA is partially resistant to monensin and a 20°C temperature block but is completely inhibited by brefeldin A, suggesting that it occurs in the Golgi complex. Monensin, brefeldin A, and a 20°C temperature block almost completely inhibit βA secretion without causing increased cellular retention of βA, suggesting that secreted βA is generated in a post-Golgi compartment. These results suggest that the metabolism of Sw-APP gives rise to intracellular and secreted forms of βA through distinct processing pathways. Pathological conditions may therefore alter both the level and sites of accumulation of βA. It remains to be determined whether the intracellular form of βA plays a role in the formation of amyloid plaques.

The deposition of β-amyloid (βA) in senile plaques is a pathological hallmark of Alzheimer’s disease (Glenner and Wong, 1984; Masters et al., 1985). βA is a normal product of the metabolism of amyloid precursor protein (APP) in the secretory pathway (Haass et al., 1992; Seubert et al., 1992; Shoji et al., 1992; Busciglio et al., 1993). Mutations in APP that are linked to autosomal dominant inheritance of Alzheimer’s disease have been found to alter the production of βA. Mutation of the two amino acids proximal to the N terminus of βA has been described in individuals from two Swedish families that develop early onset Alzheimer’s disease (Mullan et al., 1992). Expression of APP containing the Swedish mutation (Sw-APP) in transfected cells increases the production of βA by about 5-fold, suggesting a causal link between altered APP processing and the development of Alzheimer’s disease (Citron et al., 1992; Cai et al., 1993). In this report, we show that the Swedish APP mutation not only increases βA production but also results in abnormal intracellular accumulation of βA. The processing pathways that give rise to intracellular and secreted βA can be distinguished by their differential kinetics and sensitivities to metabolic inhibitors and temperature block.

EXPERIMENTAL PROCEDURES

Metabolic Labeling and Immunoprecipitation—COS-1 cells maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) were transiently transfected with expression plasmids encoding APP695, APP751, and APP751 containing the Swedish mutation (KM changed to NL immediately N-terminal to the APP695 sequence, designated Sw-APP) (Mullan et al., 1992). HEK 293 cells stably transfected with the APP695 cDNA containing the Swedish mutation were maintained in DMEM with 10% FCS and 0.25 mg/ml G418. Transfected COS cells were metabolically labeled with 125 μCi/ml [35S]methionine, and immunoprecipitations were performed as described previously (Busciglio et al., 1993). For immunoprecipitation of cell-associated βA, three semi-confluent 10-cm plates of transfected COS cells were lysed and combined, and the supernatant of the 100,000 g centrifugation was immunoprecipitated. APP was immunoprecipitated with a polyclonal antibody to the 20 C-terminal residues of APP (RG117, 1:200) and resolved by 10% Tris-glycine SDS-polyacrylamide gel electrophoresis. βA was immunoprecipitated with a polyclonal antibody to synthetic 1–40 (1:200 in culture medium and 1:30 in cell lysates) and resolved by 10–20% Tris-Tricine SDS-polyacrylamide gel electrophoresis. Gel loading was normalized to the protein content of the cell lysate (Bio-Rad protein assay), and quantitation was performed by PhosphorImager scanning. The specificity of these antibodies and the protocol for peptide preabsorption have been described previously (Busciglio et al., 1993). For pulse-chase analysis, stably transfected HEK 293 cells were labeled with 100 μCi/ml [35S]cysteine/methionine in cysteine/methionine-free DMEM with 2% dialyzed FCS for 20 min and then chased in DMEM with 2% FCS and 1 mM cysteine/methionine.

βA was immunoprecipitated with 5 μg of purified mouse monoclonal antibody 11 residues 1–40 of βA (Paganetti and Scheller, 1994). Quantitation of pulse-chase results was performed by direct counting of the gels using an InstantImager (Canberra Packard). Values were normalized for the number of cysteine and methionine residues in the different APP metabolites.

Treatments—The 4°C temperature block was performed by pulse labeling of transfected cells for 10 min at 37°C followed by a 90-min chase in unlabeled DMEM at either 4°C or 25°C. Trypsinization of cell surface βA was performed by labeling transfected cells for 16 h followed by addition of 0.25% trypsin, 0.02% EDTA for 10–20 min at 37°C. The reaction was stopped by 10-fold dilution in cold phosphate-buffered saline containing 5 μg/ml phenylmethylsulfonyl fluoride. The cells were centrifuged at 500 × g and washed with phosphate-buffered saline, and the cell pellet was lysed and immunoprecipitated. Brefeldin A and monensin were preincubated with transfected COS cells in methionine-free medium for 60 min followed by a 2-h labeling period in the presence of the drugs. The 20°C temperature block was performed by preincubation in methionine-free DMEM for 1 h at 20°C followed by labeling with 150 μCi/ml [35S]methionine for 3 h at 20°C using a 20°C water bath in a 4°C cold room.

RESULTS

COS cells were transiently transfected with wild-type APP695 and APP751 and Sw-APP751 cDNAs and metabolically labeled followed by immunoprecipitation of βA and APP from...
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The level of cell-associated βA in the medium was only slightly decreased after this incubation period (Fig. 1E). A low level of radiolabeled βA was detected in association with non-transfected cells after incubation with radiolabeled βA but did not exceed 20% of the level of cell-associated βA in Sw-APP-transfected cells (Fig. 1E). Thus, most of the intracellular βA produced from Sw-APP is not derived by uptake of βA from the medium. These results suggest that cell-associated βA is intracellular and is predominantly generated from intracellular processing of Sw-APP.

The kinetics of production of intracellular and secreted βA were examined using pulse-chase labeling of HEK 293 cells stably transfected with Sw-APP. Intracellular and secreted βA both reached a maximum of about 15% of secreted βA at 60-min chase time. We also examined the time course of appearance of the 11.5- and 9-kDa C-terminal fragments of APP, which are metabolic intermediates in the processing of APP to βA and the 3-kDa peptide, respectively (Busciglio et al., 1993; Haass et al., 1995). The 11.5-kDa fragment appeared by 15-min chase time, which slightly preceded the appearance of intracellular and secreted βA (Fig. 2, A and B). Intracellular βA reached a maximum of about 15% of secreted βA at 60-min chase time. We also examined the time course of appearance of the 11.5- and 9-kDa C-terminal fragments of APP, which are metabolic intermediates in the processing of APP to βA and the 3-kDa peptide, respectively (Busciglio et al., 1993; Haass et al., 1993; Higaki et al., 1995).

The kinetics of production of intracellular and secreted βA were examined using pulse-chase labeling of HEK 293 cells stably transfected with Sw-APP. Intracellular and secreted βA both appeared by 30 min of chase time (Fig. 2, A and B). Intracellular βA continued to accumulate up to 60- and 60-min chase times and then declined by 120 min. In contrast, βA in the medium continued to accumulate up to 120-min chase time (Fig. 2, A and B). Intracellular βA reached a maximum of about 15% of secreted βA at 60-min chase time. We also examined the time course of appearance of the 11.5- and 9-kDa C-terminal fragments of APP, which are metabolic intermediates in the processing of APP to βA and the 3-kDa peptide, respectively (Busciglio et al., 1993; Haass et al., 1993; Higaki et al., 1995).

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and transport in the secretory pathway. Pretreatment of cells with brefeldin A causes resorption of the proximal Golgi into the endoplasmic reticulum and inhibits anterograde transport and maturation of APP in the secretory pathway (Caporaso et al., 1992; Gabuzda et al., 1994). Brefeldin A completely inhibited the production of both intracellular and secreted βA from Sw-APP, suggesting that these processing steps are unlikely to occur in the endoplasmic reticulum or proximal Golgi (Fig. 3A). We also examined the effects of the ionophore monensin, which inhibits the maturation of newly synthesized proteins at the trans-Golgi and their transport past the trans-Golgi network (Tartakoff, 1983). Incubation with 7.5 μM monensin almost completely inhibited βA secretion in both Sw-APP and wild-type APP-expressing cells but only partially inhibited the generation of intracellular βA in cells expressing Sw-APP (Fig. 3A). PhosphorImager analysis showed that monensin inhibited βA secretion by 99 ± 0.2% but inhibited the generation of intracellular βA by only 49 ± 5%. A monensin dose-response analysis showed that βA secretion was almost completely inhibited by 2.5 μM monensin, whereas intracellular βA production was only partially inhibited by 2.5 μM monensin, and no further inhibition was evident up to 10 μM monensin (Fig. 3B). Cell-associated APP increased in monensin-treated cells, as previously reported (Gabuzda et al., 1994). The differential sensitivity of intracellular βA to monensin and brefeldin A is consistent with a site of generation in the trans-Golgi. In contrast, the inhibition of βA secretion by monensin, without increased cellular retention of βA, is consistent with the generation of secreted βA in a post-Golgi compartment.

To further assess the site of intracellular βA generation, we examined the effects of a 20°C temperature block, which results in protein retention in the trans-Golgi (Matlin and Simons, 1983). APP synthesis occurred at 20°C but was significantly reduced (data not shown). Hence, determinations of βA were normalized for the level of APP. The 20°C temperature block almost completely inhibited βA secretion but only partially inhibited the generation of intracellular βA (Fig. 3C). The level of intracellular βA generated at 20°C was similar to that observed in the presence of monensin (Fig. 3, B and C). These results provide additional evidence that intracellular and secreted βA are generated at distinct sites in Golgi and post-Golgi compartments, respectively.

**Fig. 2.** Pulse-chase kinetic analysis of Sw-APP metabolism to secreted and intracellular βA and C-terminal APP fragments. A, intracellular (IC) and secreted (SEC) 4-kDa βA and the 11.5-kDa C-terminal APP fragment (CTD) after 20 min of pulse labeling followed by chase in unlabeled medium for the indicated times. Shown are fluorographs of 10% Tris-Tricine (IC) or 13% Tris-Tricine (SEC) SDS gels. B, quantitative pulse-chase analysis of the appearance of intracellular (●) and secreted βA (○) and the 11.5-kDa C-terminal fragment (■). Values are the mean ± S.E.; n = 3. A and B are from separate experiments.

**Fig. 3.** Effects of monensin, brefeldin A, and a 20°C temperature block on the generation of intracellular and secreted βA. A, PhosphorImager scans of intracellular βA (IC) and secreted βA (SEC) in COS cells expressing wild-type or Swedish mutant APP under control conditions (CNTL) and after treatment with 3 μM brefeldin A (BFA) or 7.5 μM monensin (MON). B, monensin dose response of intracellular and secreted βA. Note that monensin almost completely inhibits secretion of βA but only partially inhibits generation of intracellular βA. Values are expressed as percent of IC βA in the absence of monensin (control) and represent the mean ± S.E. of three independent experiments. C, incubation at 20°C almost completely inhibits generation of secreted βA but only partially inhibits generation of intracellular βA. Values are molar ratios of βA:APP normalized to the 37°C value and represent the mean ± S.E., n = 3. * p < 0.01 relative to control by ANOVA.
DISCUSSION

These experiments suggest that APP harboring the Swedish mutation is processed to βA at an early step in the secretory pathway giving rise to a stable intracellular pool of βA. Hence, the Swedish mutation results in both increased secretion and intracellular accumulation of βA. Although intracellular βA is a small fraction of the total βA produced, it may nevertheless play a potentially important pathogenic role in plaque formation. The appearance of βA in a cell-associated form has also been observed in a neuronal cell line (Wertkin et al., 1993). Although we have not observed intracellular βA in transfected cells that overexpress wild-type APP, we cannot exclude the possibility that there is a small pool that is below the limits of resolution. Nevertheless, our results demonstrate that the Swedish mutant APP gives rise to significantly increased accumulation of intracellular βA.

Several lines of evidence suggest that the intracellular and secreted forms of βA arise through distinct processing pathways. First, the kinetics of generation of intracellular and secreted forms of βA is processed to an intracellular compartment, which is distinct from the Golgi site of generation. Secretion of βA is altered in MDCK cells expressing Swedish mutant APP (Gabuzda et al., 1994). Hence, a variety of genetic and non-genetic pathological situations can alter the processing of APP and affect both the level and cellular sites of accumulation of βA. It remains to be determined whether intracellular accumulation of βA predisposes to βA aggregation and plays a role in the formation of amyloid plaques.

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