Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 742–747, January 1999
Neurobiology

Endoplasmic reticulum and trans-Golgi network generate distinct populations of Alzheimer β-amyloid peptides

JEFFREY P. GREENFIELD*†, JULIA TSAI†, GUNNAR K. GOURAS*†, BING HAI*, GOPAL THINAKARAN‡, FREDERIC CHECLER§, SANGRAM S. SISODIA‡, PAUL GREENGARD*, AND HUAXI XU*¶

*Laboratory of Molecular and Cellular Neuroscience, and Fisher Center for Research on Alzheimer Disease, The Rockefeller University, 1230 York Avenue, New York, NY 10021; †Department of Neurology and Neuroscience, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10021; §Institute de Pharmacologie Moleculaire et Cellulaire, 06560 Valbonne, France; and ‡Department of Pharmacological and Physiological Science, University of Chicago, 947 East 58th Street, Chicago, IL 60637

Contributed by Paul Greengard, October 6, 1998

ABSTRACT **The excessive generation and accumulation of 40- and 42-aa β-amyloid peptides ($A\beta_{40}$/$A\beta_{42}$) in selectively vulnerable brain regions is a major neuropathological feature of Alzheimer's disease. Aβ, derived by proteolytic cleavage from the β-amyloid precursor protein (βAPP), is normally secreted. However, recent evidence suggests that significant levels of Aβ also may remain inside cells. Here, we have investigated the subcellular compartments within which distinct amyloid species are generated and the compartments from which they are secreted. Three experimental approaches were used: (*i*) immunofluorescence performed in intact cortical neurons; (*ii*) sucrose gradient fractionation performed with mouse neuroblastoma cells stably expressing wild-type βAPP_{695} ($N2a_{695}$); and (*iii*) cell-free reconstitution of Aβ generation and trafficking from $N2a_{695}$ cells. These studies demonstrate that: (*i*) $A\beta_{40}$ ($A\beta_{1-40}$ plus $A\beta_{x\text{-}40}$, where x is an NH_2-terminal truncation) is generated exclusively within the trans-Golgi Network (TGN) and packaged into post-TGN secretory vesicles; (*ii*) $A\beta_{x\text{-}42}$ is made and retained within the endoplasmic reticulum in an insoluble state; (*iii*) $A\beta_{42}$ ($A\beta_{1-42}$ plus $A\beta_{x\text{-}42}$) is made in the TGN and packaged into secretory vesicles; and (*iv*) the amyloid peptides formed in the TGN consist of two pools (a soluble population extractable with detergents and a detergent-insoluble form). The identification of the organelles in which distinct forms of Aβ are generated and from which they are secreted should facilitate the identification of the proteolytic enzymes responsible for their formation.**

Alzheimer's disease (AD), the most common form of dementia in the elderly, is characterized clinically by the insidious onset and inexorable progression of dementia, and pathologically by the abnormal accumulation of amyloid plaques and neurofibrillary tangles in vulnerable brain regions. Plaques are composed of variously sized β-amyloid peptides (Aβ) derived through proteolytic processing of the β-amyloid precursor protein (βAPP). Mutations within βAPP were discovered to cause autosomal dominant familial AD (FAD) (1, 2), implicating βAPP in the etiology of this disease. In addition, early-onset FAD also segregates with two other genes, the presenilin 1 (*PS1*) gene (3) and the presenilin 2 (*PS2*) gene (4), which appear to cause FAD by increasing the ratio of Aβ ending with amino acid 42 ($A\beta_{42}$) versus Aβ ending with amino acid 40 ($A\beta_{40}$) (5, 6). $A\beta_{42}$ is more highly amyloidogenic than $A\beta_{40}$ (7) and is believed to form the core of the amyloid plaques, despite being produced far less abundantly than $A\beta_{40}$. Any one of several diverse molecular anomalies of βAPP metabolism may lead to AD.

βAPP, an integral membrane glycoprotein, matures through the secretory pathway and is metabolically processed by at least two distinct pathways. Cleavage of βAPP by an enzyme, α-secretase, in a late secretory compartment, or at the cell surface, generates a large βAPP fragment ($s\beta APP_{\alpha}$). This cleavage, within the Aβ coding region, precludes formation of Aβ. Alternatively, cleavage by two enzymes, β- and γ-secretase, is believed to generate the majority of β-amyloid variants (8). βAPP is initially synthesized and cotranslationally inserted into the endoplasmic reticulum (ER). Recently, evidence has been obtained for the presence of $A\beta_{42}$ within the ER (9–12), but it was not determined whether the peptides found in the ER were packaged into vesicles for trafficking through the late secretory pathway. In addition, finding Aβ species in the ER does not demonstrate that they were generated there; retrograde transport of proteins from the Golgi complex and trans-Golgi Network (TGN) to the ER has been documented (13, 14). Finally, reports of $A\beta_{42}$ in the ER have been based primarily on ELISA assays using C-terminal specific antibodies. These assays cannot distinguish between the N termini of captured peptides and thus do not distinguish between full-length and N-truncated species both of which may end at amino acid 42.

The majority of βAPP molecules are transported through the secretory pathway to the TGN. The TGN has many known cellular functions including sorting secretory proteins, lysosomal enzymes, and plasma membrane proteins, and proteolytically cleaving prohormones (15). These functions, the localization of βAPP within the TGN, and the TGN′s acidic pH, which is optimal for the activity of many processing enzymes, suggested that Aβ generation might occur within the TGN or late Golgi (16). Generation of Aβ peptides within the TGN first was demonstrated in a cell-free system derived from cells expressing βAPP harboring the Swedish double-point mutation (17). The identity of the Aβ found in the TGN was not established.

In this study we were able to determine the sites of generation and identities of several Aβ species by using a well-characterized (15, 18) cell-free assay. In addition, we determined which species contributed to the secreted pool of peptides and which were retained intracellularly. Finally, we demonstrated that within the ER and TGN Aβ can be separated into biochemically distinct pools based on their solubility. These *in vitro* observations were consistent with results obtained by performing cell fractionation and immunofluorescence studies in intact cells.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

PNAS is available online at www.pnas.org.

Abbreviations: Aβ, β-amyloid peptides; βAPP, β-amyloid precursor protein; TGN, trans-Golgi network; ER, endoplasmic reticulum; AD, Alzheimer's disease; PS, presenilin; BIP, ER-restricted binding protein.

¶To whom reprint requests should be addressed. e-mail: xuh@rockvax.rockefeller.edu.

MATERIALS AND METHODS

Cell Lines. Neuroblastoma (N2a) cells doubly transfected with human βAPP_{695} and human wild-type *PS1* were maintained in medium containing 50% DMEM, 50% Opti-MEM, supplemented with 5% fetal bovine serum, 200 μg/ml of G418, and antibiotics (GIBCO/BRL). Cells were induced with 10 mM *n*-butyric acid (Sigma) to stimulate βAPP transcription 12 h before performing metabolic labeling experiments.

Neuronal Cultures. Primary rat neuronal cultures were derived from the cerebral cortices of embryonic day 17 embryos obtained from timed-pregnant Sprague–Dawley rats (Charles River Breeding Laboratories) as described (19). Brains were removed, and cortices and meninges were excised from the remaining brain. Cortices were triturated in glass pipettes until cells were dissociated. Cells were counted in a hemocytometer and plated in equal amounts in serum-free Neurobasal media with N2 supplement (GIBCO), 25 mM glutamate, and 0.5 mM L-glutamine on poly-D-lysine-treated (0.1 mg/ml; Sigma) 100-mm dishes (Fisher) (approximately $8–10 \times 10^6$ cells per plate) for biochemical analyses, or on microscope cover slides (Fisher) at low concentrations for immunofluorescence studies. More than 95% of cells in the preparations were neurons (19).

Antibodies. Polyclonal antibody 369 recognizes the C terminus of βAPP; FCA3340 recognizes Aβ_{40}, but not Aβ_{42}; FCA3542 recognizes Aβ_{42}, but not Aβ_{40}; FCA18 recognizes A$\beta_{1\text{-}x}$, where x is an NH_2-terminal truncation (20); anti-TGN38 was obtained from Affinity BioReagents, Golden, CO; anti-calnexin and anti-ER-restricted binding protein (BIP) were obtained from StressGen Biotechnologies, Victoria, Canada; anti-EEA1 was obtained from Transduction Laboratories, Lexington, KY; fluorescent secondary antibodies Alexa 488 goat anti-mouse and Alexa 594 goat anti-rabbit were obtained from Molecular Probes.

Enzymes and Drugs. Complete protease inhibitor mixture tablets, adenosine-5′-triphosphate, guanosine-5′-triphosphate, creatine phosphate, and creatine phosphokinase were obtained from Boehringer Mannheim.

Immunofluorescence. Immunofluorescence was performed on embryonic day 17 primary neuronal cultures that had been grown for 3 days on poly-D-lysine-coated no. 1 round glass coverslips (Fisher). Cells were washed three times with PBS containing 0.2 mM $CaCl_2$ and 2 mM $MgCl_2$ (PBS/CM) on ice, fixed with prechilled (−80°C) methanol for 25 min at −20°C, and rehydrated with 3× 5-min washes of PBS/CM at room temperature. Cells were quenched with 50 mM NH_4Cl in PBS/CM for 10 min and rinsed with PBS/CM before being permeabilized and blocked with 0.075% saponin in PBS/CM/0.2% BSA buffer for 30 min at room temperature. Primary antibodies diluted in the PBS/CM/BSA/Saponin buffer (buffer A) were added for 1 h at room temperature, followed by 3× 10-min washes with buffer A. Appropriate fluorescent species-specific secondary antibody conjugates, diluted in buffer A, were added, and incubations were carried out for 1 h at room temperature. After 3× 10-min washes with buffer A, coverslips were mounted with a ProLong Antifade Kit (Molecular Probes) on glass slides. Antibody dilutions were: FCA3340, 1:750; FCA3542, 1:750; FCA18, 1:750; anti-TGN38, 1:500; anti-calnexin, 1:1,000; anti-EEA1, 1:500; fluorescent secondary antibodies Alexa 488 goat anti-mouse and Alexa 594 goat anti-rabbit, 1:500. In each experiment fluorescence was shown to be negligible in the absence of primary antibody.

Confocal Microscopy. Images were collected and analyzed on a confocal laser scanning microscope (model LSM 510, Zeiss) using a 63× water immersion lens and rhodamine or fluorescein isothiocyanate filters. Images were converted into TIFF format and processed by using Adobe Photoshop Software (version 3.0.5, Mountain View, CA) and printed on a color laser printer.

Sucrose Gradients. To separate and enrich TGN and ER membranes, cells were homogenized by using a stainless steel ball-bearing homogenizer in 0.25 M sucrose, 10 mM Tris·HCl (pH 7.4), 1 mM $MgAc_2$, and a protease inhibitor mixture in a final concentration of 1 vol of cell pellet per 5 vol of homogenizing medium. The homogenate was loaded on top of a step gradient comprised of 1 ml of 2 M sucrose, 4 ml of 1.3 M sucrose, 3.5 ml of 1.16 M sucrose, and 2.0 ml of 0.8 M sucrose. All solutions contained 10 mM Tris·HCl, pH 7.4, and 1 mM $MgAc_2$. The gradients were centrifuged for 2.5 h at 100,000 × *g* in a Beckman SW41Ti rotor. Twelve 1-ml fractions were collected from the top of each gradient and assayed for total protein by the method of Bradford. TGN-38, BIP, and βAPP were assayed by running fractions on 4–12% SDS/PAGE, transferring the proteins onto poly(vinylidene difluoride) membranes, and performing Western blot analysis. Aβ_{40}, Aβ_{42}, and A$\beta_{1\text{-}x}$ were assayed by metabolic labeling-immunoprecipitation with FCA3340, FCA3542, and FCA18, respectively.

Preparation of Permeabilized N2a Cells. It is well established that incubation of cells at 15°C (18) or 20°C (15, 24) leads to an accumulation of membrane and secretory proteins in the ER and TGN, respectively. To assay Aβ generation in the TGN, cells were pulse-labeled with [^{35}S]methionine (500 μCi/ml) for 15 min at 37°C, washed with PBS (prewarmed to 20°C), and chased for 2 h at 20°C in complete media prewarmed to 20°C. To assay for Aβ generation in the ER, cells were pulse-labeled with [^{35}S]methionine (500 μCi/ml) for 4 h at 15°C. For both types of preparation, cells were permeabilized at the termination of the incubation. For this purpose, cells were incubated at 4°C in swelling buffer (10 mM KCl/10 mM Hepes, pH 7.2) for 10 min. The buffer was aspirated and replaced with 1 ml of breaking buffer (90 mM KCl/10 mM Hepes, pH 7.2), after which the cells were broken by scraping with a rubber policeman. The cells were centrifuged at 800 × *g* for 5 min, washed in breaking buffer, and resuspended in 5 vol of breaking buffer. This procedure resulted in >95% cell breakage evaluated by staining with trypan blue. Broken cells (approx. 2×10^6 cells) were incubated in a final volume of 300 μl containing 2.5 mm $MgCl_2$, 0.5 mM $CaCl_2$, 110 mM KCl, and an energy-regenerating system consisting of 1 mM ATP, 0.02 mM GTP, 10 mM creatine phosphate, 80 μg/ml of creatine phosphokinase, and a protease inhibitor cocktail. Incubations were carried out at 15°, 20°, or 37°C as indicated. Each experiment was performed at least three times.

Formation of Nascent Secretory Vesicles in Permeabilized Cells. After incubation of broken cells, vesicle and membrane fractions were separated by centrifugation at 14,000 rpm for 15 sec at 4°C in a Brinkman centrifuge. Vesicle (supernatant) and membrane (pellet) fractions were extracted with a cell lysis buffer containing 0.5% Nonidet P-40 and 0.5% deoxycholate. In some experiments, membrane fractions were extracted further with 70% formic acid and neutralized with 2 M Tris·HCl, pH 8.3.

Immunoprecipitation. Extracted proteins from the various fractions were brought to 0.5% SDS and heated for 3 min at 75°C. Samples were treated with IP buffer (10 mM sodium phosphate, pH 7.4/100 mM sodium chloride/1% Triton X-100), and appropriate antibody was added. After incubating overnight, samples were treated with protein-A Sepharose, and the immunoprecipitable material was analyzed by SDS/PAGE using 10–20% Tricine gels (for Aβ species) or 4–12% Tris-glycine gels (for full-length βAPP).

Densitometry. Band intensities were analyzed and quantified by using NIH IMAGE QUANT software, version 1.52.

RESULTS AND DISCUSSION

Localization of Aβ_{40}, Aβ_{42}, and A$\beta_{1\text{-}x}$ by Double Immunofluroescence Confocal Microscopy. Cortical cultures derived

from embryonic day 17 fetal rat brains were used to examine the subcellular distribution of Aβ terminating at amino acid 40 by using FCA3340. $A\beta_{40}$ immunoreactivity was limited to an area of the cells that corresponded with the localization of a TGN protein, TGN-38 (Fig. 1*a*). The distribution of $A\beta_{40}$ was distinct from that of calnexin, an ER-restricted protein (Fig. 1*b*). These findings confirm previous biochemical results that suggested the TGN as the primary intracellular site within which $A\beta_{40}$ exists under steady-state conditions (9, 17).

Although $A\beta_{40}$ does not appear to be generated within an early compartment of the secretory pathway, accumulating evidence has indicated that $A\beta_{42}$ may be found within an early compartment (9–12). When we performed immunofluorescence with FCA3542, $A\beta_{42}$ was detected in a pattern that partially overlapped with calnexin (Fig. 1*d*), providing evidence that $A\beta_{42}$ is present in the ER under steady-state conditions. $A\beta_{42}$ also was localized within the TGN (Fig. 1*c*). These experiments provide evidence that $A\beta_{40}$ is present in a late Golgi compartment, whereas $A\beta_{42}$ is present in both early and late organelles of the secretory pathway.

To evaluate the subcellular distribution of non-N-terminal truncated Aβ, immunofluorescence studies were performed by using FCA18. $A\beta_{1\text{-}x}$ was restricted to a circumscribed region of the cells that matched the localization of TGN-38 (Fig. 1*e*), but not calnexin (Fig. 1*f*). These studies suggest that $A\beta_{42}$ species seen within the ER may not be full-length $A\beta_{1-42}$ but rather a truncated $A\beta_{x\text{-}42}$ species.

To evaluate a possible localization of Aβ within endosomal compartments (21, 22), we compared the localization of EEA1, a protein that is restricted to endocytic endosomal compartments, with that of $A\beta_{40}$ and $A\beta_{42}$. A punctate pattern of staining was found for EEA1 (Fig. 1*g*), distinct from that

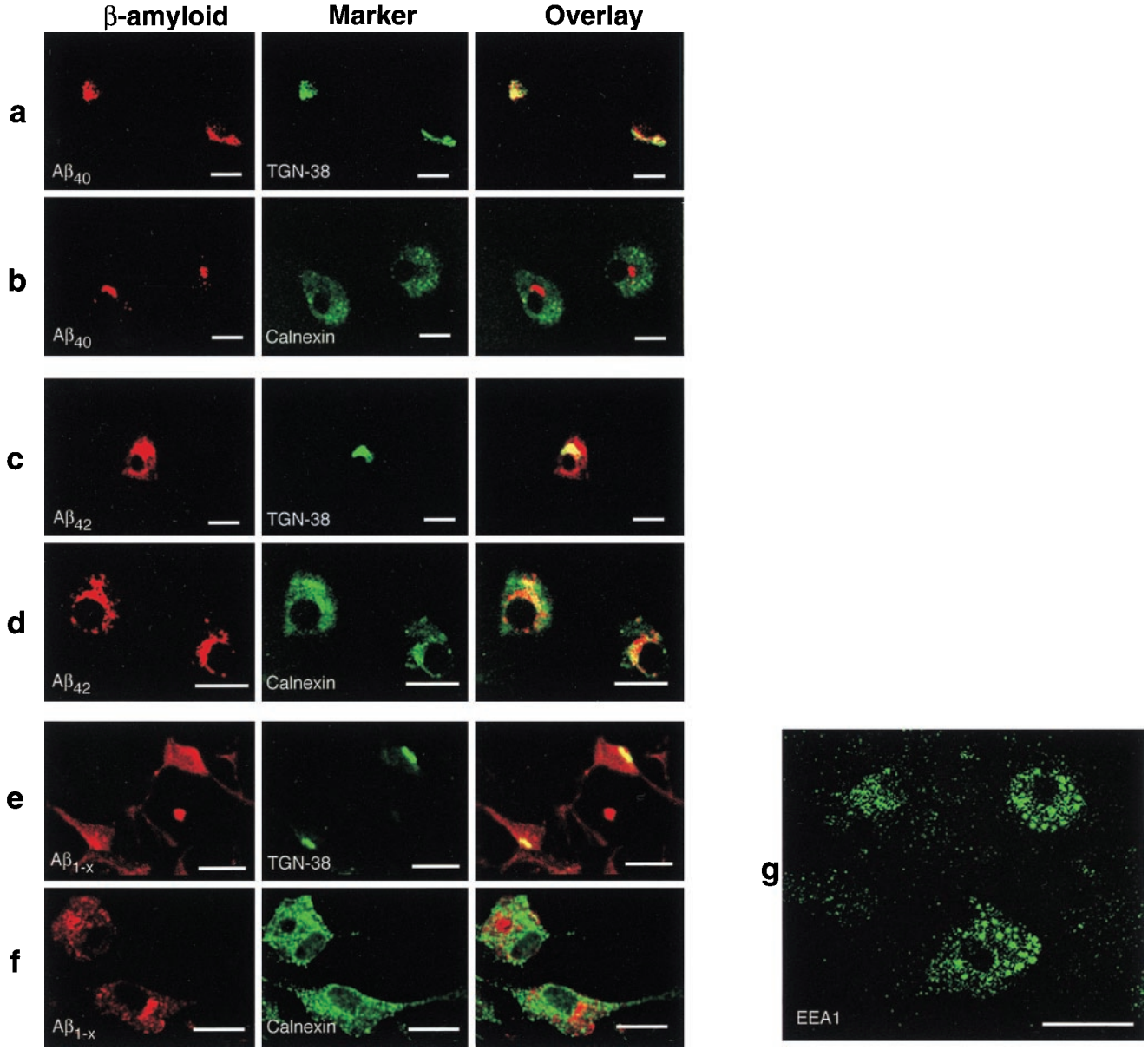

FIG. 1. Localization of Aβ in rat primary neurons by double immunofluorescence confocal microscopy. (*a* and *b*) $A\beta_{40}$ colocalizes with TGN38, but not with calnexin. (*c* and *d*) $A\beta_{42}$ colocalizes with TGN-38 and calnexin. (*e* and *f*) $A\beta_{1\text{-}x}$ colocalizes with TGN38, but not calnexin. (*g*) EEA1 has a punctate, cytoplasmic localization, different than that of $A\beta_{40}$, $A\beta_{42}$, and $A\beta_{1\text{-}x}$. Aβ were visualized by incubation with primary antibody followed by rhodamine-conjugated (red fluorescence) secondary antibody. TGN-38, calnexin, and EEA1 were used as markers for TGN, ER, and endsomes respectively, and were visualized by incubation with primary antibody followed by fluorescein isothiocyanate-conjugated (green fluorescence) secondary antibody. Overlays represent digitally merged images. Yellow fluorescence indicates colocalization of β-amyloid with marker protein. (Bar = 10 μm.)

observed for Aβ_{40} (Fig. 1 *a* and *b*), Aβ_{42} (Fig. 1 *c* and *d*), and A$\beta_{1\text{-}x}$ (Fig. 1 *e* and *f*). These findings do not, however, rule out a role for the endocytic pathway in Aβ generation. For instance, a pathway recently has been described (23) through which cell-surface proteins can be delivered to the TGN after trafficking through an endocytic recycling compartment. This pathway could contribute to a lesser extent to Aβ generation in the TGN.

Subcellular Distribution of Aβ_{40} and Aβ_{42} by Using Sucrose Gradient Fractionation. The localization of Aβ also was determined by subcellular fractionation by using TGN-38 and βAPP as markers for the TGN and BIP as a marker for the ER. For determination of Aβ, mouse neuroblastoma cells doubly expressing human βAPP$_{695}$ and wild-type *PS1* were metabolically labeled with [^{35}S]methionine for 4 h. Proteins present in subcellular fractions prepared from these cells were immunoprecipitated with FCA3340 and FCA3542 and analyzed by SDS/PAGE. The distribution of marker proteins was determined by immunoblotting subcellular fractions from unlabeled cells. TGN-38 was localized within fractions 3 and 4 (Fig. 2*a*), whereas BIP was found mainly in fractions 11 and 12, and to a lesser extent in fractions 8 and 9 (Fig. 2*b*), presumably representing heavy and light fractions of ER membranes, respectively. βAPP was found in all but the lightest of fractions, but was most heavily concentrated within the TGN-enriched fractions. Immature βAPP was found in both the ER and Golgi fractions, whereas mature βAPP was localized to the Golgi fractions (Fig. 2*c*).

A$\beta_{1\text{–}40}$ and A$\beta_{x\text{-}40}$ were immunoprecipitated from the TGN fractions of labeled cells (Fig. 2*d*). Both A$\beta_{1\text{–}42}$ and a 3-kDa Aβ, A$\beta_{x\text{-}42}$, were detected in the TGN (Fig. 2*e*). A$\beta_{x\text{-}42}$ also was found in the ER (fraction 11) (Fig. 2*d*). In parallel experiments, A$\beta_{1\text{-}x}$ was not detected within the ER, whereas a single 4-kDa species presumably representing A$\beta_{1\text{–}40}$ and A$\beta_{1\text{–}42}$ was detected within TGN fractions (data not shown). Thus the results of the sucrose gradient fractionation studies were in good agreement with results obtained by using double immunofluorescence confocal microscopy.

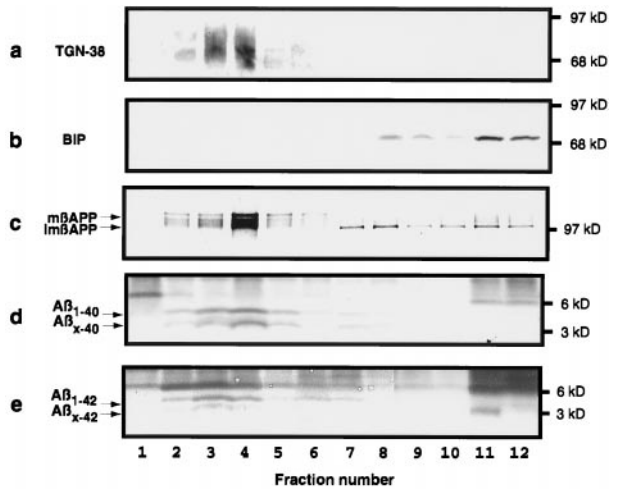

FIG. 2. Localization of Aβ by sucrose gradient fractionation. Rat primary neurons (*a*) and N2a cells doubly expressing human βAPP695 and wild-type *PS1* (*b* and *c*) were homogenized, and a postnuclear supernatant was fractionated on an equilibrium flotation sucrose gradient (see *Materials and Methods*). Proteins from each fraction were precipitated with trichloroacetic acid and separated by 4–12% SDS/PAGE, followed by immunoblotting using antibodies against either (*a*) TGN-38, (*b*) BIP, or (*c*) βAPP. (*d* and *e*) N2a cells were labeled with [^{35}S]methionine for 4 h at 37°C followed by fractionation as for *a-c*. Each fraction was sequentially immunoprecipitated with (*d*) FCA3340 (anti-Aβ_{40}) and (*e*) FCA3642 (anti-Aβ_{42}) and analyzed by 10–20% Tricine SDS/PAGE. Arrows indicate mature (m) and immature (im) forms of βAPP, A$\beta_{1\text{–}40}$, A$\beta_{x\text{-}40}$, A$\beta_{1\text{–}42}$, and A$\beta_{x\text{-}42}$.

Reconstitution of βAPP Trafficking from ER and TGN in a Cell-Free System. Microscopy and fractionation studies do not allow conclusions concerning whether the compartments within which specific Aβ were located were the same compartments within which they were generated. This issue is important because the compartment within which the peptides are generated presumably represents the location of the secretases, the major, as yet-unidentified biological targets for AD therapeutics. We attempted to resolve these questions by designing a cell-based *in vitro* assay to identify the compartments within which specific peptides are generated and to determine whether those peptides are secreted from those intracellular compartments.

To determine the reliability of this cell-free system for the study of ER- and TGN-specific events, we examined the intracellular trafficking of βAPP. Cells were labeled to accumulate βAPP within either the TGN or ER (see *Materials and Methods*), permeabilized, and incubated at 37° for 90 min. Only immature βAPP could be recovered from the ER (Fig. 3, lanes 1 and 2), whereas both mature and immature βAPP could be recovered from the TGN (Fig. 3, lanes 5 and 6). To validate the integrity of protein trafficking within this system, βAPP was immunoprecipitated from post-ER and post-TGN vesicles (Fig. 3, lanes 3, 4, 7 and 8). Under complete conditions (see *Materials and Methods*), ER (Fig. 3, lane 4) and TGN (Fig. 3, lane 8) vesicles each contain approximately 20% of the material found in the membrane (donor) fraction. Vesicle budding from the ER and the TGN are arrested at 15°C and 20°C, respectively (18, 24). Under these conditions, budding of vesicles containing βAPP was almost entirely abolished (Fig. 3, lanes 3 and 7), demonstrating the temperature dependence of the assay. Elimination of the energy-regenerating system reduced vesicle budding by nearly 90% (data not shown). These results indicated that a cell-free assay reconstituted from βAPP$_{695}$-expressing neuroblastoma cells was a reliable system in which to study the *in vitro* generation and trafficking of relevant Aβ within and from distinct intracellular compartments.

Reconstitution of Aβ_{40} Generation and Trafficking from ER and TGN. In permeabilized cells in which labeled βAPP had accumulated in the ER, Aβ_{40} was not detected in either detergent or formic acid extractions (Fig. 4*a*, lanes 1–4) or in vesicles derived from ER membranes (Fig. 4*a*, lanes 5 and 6) after *in vitro* incubations, confirming the immunofluorescence and cell fractionation studies. The absence of Aβ_{40} is not attributable to a lack of substrate, because full-length βAPP is

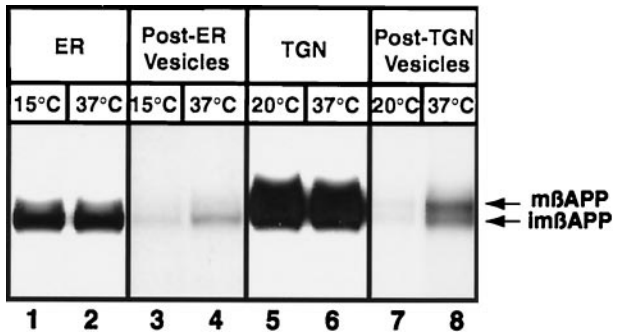

FIG. 3. Cell-free formation of post-ER and post-TGN vesicles containing βAPP. N2a cells were labeled with [^{35}S]methionine either at 15°C for 4 h or at 37°C for 15 min followed by a 2-h chase at 20°C to accumulate labeled βAPP in the ER and TGN, respectively. Cells were permeabilized and incubated (see *Materials and Methods*) for 90 min with an energy-regenerating system at 15°C (lanes 1 and 3), 20°C (lanes 5 and 7), or 37°C (lanes 2, 4, 6, and 8). After incubation, samples were centrifuged, pellets (lanes 1, 2, 5, and 6) and supernatants (lanes 3, 4, 7, and 8) were immunoprecipitated with polyclonal antibody 369 and separated by SDS/PAGE (4–12%). Arrows indicate the positions of mature and immature βAPP.

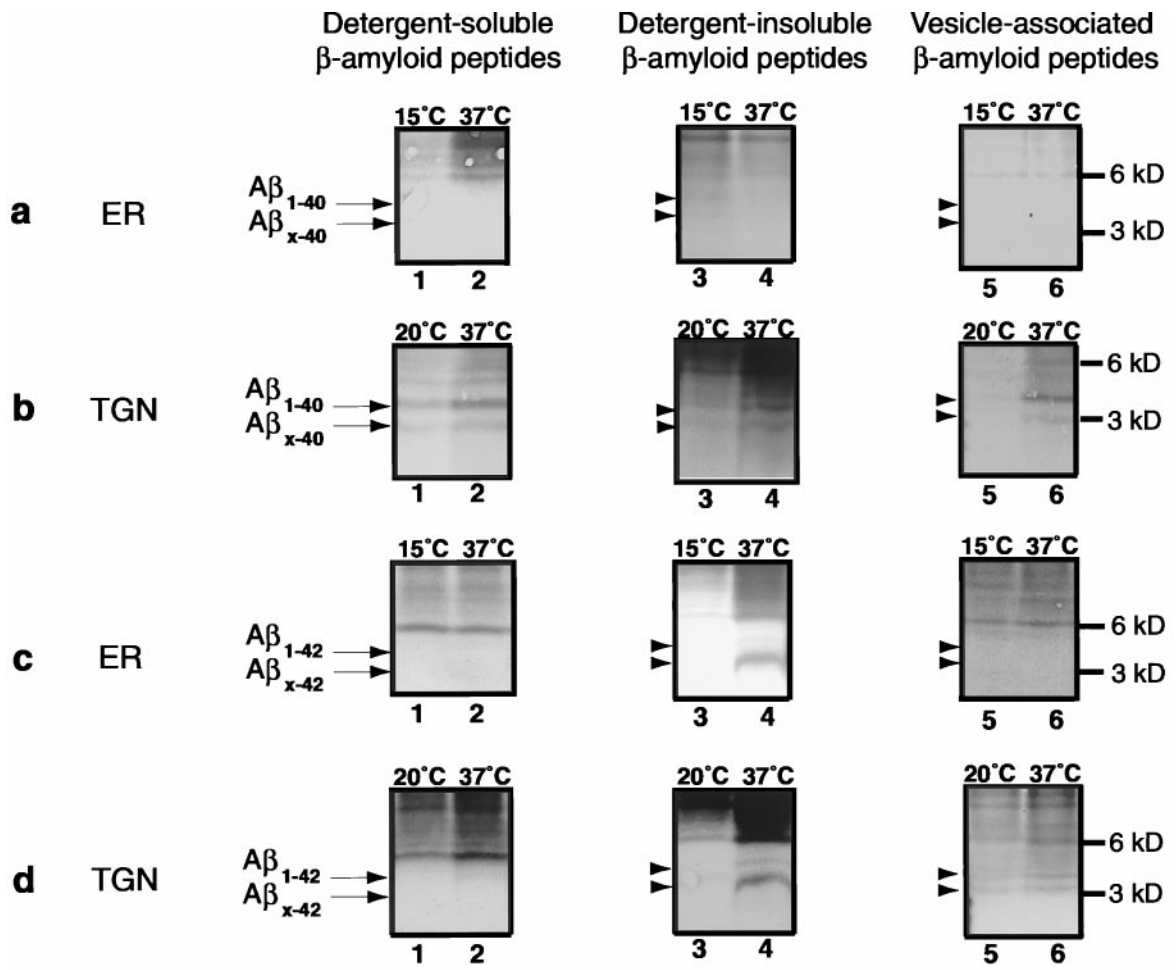

FIG. 4. Formation of Aβ_{40} and Aβ_{42} in a cell-free system. Experiments were performed as described in the legend to Fig. 3. except that, after detergent extraction, insoluble pellets were re-extracted by using 70% formic acid. Detergent-soluble (lanes 1 and 2), detergent-insoluble (lanes 3 and 4), and vesicle-associated (lanes 5 and 6) fractions were analyzed for Aβ_{40} (*a* and *b*) and Aβ_{42} (*c* and *d*) by sequential immunoprecipitation with FCA3440 followed by FCA3642. Immunoprecipitated proteins were subjected to Tricine SDS/PAGE (10–20%) analysis. Arrows indicate the positions of 4-kDa Aβ_{1-40}, 3-kDa Aβ_{x-40}, 4-kDa Aβ_{1-42}, and 3-kDa Aβ_{x-42}.

found in the ER, and the absence of vesicle-associated Aβ is not attributable to deficits in vesicle formation because βAPP is detected in post-ER vesicles (Fig. 3).

In permeabilized cells in which labeled βAPP accumulated in the TGN, detergent extraction, followed by immunoprecipitation with FCA3340 revealed Aβ_{40} of ≈4 and ≈3 kDa (Fig. 4*b*, lane 2). The formation of these Aβ species was inhibited at 20°C (Fig. 4*b*, lane 1). Further extracting the membranes with 70% formic acid followed by immunoprecipitation with FCA3340 revealed a detergent-insoluble pool of Aβ_{40} (Fig. 4*b*, lanes 3 and 4). Peptides of 3 and 4 kDa also were detected in post-TGN vesicles at 37°C but not at 20°C (Fig. 4*b*, lanes 5 and 6). Further experiments will be needed to determine the proportion of vesicle-associated peptides derived from the soluble versus insoluble pools of Aβ_{40}.

Reconstitution of Aβ_{42} Generation and Trafficking from ER and TGN. Detergent-soluble Aβ_{42} was not detected within the ER (Fig. 4*c*, lanes 1 and 2). However, a large pool of detergent-insoluble Aβ_{x-42} was revealed by formic acid extraction (Fig. 4*c*, lanes 3 and 4). Aβ_{x-42} produced within the ER was not packaged into ER-derived vesicles (Fig. 4*c*, lanes 5 and 6), indicating that insoluble Aβ_{42} cannot serve as a source for secreted Aβ. These results are consistent with a previous report that insoluble Aβ_{42} accumulates intracellularly with age in NT2N cells, a neuronal-like cell line (12). Aβ_{1-42}, and more abundantly Aβ_{x-42}, were found in detergent-insoluble (Fig. 4*d*, lanes 3 and 4) but not detergent-soluble (Fig. 4*d*, lanes 1 and 2) extracts of TGN. Both Aβ_{1-42} and Aβ_{x-42} also were found in vesicles derived from the TGN (Fig. 4*d*, lanes 5 and 6). The intracellular sites of Aβ_{1-x} generation were similar to those of Aβ_{1-40} generation (data not shown). Thus, detectable levels of Aβ_{1-x} were neither generated within ER membranes (either soluble or insoluble fractions) nor within vesicles derived from ER membranes. However, Aβ_{1-x} of 4 kDa were detected in both soluble and insoluble extracts of TGN and in vesicles derived from the TGN.

A schematic diagram of βAPP metabolism that can account for recent relevant literature, plus the data derived from the immunofluorescence, sucrose gradient fractionation, and cell-free reconstitution studies, is shown in Fig. 5. It was demonstrated in the present investigation that Aβ_{1-40} and Aβ_{x-40} are generated within the TGN and packaged into post-TGN secretory vesicles. In conjunction with our earlier findings (15, 17) and evidence from other laboratories (9, 16), these current studies suggest that the TGN is the major, if not exclusive, intracellular compartment within which the 40-specific γ-secretase is active. In addition, we confirmed that a population of insoluble Aβ_{x-42} is generated within the ER and cannot be secreted. Thus, the source of the constitutively secreted population of Aβ_{42}, which is believed to be deposited as extracellular insoluble amyloid plaques, must reside elsewhere. The present results indicate that both Aβ_{x-42} and Aβ_{1-42} are formed within the TGN, and that these peptides, combined with TGN-generated Aβ_{x-40} and Aβ_{1-40}, constitute the major

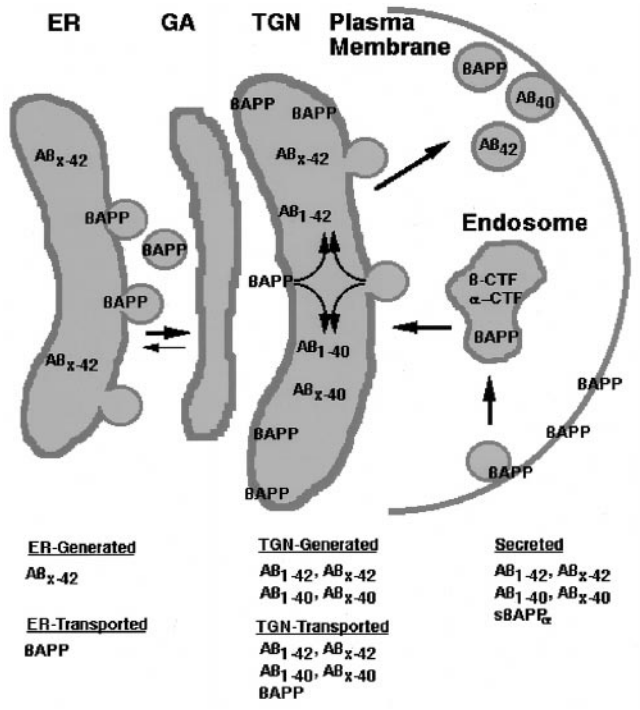

FIG. 5. Proposed sites of Aβ generation in the secretory pathway. When βAPP is translocated into the lumen of the ER, an N-terminally truncated $A\beta_{42}$ is formed by the actions of both a β-like secretase (s) and γ_{42}-secretase. This $A\beta_{42}$ remains in the ER in an insoluble state whereas uncleaved βAPP molecules are packaged into post-ER vesicles and travel through the Golgi apparatus (GA) to the TGN where most of them reside. Both β- and β-like secretases together with γ_{40}- and γ_{42}-secretases cleave βAPP within the TGN. Detergent-insoluble Aβ molecules aggregate and remain within the TGN. Full-length βAPP, β-CTFs, and soluble Aβ are packaged into post-TGN secretory vesicles. Full-length βAPP can be proteolyzed by α-secretase late within the secretory pathway/cell surface to release $s\beta APP_{\alpha}$. Some uncleaved βAPP molecules as well as C-terminal fragments can be internalized via a clathrin-coated endocytic pathway to the endosome or lysosome where βAPP processing enzymes also might occur. A recently characterized pathway (23) could deliver those molecules to the TGN for an additional round of Aβ generation.

reservoir of peptides from which the constitutively secreted pool of Aβ is recruited. Cell-free Aβ generation assays may provide an opportunity to delineate the contributions of intracellular insoluble peptides versus secreted soluble peptides to amyloid plaque formation.

We thank Dr. Ching-Hwa Sung for technical advice on immunofluorescence studies. This work was supported by National Institutes of Health/National Institute on Aging Grant AG09464, and an Alzheimer Association Grant (G.K.G.).

1. Goate, A., Chartier-Harlin, M., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L., *et al*. (1991) *Nature (London)* **349,** 704–706.
2. Murrell, J., Farlow, M., Ghetti, B. & Benson, M. D. (1991) *Science* **254,** 97–99.
3. Sherrington, R., Rogaev, E., Liang, Y., Rogaeva, E., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K., *et al*. (1995) *Nature (London)* **375,** 754–760.
4. Levy-Lahad, E., Wasco, W., Poorkaj, P., Romano, D., Oshima, J., Pettingel, W. H., Yu, C.-E., Jondro, P. D., Schmidt, S. D., Wang, K., *et al*. (1995) *Science* **269,** 973–977.
5. Duff, K., Eckman, C., Zehr, C., Yu, X., Prada, C., Perez-tur, J., Hutton, M., Buee, L., Harigaya, Y., Yager, D., *et al*. (1996) *Nature (London)* **383,** 710–713.
6. Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T., Hardy, J., Hutton, M., Kukull, W., *et al*. (1996) *Nat. Med.* **2,** 864–870.
7. Pike, C. J., Overman, M. J. & Cotman, C. W. (1995) *J. Biol. Chem.* **270,** 23895–23898.
8. Greenfield, J. P., Gouras, G. K. & Xu, H. (1998) *Front. Biosci.* **3,** 399–407.
9. Hartmann, T., Bieger, S. C., Bruhl, B., Tienari, P. J., Ida, N., Allsop, D., Roberts, G. W., Masters, C. L., Dotti, C. G., Unsicker, K. & Beyreuther, K. (1997) *Nat. Med.* **3,** 1016–1020.
10. Cook, D. G., Forman, M. S., Sung, J. C., Leight, S., Kolson, D. L., Iwatsubo, T., Lee, V. M. & Doms, R. W. (1997) *Nat. Med.* **3,** 1021–1023.
11. Wild-Bode, C., Yamazaki, T., Capell, A., Leimer, U., Steiner, H., Ihara., Y. & Haass, C. (1997) *J. Biol. Chem.* **272,** 16085–16088.
12. Skovronsky, D. M., Doms, R. W. & Lee, V. M. (1998) *J. Cell. Biol.* **141,** 1031–1039.
13. Lippincotz-Schwart, J., Cole, N. B. & Donaldson, J. G. (1998) *Histochem. Cell. Biol.* **109,** 449–462.
14. Drecktrah, D., Figueiredo, P., Mason, R. M. & Brown, W. J. (1998) *J. Cell. Sci.* **111,** 951–965.
15. Xu, H. & Shields, D. (1993) *J. Cell. Biol.* **122,** 1169–1184.
16. Thinakaran, G., Teplow, D. S., Siman, R., Greenberg, B. & Sisodia, S. S. (1996) *J. Biol. Chem.* **271,** 9390–9397.
17. Xu, H., Sweeney, D., Wang, R., Thinakaran, G., Lo, A., Sisodia, S. S., Greengard, P. & Gandy, S. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 3748–3752.
18. Beckers, C. J. M. & Balch, W. E. (1989) *J. Cell. Biol.* **108,** 1245–1256.
19. Gouras, G. K., Xu, H., Jovanovic, J. N., Buxbaum, J. D., Wang, R., Greengard, P., Relkin, N. R. & Gandy, S. (1998) *J. Neurochem.* **71,** 1920–1925.
20. Barelli, H., Lebeau, A., Vizzavona, J., Delaere, P., Chevallier, N., Drouot, C., Marambaud, P., Ancolio, K., Buxbaum, J. D., Khorkova, O., *et al*. (1997) *Mol. Med.* **3,** 695–707.
21. Haass, C., Koo, E., Mellon, A., Hung, A. & Selkoe, D. (1992) *Nature (London)* **357,** 3116–3123.
22. Koo, E. & Squazzo, S. L. (1994) *J. Biol. Chem.* **269,** 17386–17389.
23. Ghosh, R., Mallet, W. G., Soe, T. T., McGraw, T. E. & Maxfield, F. R. (1998) *J. Cell. Biol.* **142,** 923–936.
24. DeCurtis, I. & Simons, K. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8052–8056.