The Alzheimer β-amyloid protein precursor/protease nexin-II is cleaved by secretase in a *trans*-Golgi secretory compartment in human neuroglioma cells

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Alzheimer β-amyloid protein precursor (βAPP) is expressed endogenously and abundantly by human neuroglioma (H4) cells. Its secretory processing has been shown to involve discrete proteolysis within the βA4 region, thus preventing β-amyloid formation, by an enzyme which has been referred to as 'βAPP secretase'. This cleavage results in secretion of a soluble N-terminal 135 kDa protein and retention of an integral membrane C-terminal fragment within the cell. The membrane-associated C-terminal fragment is sorted to lysosomes where it undergoes limited degradation. We show here that most newly synthesized βAPP is degraded via a non-lysosomal pathway before maturation in H4 cells, and most mature βAPP is processed predominantly by the so-called secretase. The rapid kinetics of appearance/disappearance of a cleaved 135 kDa protein within a microsomal fraction and the slow accumulation of this form in the extracellular medium indicated that secretase cleaves βAPP in an intracellular compartment. Low-temperature block (20 °C) was used to demonstrate that βAPP is cleaved within a late Golgi compartment after sulphation which occurs in the *trans*-Golgi network (TGN). This is consistent with (1) the immunolocalization of most of the βAPP within a Golgi compartment that reacts with wheat germ agglutinin, (2) the fact that less than 1.5% of the total mature full-length βAPP is present at the plasma membrane and (3) subcellular fractionation studies which showed that the mature full-length and intracellular cleaved βAPPs co-sediment with a membrane fraction that is slightly more dense than the plasma membrane. This study provides evidence that most of the βAPP secretase in H4 cells is intracellular, and confirms that the resulting C-terminal fragment is delivered to lysosomes immediately after cleavage. These results are discussed with regard to the possibility that mature full-length βAPP escapes secretase cleavage and is delivered directly from the TGN to the lysosome without passing through the plasma membrane. Either pathway will result in the generation of amyloidogenic fragments.

INTRODUCTION

Alzheimer's disease is a human neurodegenerative disorder distinguished by intraneural neurofibrillary tangles and extracellular β-amyloid deposits within affected neuronal circuits. These occur primarily, but not exclusively, within the hippocampus and associated regions of the cortex [reviewed by Greenberg et al. (1991) and Joachim and Selkoe (1992)]. The exact role of β-amyloid in Alzheimer's disease is not known and understanding its generation has been a major focus in research of this disease. Amyloid β-protein or βA4 peptide is a 39–42-amino acid peptide derived from the β-amyloid protein precursor (βAPP) (Kang et al., 1987). It has been suggested that aberrant proteolysis of βAPP results in generation of βA4 and subsequent neuronal death (Selkoe, 1989; Greenberg et al., 1991); however, how this fragment is externalized remains unexplained.

βAPP is an integral membrane glycoprotein which exists in multiple forms as a result of alternative splicing of mRNA encoded by a unique gene located on human chromosome 21 (Figure 1) (Greenberg et al., 1991; Joachim and Selkoe, 1992). The 695-amino acid βAPP (APP-695) is found in cerebrospinal fluid and appears to be the predominant form expressed within the central nervous system (Palmert et al., 1989). The 751 (APP-751)- and 770 (APP-770)-amino acid βAPPs contain a Kunitz-type protease inhibitor domain, and the secreted form of these longer βAPPs was identified as protease nexin II which is implicated in wound repair as a protease inhibitor (Oltersdorf et al., 1989; Van Nostrand et al., 1990). The function of APP-695 is not known and its origin within the central nervous system appears to be from several cell types including neurons and glial cells (Shivers et al., 1988; Berkenbosch et al., 1990; Haass et al., 1991).

After synthesis, βAPP is *N*- and *O*-glycosylated and sulphated during transit from the endoplasmic reticulum (ER) to the cell surface (Weidemann et al., 1989; Oltersdorf et al., 1990). At points during its maturation, βAPP is cleaved by one of several proteolytic pathways (Anderson et al., 1991; Estus et al., 1992; Fukuchi et al., 1992; Golde et al., 1992; Haass et al., 1992; Knops et al., 1992; Tamaoka et al., 1992). Some of the resulting fragments are secreted and others remain cell associated. The most common secretory cleavage is directed by a so-called 'βAPP secretase' within the βA4 region, thus precluding formation of intact βA4 peptide. This cleavage results in the rapid release of an N-terminal fragment, and the retention of an approx. 10 kDa fragment within the cell, consisting of the cytoplasmic tail, the transmembrane domain and part of the βA4 domain (Figure 1) (Selkoe et al., 1988; Esch et al., 1990; Sisodia et al., 1990; Overly et al., 1991). The βAPP secretase has not yet been identified, nor has the exact cellular site of cleavage been determined.

Abbreviations used: βAPP, β-amyloid protein precursor; ER, endoplasmic reticulum; TGN, *trans*-Golgi network; MEM, minimal essential medium; FBS, fetal bovine serum; TRITC, tetramethylrhodamine-5-isothiocyanate; FITC, fluorescein isothiocyanate; MMLV-RT, Moloney murine leukaemia virus reverse transcriptase; CMF-PBS, Ca^{2+}- and Mg^{2+}-free PBS; WGA, wheat germ agglutinin; Endo H, endoglycosidase H.

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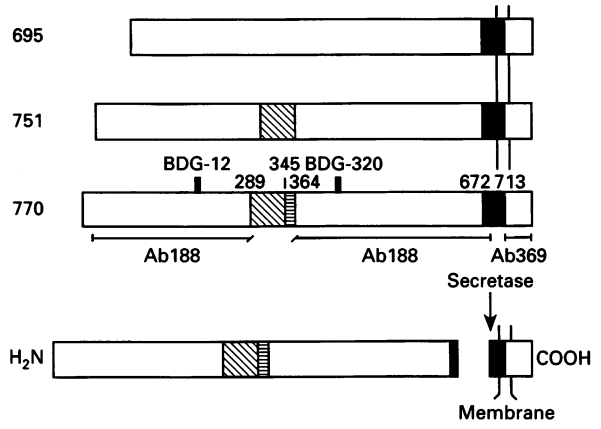

Figure 1 The three forms of βAPP consisting of 695, 751 and 770 amino acids

APP-751 and APP-770 contain a Kunitz-like protease inhibitor (KPI) (▨) domain, and APP-770 contains a Kitaguchi or OX2 (▤) domain. All forms have the β-protein region or βA4 peptide (■) within which is the cleavage site for the secretase. Cleavage releases the N-terminal 135 kDa fragment of APP-770, and the membrane-inserted C-terminal 15 kDa fragment remains cell associated. BDG-12 and BDG-320 are the oligonucleotides used in PCR and the regions recognized by antibodies Ab188 and Ab369 are shown.

In this paper, βAPP is shown to be located predominantly within a late Golgi compartment of a human neuroglioma cell which expresses relatively abundant amounts of Kunitz-containing βAPP. Low-temperature block, kinetics of maturation, sulphation and subcellular fractionation are used to show that mature full-length βAPP is constitutively cleaved in H4 cells probably within a compartment in the *trans*-Golgi network (TGN) (Griffiths and Simons, 1986). After cleavage, the membrane-associated C-terminal fragment is transferred to lysosomes via an undefined pathway, and the N-terminal βAPP fragment or protease nexin II is secreted. These results suggest that the βAPP secretase is located intracellularly and that missorting of the full-length βAPP, whereby the protease is avoided, could be the first step in an amyloidogenic pathway (Nordstedt et al., 1991).

EXPERIMENTAL

Reagents

Human neuroglioma (H4) cells were obtained from the American Type Culture Collection (HTB148). Cell culture materials including minimal essential medium (MEM), methionine-free αMEM (formula 84-5159) and fetal bovine serum (FBS) were purchased from Gibco-BRL, Grand Island, NY, U.S.A. $Na^{125}I$ (16.3 mCi/μg of I^-), L-[U-^{14}C]leucine (325 mCi/mmol) and [^{35}S]methionine (1000–1396 Ci/mmol) were obtained from Amersham Corp., Arlington heights, IL, U.S.A. Enlightning and $Na_2{}^{35}SO_4$ (43 Ci/mg of S at 100% isotopic enrichment) were purchased from NEN Research Products, Boston, MA, U.S.A. and ICN Radiochemicals, Irvine, CA, U.S.A. respectively. Protein A–Sepharose CL-4B, Percoll, TMP *p*-nitrophenyl ester, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, monensin, chloroquine, normal goat and horse serum, anti-(amoeba actin) mouse monoclonal antibody, tetramethylrhodamine-5-isothiocyanate (TRITC)–wheat germ agglutinin (WGA) and BSA fraction V were from Sigma Chemical Co., St. Louis, MO, U.S.A. Endoglycosidase H and sheep anti-(cathepsin B) antibody were purchased from ICN ImmunoBiologicals, Lisle, IL, U.S.A. The mouse monoclonal antibody anti-(human transferrin receptor) (B3/25) was from Hybritech, San Diego, CA, U.S.A. Affinity-purified secondary antibodies, rabbit anti-(mouse IgG), fluorescein isothiocyanate (FITC)–goat anti-(rabbit IgG), TRITC-sheep anti-(mouse IgG) and TRITC-donkey anti-(sheep IgG) were from Accurate Chem. & Sci. Corp., Westbury, NY, U.S.A. Lab-Tek Permanox chamber slides by Nunc, Naperville, IL, U.S.A. were purchased through Baxter Diagnostics, Scientific Products, McGraw Park, IL, U.S.A. Centricon-30 microconcentrators were purchased from Amicon, Beverly, MA, U.S.A. X-OMAT film is a product of Eastman Kodak Co., Rochester, NY, U.S.A. Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) was from Bethesda Research Laboratories, Bethesda, MD, U.S.A. PCR reagents were from Perkin–Elmer Cetus, Norwalk, CT, U.S.A. Sep-Pak C_{18} columns were purchased from Waters Chromatography Division, Millipore, Milford, MA, U.S.A. RNazol kits were from Cinna/Biotecx Lab., Friendswood, TX, U.S.A. Agarose–anhydrotrypsin, Immunopure immobilized avidin and sulpho-NHS-SS-biotin were purchased from Pierce Chemical Co., Rockford, IL, U.S.A.

Table 1 List of polyclonal rabbit antibodies, their specificities and the peptides used to block their binding

Antibody	Specifity*	Blocking peptide*
188	N-terminus of APP-695, amino acids 18–624	Residues 18–624
369	C-terminus of APP-695, amino acids 645–694	Residues 645–694
Preimmune	–	

* Amino acid numbering based on APP-695 (see Figure 1 for the corresponding regions on APP-770).

Cell culture

H4 cells were grown in MEM containing 10% (v/v) heat-inactivated FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.025 μg/ml amphotericin B, 0.01 mM MEM non-essential amino acids, 0.1 mM sodium pyruvate and 2 mM additional L-glutamine at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Anti-βAPP antibodies

The rabbit anti-βAPP serum (Ab188) was raised against a truncated recombinant human APP-695 (residues 18–624) expressed in Sf9 cells (Lowery et al., 1991). The rabbit anti-(C-terminal) serum or Ab369 (kindly provided by Dr. Samuel E. Gandy, Rockefeller University, New York, NY, U.S.A.) was raised to a synthetic peptide of residues 645–694 corresponding to the C-terminal region of APP-695 (Buxbaum et al., 1990). Preimmune Ab188 was used as a negative control. The antibodies and their specificities are summarized in Table 1 and shown in Figure 1.

Immunofluorescence microscopy

Cells grown in chamber slides were fixed with either 4% (w/v) paraformaldehyde in Ca^{2+}- and Mg^{2+}-free PBS (CMF-PBS) for 20 min at 25 °C or methanol for 10 min at −20 °C. All subsequent incubations were carried out at 25 °C using CMF-PBS containing 0.1% (v/v) Nonidet P40 to permeabilize the cell membrane and protein [1% (w/v) BSA, 2.5% (v/v) horse serum

and 5% (v/v) normal goat serum] to block non-specific binding of the antibodies. After a 30 min preincubation, the cells were treated for 2 h with 1:50–1:500 dilutions of primary antiserum [Ab188, Ab369, sheep anti-(cathepsin B), mouse anti-(human transferrin receptor)] followed by 1 h with 50 μg/ml fluorescently labelled secondary antibody [FITC–goat anti-(rabbit IgG) TRITC–donkey anti-(sheep IgG), TRITC–sheep anti-(mouse IgG)] or with 10 μg/ml TRITC–WGA. After being washed with and without Nonidet P40, the samples were placed in 75% (v/v) glycerol in CMF-PBS, covered by a coverslip and viewed with epifluorescence. Control samples were incubated with pre-immune Ab188, a corresponding normal serum or a non-relevant monoclonal antibody.

Metabolic labelling

H4 cells at 95–100% confluence were preincubated for 45 min at 37 °C in serum-free and methionine-free αMEM containing 25 mM Hepes at pH 7.2. [^{35}S]Methionine was added at 10 μCi/cm^2 to cells in a small volume of methionine-free αMEM, typically 1.4, 4 and 12 ml per 60, 100 and 150 mm dish respectively. Alternatively, $Na_2{}^{35}SO_4$ was added at 15.4 μCi/cm^2 to cells in PBS containing 1 mg/ml D-glucose and 25 mM Hepes after a 40 min preincubation. Incubation was at 37 °C with 5% CO_2 for steady-state labelling or in a circulating-water-bath for short-pulse or reduced-temperature experiments. At the end of the labelling period, the culture supernatant was collected, the cell monolayer washed with PBS at 37 °C before the chase or 4 °C before lysis and the cells chased in growth medium containing 1% (v/v) FBS. The cell monolayers were solubilized by scraping the cells into lysis buffer (0.05 M Tris/HCl, pH 7.2, 0.6 M KCl, 0.5% Nonidet P40) containing 1 mM EDTA and protease inhibitors (0.2 trypsin inhibitor unit/ml aprotinin, 1 μg/ml leupeptin and 1.5 mM phenylmethanesulphonyl fluoride), 200 and 400 μl per 60 and 100 mm dishes respectively. Solubilized proteins were recovered after centrifugation at 10000 *g* for 10 min. Supernatants were concentrated to approx. 200 μl in Centricon-30 microconcentrators and diluted with 300 μl of lysis buffer.

For perturbation studies, H4 cells were allowed to incorporate label after a 20–30 min preincubation in either 50–250 μM chloroquine, 1–10 μM monensin or 25 mM NH_4Cl. The effects of chloroquine (0.05–1 mM) and monensin (1–500 μM) on protein synthesis were examined by incorporation of [^{3}H]leucine into trichloroacetate-insoluble material. Perturbant was omitted as a control.

Quantifying cell surface βAPP

H4 cells grown to confluence in 60 mm dishes were labelled with [^{35}S]methionine for 105 min as described. After five washes with ice-cold PBS, the cells were incubated with 0.5 mg/ml sulpho-NHS-SS-biotin in PBS for 25 or 30 min at 4 °C (Lisanti et al., 1988). Control cells were incubated in PBS. All subsequent steps were carried out on ice. The cells were washed once with MEM and five times with PBS and then lysed. After preclearance of the lysate with preimmune Ab188, βAPP was recovered by immunoprecipitation with Ab369 as described. βAPP from control cells was solubilized in one-dimensional SDS/PAGE buffer. Biotinylated βAPP was released from the immunobeads in 100 μl of 0.0625 M Tris/HCl, pH 6.8, with 1% (w/v) SDS and diluted to 1 ml with Tris/HCl, pH 6.8, to reduce the SDS concentration. The biotinylated subset of βAPP was recovered by incubation with 50 μl of Immunopure-immobilized avidin (Carlsson and Fukuda, 1992) and solubilized with 100 μl of one-dimensional SDS/PAGE buffer. After electrophoresis and autoradiography, the results were quantified by phosphorimaging as described below.

To examine the specificity of the biotinylation towards extracellularly exposed cell proteins, actin was also immunoprecipitated with 20 μl of anti-(amoeba actin)monoclonal antibody and rabbit anti-(mouse IgG)-coated Protein A–Sepharose and the results were quantified as described.

Subcellular fractionation

Subcellular fractionation was carried out with Percoll density gradients as described elsewhere (Green et al., 1987). H4 cells that had been labelled with [^{35}S]methionine for 105 min were scraped into buffer (0.25 M sucrose, 10 mM Hepes and 1 mM EDTA) and homogenized with 60 strokes on ice in a 1 ml Dounce homogenizer fitted with a glass-type A pestle. Cells from one 150 mm dish homogenized in 3 ml of buffer were used per gradient. The post-nuclear supernatant, recovered after centrifugation at 750 *g* for 10 min, was diluted with additional buffer and made 27% (v/v) in Percoll with a final volume of 10.5 ml. The gradient was formed by centrifugation of this mixture, layered over a 1 ml cushion of 2.5 M sucrose, at 29000 *g* for 105 min at 4 °C in a Sorvall SS-34 rotor (Du Pont Company, Wilmington, DE, U.S.A.). Parallel gradients were run for enzyme assays and for identification of plasma membrane by using ^{125}I-labelled cells. For the latter, cells were lifted from a 100 mm dish with EDTA and were iodinated by the lactoperoxidase method (Hubbard and Cohn, 1975) using 0.5 mCi $Na^{125}I$.

Gradient fractions (approx. 0.5 ml) were collected from the bottom of the tube using a Minipuls 2 pump (Gilson Medical Electronics, Middleton, WI, U.S.A.). The fractions were diluted to 1 ml with buffer and centrifuged at 180000 *g* for 30 min (TLA 100.2 rotor, Beckman Instruments, Palo Alto, CA, U.S.A.) to remove most of the Percoll. The supernatant was aspirated down to the membrane layer and the membranes were collected and either solubilized (lysis buffer) for use in immunoprecipitations or dispersed in homogenizing buffer for enzyme assays. The amount of ^{125}I-labelled total plasma-membrane proteins per fraction was measured by γ counting after trichloroacetate precipitation.

Enzyme assays

The gradient fractions were assayed spectrophotometrically for β-hexosaminidase (i.e. lysosomes) using the substrate *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Hall et al., 1978) and for alkaline phosphodiesterase I activity (i.e. plasma membrane) (Aronson and Touster, 1974) using TMP *p*-nitrophenyl ester. For the latter assay, 25 mM $CaCl_2$ was included and the samples were read after 2 h.

Triton X-114 phase separation

Cells labelled with [^{35}S]methionine were solubilized in ice-cold 4% (v/v) Triton X-114 (precondensed) in 0.01 M Tris/HCl, pH 7.2, and 0.5 M NaCl and phase-partitioned as described by Bourdier (1981) and modified by Alcaraz et al. (1984). Antigen was immunoprecipitated from the detergent-soluble and -insoluble membrane fractions and analysed by SDS/PAGE as described below.

Carbonate extraction

Cells labelled with [^{35}S]methionine were homogenized as described above and the post-nuclear supernatant was centrifuged at 100000 *g* for 60 min (TLA 100.2 rotor). The cytosol fraction (supernatant) was recovered and the membrane pellet was

extracted on ice for 40 min with mixing in 1 ml of 0.1 M Na_2CO_3 at pH 11.5 (Fujiki et al., 1982). After a second 100000 *g* centrifugation, the carbonate supernatant was neutralized with 1 M HCl and the membrane pellet was solubilized in lysis buffer. Antigen was immunoprecipitated from the cytosol, carbonate solution and solubilized membrane fraction and analysed by SDS/PAGE as described below.

Endoglycosidase H

Endo-β-*N*-acetylglucosaminidase H (Endo H) was reconstituted as a 0.443 munit/μl stock in CMF-PBS. Protein A–Sepharose-bound immunocomplexes were boiled for 5 min in 75 μl of pH 5.5 citrate/phosphate buffer, cooled and the reaction initiated by the addition of 1 μl of phenylmethanesulphonyl fluoride (150 mM stock in propan-2-ol) and 4 μl of Endo H or water (control). After an overnight incubation at 37 °C with slow mixing, the reaction was stopped by the addition of 20 μl of 3 × SDS/PAGE buffer (see below) and boiling for 5 min. The enzyme digests were analysed by SDS/PAGE.

Immunoprecipitation and electrophoresis

Lysates and supernatants were precleared by overnight incubation at 4 °C with 12 μl of preimmune Ab188 and slow mixing followed by immunoglobulin removal with Protein A–Sepharose. The samples were then incubated with 12 μl of Ab188 or 1 μl of Ab369 and the specific immunocomplexes recovered on Protein A–Sepharose as above (3–4 h). The immunoprecipitates were washed with buffer (0.05 M NaCl, 0.05 M Tris/HCl, 0.5% Nonidet P40, 0.1% SDS, pH 7.2), solubilized in 65 μl of SDS/PAGE buffer [0.0625 M Tris/HCl, pH 6.8, 10% (w/v) glycerin, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.05% Bromophenol Blue) and boiled for 5 min. Samples were analysed by electrophoresis on SDS/polyacrylamide gels (6 and 15% gels) (Laemmli, 1970). Fixed and Coomassie-stained gels were treated with Enlightning before drying and exposure to X-OMAT AR film at −70 °C. Autoradiograms from pulse–chase experiments were analysed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The data expressed as pixels relative to the amount of ^{35}S present were analysed by non-linear least-squares curve-fitting (Yamaoka et al., 1981).

Analysis of βAPP forms expressed by H4 cells

PCR

Oligonucleotides 5′-GCGGAGGAGGATGACTCGGA-3′ (BDG-12) and 5′-AAAGAT-TCCACTTTCTCCTG-3′ (BDG-320) were synthesized on an Applied Biosystems 380A synthesizer (Foster City, CA, U.S.A.), purified by elution from polyacrylamide gels and desalted on Sep-Pak C_{18} columns. Total RNA was isolated by standard methods (Chomczynski and Sacchi, 1987) using an RNazol kit. Total RNA (2 μg) was mixed with 20 pmol of oligonucleotide BDG-320 and reverse-transcribed with MMLV-RT. PCR was performed on the resulting cDNAs as described by Saiki et al. (1988). Thirty PCR cycles were performed using BDG-12 and BDG-320 as primers, and portions of the reaction mixture were analysed by electrophoresis on 2% agarose gels containing 0.5 μg/ml ethidium bromide.

Precipitation with immobilized anhydrotrypsin

Recombinant APP-695 and -770 in Chinese hamster ovary cells (D. Palermo, D. Fischer, M. DeGraaf, S. Cleary and M. Babcock, unpublished work) and H4 cell βAPP were radiolabelled with [^{35}S]methionine as described above. The βAPP was either immunoprecipitated from the culture medium using Ab188 or precipitated by agarose-bound anhydrotrypsin, which binds to the Kunitz protease inhibitor domain in APP-751 and -770 (Figure 1). Culture media containing [^{35}S]methionine-labelled proteins (approx. 10^7 c.p.m.) were incubated for 4 h at 4 °C with enough agarose–anhydrotrypsin to give a 100 μl packed pellet. After several washes with buffer containing 0.05 M Tris/HCl, pH 8.2, and 0.05 M NaCl, the bound proteins were analysed by SDS/PAGE and autoradiography.

RESULTS

H4 cells express mostly Kunitz-containing βAPP mRNA and protein

Total RNAs were extracted and converted into single-stranded

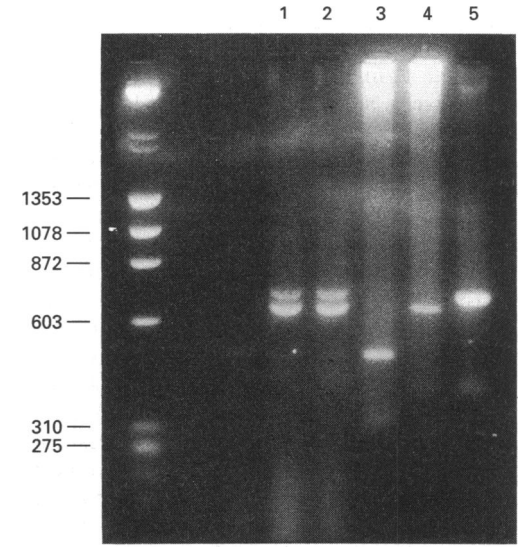

Figure 2 Reverse transcription/PCR analysis of H4 βAPP mRNA

Total mRNAs from H4 cells (lanes 1 and 2) were reverse-transcribed using BDG-320 as primer. Single-stranded cDNA was amplified with BDG-12 and BDG-320 as primers to generate double-stranded cDNAs which cross the βAPP junction region to reveal which variably spliced βAPP mRNAs are transcribed in H4 cells. Lane 3 shows PCR products from a recombinant plasmid containing full-length APP-695 cDNA revealing the 500 bp band expected for that form. Lane 4 shows the expected 668 bp band amplified from a APP-751 cDNA and lane 5 shows the 725 bp band expected from a APP-770 cDNA. The left lane contains a mixture of PhiX174-*Hae*III and Lambda-*Hin*dIII base pair size standards. This gel reveals that H4 cells produce APP-751 and -770, but not APP-695.

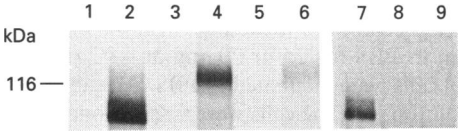

Figure 3 Synthesis of Kunitz-containing βAPP by H4 cells

βAPP was recovered from culture medium of cells labelled with [^{35}S]methionine for 120 min by immunoprecipitation with Ab188 (lanes 2, 4 and 6). Lanes 1, 3 and 5 are cell media that were incubated with preimmune Ab188 as a negative control. Lane 2 is APP-695 expressed in CHO cells. Lane 4 is APP-770 expressed in CHO cells. Lane 6 is βAPP expressed by H4 cells. The above culture media containing [^{35}S]methionine-labelled βAPP were incubated first with agarose–anhydrotrypsin, to specifically remove Kunitz-containing βAPP, i.e. APP-770 and -751, and then with Ab188 to immunoprecipitate the βAPP remaining (lanes 7–9). As expected, APP-695 from CHO cells (lane 7) did not bind to anhydrotrypsin, whereas APP-770 from CHO cells (lane 8) and βAPP from H4 cells (lane 9) were removed.

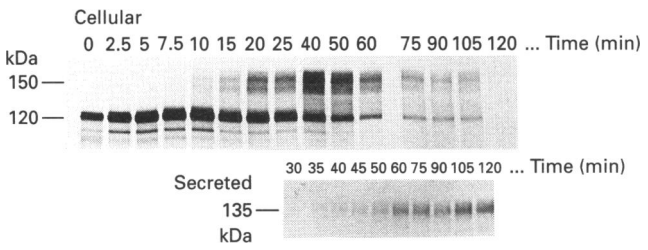

Figure 4 Synthesis of βAPP by H4 cells and secretion of the cleaved 135 kDa N-terminal fragment

Cells were pulse-labelled for 4 min with [^{35}S]methionine and chased for 3 h in complete medium. Cell lysates and culture medium were precleared with preimmune serum and sequentially incubated with Ab188 and Protein A–Sepharose to immunoprecipitate βAPP. The immunoprecipitates were subjected to SDS/PAGE using a 6% separating gel, and autoradiograms were obtained by exposure for 19 h to X-ray film. These autoradiograms are representative of the results from two experiments. The 110 kDa species is a specific cellular protein which might be a degradative fragment of the immature 120 kDa βAPP.

cDNAs by reverse transcription. Thirty PCR cycles were applied to these single-stranded cDNAs to evaluate which of the βAPP mRNAs are expressed by this cell type. The primers BDG-12 and BDG-320 should amplify a segment of the βAPP that contains the alternatively spliced insert sites (Figure 1) [reviewed in Greenberg et al. (1991)]. The expected fragment sizes bounded by these oligonucleotides are 500 bp for APP-695 (Kang et al., 1987), 668 bp for APP-751 (Ponte et al., 1988; Tanzi et al., 1988) and 725 bp for APP-770 (Kitaguchi et al., 1988). As shown in Figure 2, H4 cells express mRNAs for APP-751 and -770, with no evidence of APP-695 mRNA. Under identical conditions, only APP-695 mRNA was amplified from mouse brain extracts.

βAPP recovered from H4-cell-conditioned media was analysed by gel migration relative to recombinant standards and by binding to anhydrotrypsin. H4-cell βAPP migrated with a mobility similar to that of recombinant APP-770 expressed in CHO cells (Figure 3). Precipitation of [^{35}S]methionine-labelled βAPP by immobilized anhydrotrypsin further supported the finding that H4 cells produce one or both of the Kunitz-containing βAPP forms (Figure 3).

Kinetics of βAPP maturation, cleavage and secretion

Pulse–chase experiments ($n = 3$) were used to determine the kinetics of βAPP processing within H4 cells. Cells were labelled for 4 min with [^{35}S]methionine and chased in complete medium without radiolabel for 3 h. The medium and detergent cell lysates were immunoprecipitated with Ab188, and autoradiographs after SDS/PAGE were obtained (Figures 4 and 5a) and quantified by phosphorimaging (Figure 5b). All lysates were precleared with preimmune or normal serum, and specificity of the antisera was confirmed by inhibiting precipitation with competing antigen

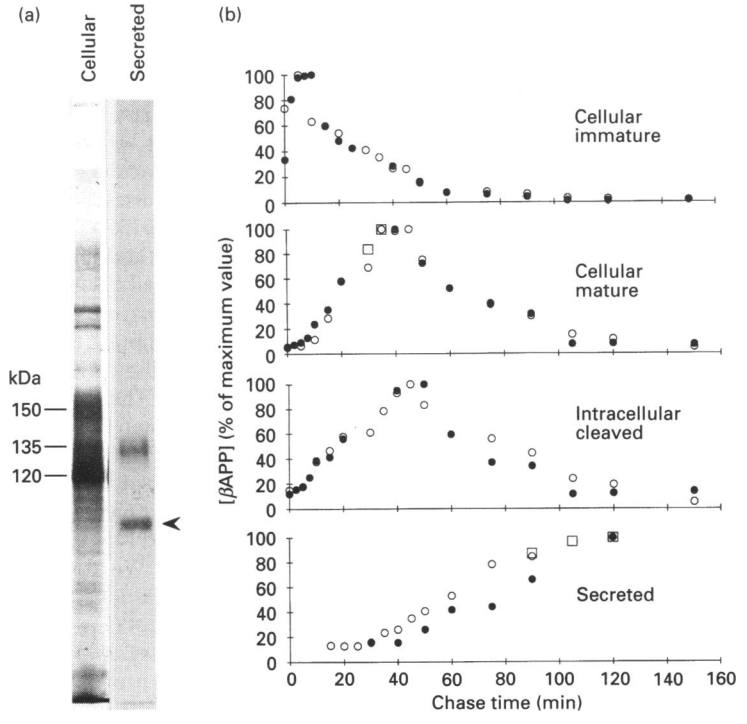

Figure 5 Kinetics of βAPP processing obtained by quantifying the amounts of the various βAPP forms recovered by immunoprecipitation from the pulse–chase experiments

(**a**) Representative autoradiograms identify the three βAPP species quantified for kinetics. The full-length immature 120 kDa and mature 150 kDa βAPPs are recovered by immunoprecipitation as described in Figure 4 from the cell lysates. The cleaved 135 kDa species appears in the cell lysate and in the culture medium. A non-specific protein (arrow) was immunoprecipitated from the medium (see Figure 6). (**b**) Data from two replicate experiments (○, ●) are plotted including that of a third experiment (□) to confirm the maxima. The amount of each protein species in each lane was measured by phosphorimaging and plotted as a fraction of maximal intensity versus time of chase. Changes in amounts of the 120 kDa, the 150 kDa and the intracellular cleaved and secreted 135 kDa βAPPs are temporally related. In these experiments, the cellular 135 kDa βAPP was quantified from the diffuse band between the immature and mature species (see Figure 4). Its identity was confirmed by extraction of crude microsomes (see Figure 8) and sedimentation in Percoll gradients (see Figure 11b).

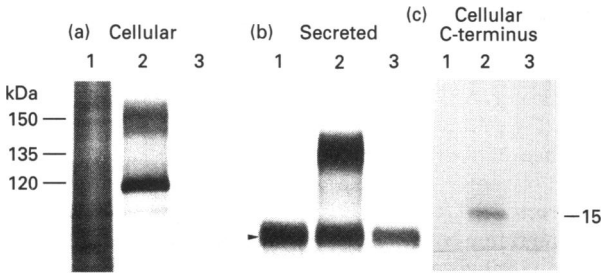

Figure 6 Specificities of the antisera used in this study shown by immunoprecipitation of cell lysate and medium with preimmune Ab188 antisera (lane 1), Ab188 (lane 2 of a and b) or Ab369 (lane 2 of c)

Lane 3 used immune sera to which was added either APP-695 residues 18–624 (**a** and **b**) or C-terminal peptide of APP-695 residues 645–694 (**c**) (see Table 1 and Figure 1). All samples except lane 1 of (**a**) were preabsorbed with preimmune Ab188 to reduce non-specific background. The lower molecular-mass protein (arrow) from the medium is not related to βAPP, as it was immunoprecipitated by preimmune Ab188 and by Ab188 in the presence of blocking peptide.

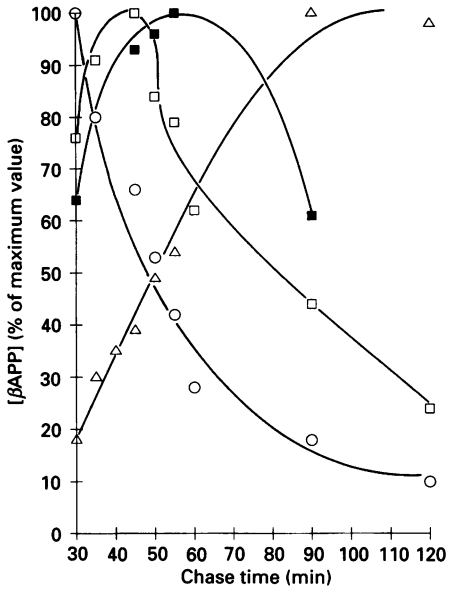

Figure 7 Kinetics of βAPP processing where the intracellular secreted βAPP fraction was recovered

The experiment was carried out as described in Figures 4 and 5 except that a total microsomal fraction was isolated as described in Figure 8 and carbonate-extracted to recover intracellular secreted βAPP. The full-length immature (○), mature (□), intracellular cleaved (■) and extracellular secreted (△) βAPPs were recovered after a 30 min chase by immunoprecipitation and the amounts quantified by phosphorimaging. The data are expressed as a percentage of the maximal value recovered.

(Figure 6). Label was incorporated initially into a 120 kDa precursor protein form which is chased with time into a family of proteins of 145–155 kDa. Decay of the 120 kDa protein or immature βAPP proceeded with first-order kinetics with a half-time of 18 ± 1 min ($n = 2$). That this form is immature βAPP was confirmed by sensitivity to Endo H which cleaves N-linked high-mannose oligosaccharides and not terminally glycosylated forms (results not shown). Mass balance of the radioactivity associated with the various fractions as measured by phosphorimaging

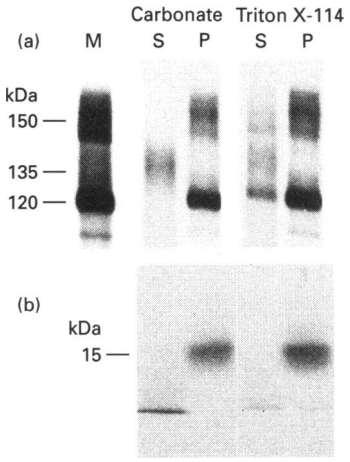

Figure 8 Crude microsomes (M), prepared by fractionation of [^{35}S]methionine-labelled cells in isotonic sucrose and treated with either Na_2CO_3 at pH 11 or Triton X-114

Soluble extracts (S) and membrane pellets (P) were recovered by centrifugation. Each was incubated with Ab188 (**a**) or Ab369 (**b**) to immunoprecipitate full-length βAPPs and the C-terminal fragment respectively. Ab188 and Ab369 immunoprecipitates were separated by SDS/PAGE (6% and 15% gels respectively) and autoradiograms obtained.

indicated that recoveries decreased rapidly with time. Recovery of only 20–30% of the total radioactivity incorporated suggested that 70–80% of the synthesized immature βAPP is degraded. Exact values were difficult to calculate beyond 45 min because we did not quantitatively recover the secreted forms nor did we account for the C-terminal fragment remaining after secretase cleavage. From experiments described below, loss of immature βAPP was not affected by the presence of chloroquine, NH_4Cl or monensin (results not shown), suggesting that its degradation does not occur in an acidic compartment, e.g. lysosomes.

Analysis of the pulse–chase experiments showed that appearance of the 145–155 kDa proteins or mature full-length βAPP coincided with the appearance of a 135 kDa protein which was detected in the cell lysate as a diffuse band at 40 min (see Figure 4). Both the mature and cell-associated 135 kDa βAPP species represented approx. 30% of the total recovered βAPP (approx. 8–10% of the total βAPP synthesized) at maximum (Figures 5b and 7). That this 135 kDa protein in the lysate represented intracellular cleaved βAPP was confirmed by isolating a microsomal fraction of cells broken in isotonic sucrose (Figure 8). Treatment of the microsomes with Na_2CO_3 released the 135 kDa protein showing that it was not membrane bound. In contrast, the 120 and 145–155 kDa βAPPs remained membrane associated (Figure 8). We also found that these two membrane-associated forms partitioned into Triton X-114 whereas the 135 kDa protein did not (Figure 8), confirming that the 135 kDa protein was not an integral membrane protein. Furthermore, the intracellular 135 kDa protein did not react with the C-terminal-specific antibody Ab369 (results not shown), indicating that it had been cleaved by βAPP secretase. Repeating the pulse–chase experiment and isolating the intracellular 135 kDa protein suggested that accumulation of cleaved βAPP within the microsomal fraction was proportional to and followed accumulation of mature full-length βAPP but, as in the previous experiments, preceded its accumulation in the extracellular medium (Figure 7).

As the mature full-length and cell-associated cleaved βAPP decayed with a half-time of 20–25 min, the cleaved βAPP

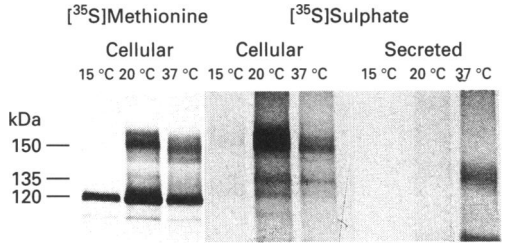

Figure 9 Sulphation and processing of βAPP in H4 cells after perturbation with temperature

Cells were labelled for 2 h at 15, 20 or 37 °C with either [^{35}S]methionine or [^{35}S]sulphate. βAPP was immunoprecipitated from cell lysates or medium using Ab188.

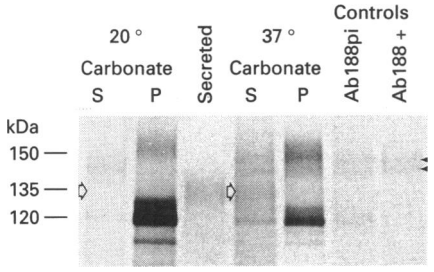

Figure 10 Inhibition of secretase cleavage of βAPP by incubation at 20 °C

Cells were labelled with [^{35}S]methionine for 4 h at 20 °C or 1.75 h at 37 °C and the βAPP forms were recovered by immunoprecipitation with Ab188. Crude microsomes were treated with Na_2CO_3 yielding the soluble extract (S) and membrane pellet (P) as described in Figure 8. The soluble 135 kDa βAPP, secreted into the medium (see lane 3 for reference), is released from carbonate-treated microsomes of cells incubated at 37 °C, but is absent from those from cells maintained at 20 °C (compare the open arrowheads). Cells labelled at 37 °C were chased for 3 h and βAPP was immunoprecipitated from whole lysates with preimmune Ab188 (Ab188pi) or with Ab188 preabsorbed with APP-695 amino acids 18–624 (Ab188+). The solid arrowheads mark a doublet that is non-specific.

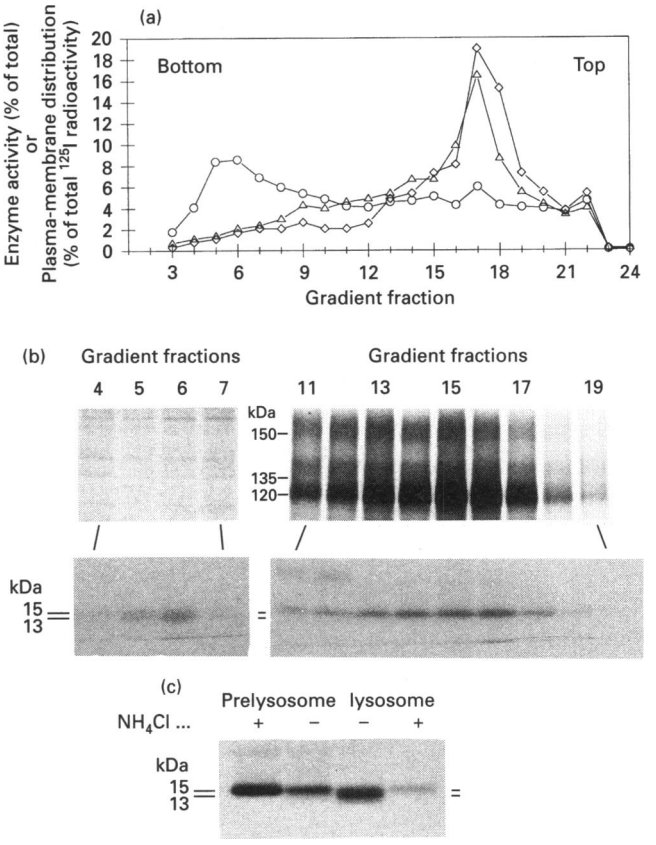

Figure 11 Examination of subcellular distribution of βAPP and its cleaved fragments using Percoll gradient sedimentation

H4 cells were labelled with [^{35}S]methionine for 105 min, broken in isotonic sucrose, and the post-nuclear homogenate was fractionated in a 27% Percoll gradient. (**a**) Activities of β-hexosaminidase (○) and alkaline phosphodiesterase (◇) are shown each as a fraction of total activity in the homogenate. Distribution of plasma membrane is also shown by labelling total cell surface proteins with ^{125}I by using lactoperoxidase (△). (**b**) Gradient fractions were centrifuged to collect the membranes, and βAPP (and βAPP fragments) was recovered by immunoprecipitation with Ab188 or Ab369. The full-length and cleaved βAPP forms were recovered in buoyant membrane vesicles (fractions 11–17) and none were detected in lysosomes (fractions 4–7). The 15 kDa fragment was recovered in the buoyant membrane fractions and the lysosomes, but at a slightly lower molecular mass in the latter. (**c**) The fate of the 15 kDa fragment after secretase cleavage in a 'pre' lysosomal compartment was examined. Treatment with 25 mM NH_4Cl before cell fractionation on Percoll gradients blocked proteolysis of the 15 kDa to the 13 kDa species after delivery from the prelysosomal compartment to the lysosome. The decreased amount of 15 kDa fragment in the lysosome and the increase in the amount of 15 kDa fragment in the prelysosome suggests that its delivery to lysosomes is slower in the presence of NH_4Cl.

accumulated slowly in the extracellular medium (Figures 4, 5b and 7). Assuming a first-order dependence, there was good theoretical proportionality between the kinetics of appearance of the 135 kDa protein in the extracellular medium and disappearance kinetics of intracellular βAPP (Figures 5b and 7). As observed with the cell-associated 135 kDa species, the medium-associated form also failed to react with Ab369 (results not shown) confirming that these forms lacked the cytoplasmic domain of βAPP. The amount of cleaved βAPP released into the medium reached a maximum at approx. 90 min with a half-life of appearance of 30 ± 8 min ($n = 2$).

βAPP is cleaved within the secretory pathway in a post-Golgi compartment

The pulse–chase kinetics indicated a product–precursor relationship between the various βAPP forms. Yet, the actual intracellular compartments in which these forms reside were not clearly distinguished because of the rapid kinetics of secretase cleavage of the mature full-length βAPP. We used temperature blocks and sulphation kinetics to map the transit of βAPP through the secretory pathway. Incubation of cells at 15 °C blocks ER-to-Golgi transport (Kuismanen and Saraste, 1989). Labelling H4 cells at 15 °C with [^{35}S]methionine for up to 4 h resulted in accumulation of immature βAPP (Figure 9).

Incubation of cells at 20 °C blocks post-Golgi transit in some cell types but permits ER-to-Golgi transport (Kuismanen and Saraste, 1989). In H4 cells, this treatment resulted in accumulation of βAPP that migrated at a slightly higher molecular mass than the mature full-length βAPP observed at 37 °C (Figure 9). This high-molecular-mass βAPP was labelled with [^{35}S]sulphate at 20 °C (Figure 9), indicating it had entered the TGN (Baeurle and Huttner, 1987; Rosa et al., 1992). Moreover, secretase cleavage of the mature full-length βAPP was also inhibited at 20 °C, as neither the medium-associated (Figure 9) nor the intracellular 135 kDa proteins were observed (Figure 10).

Several pharmacological agents known to interfere with protein traffic as well as protease activity by raising pH (Maxfield,

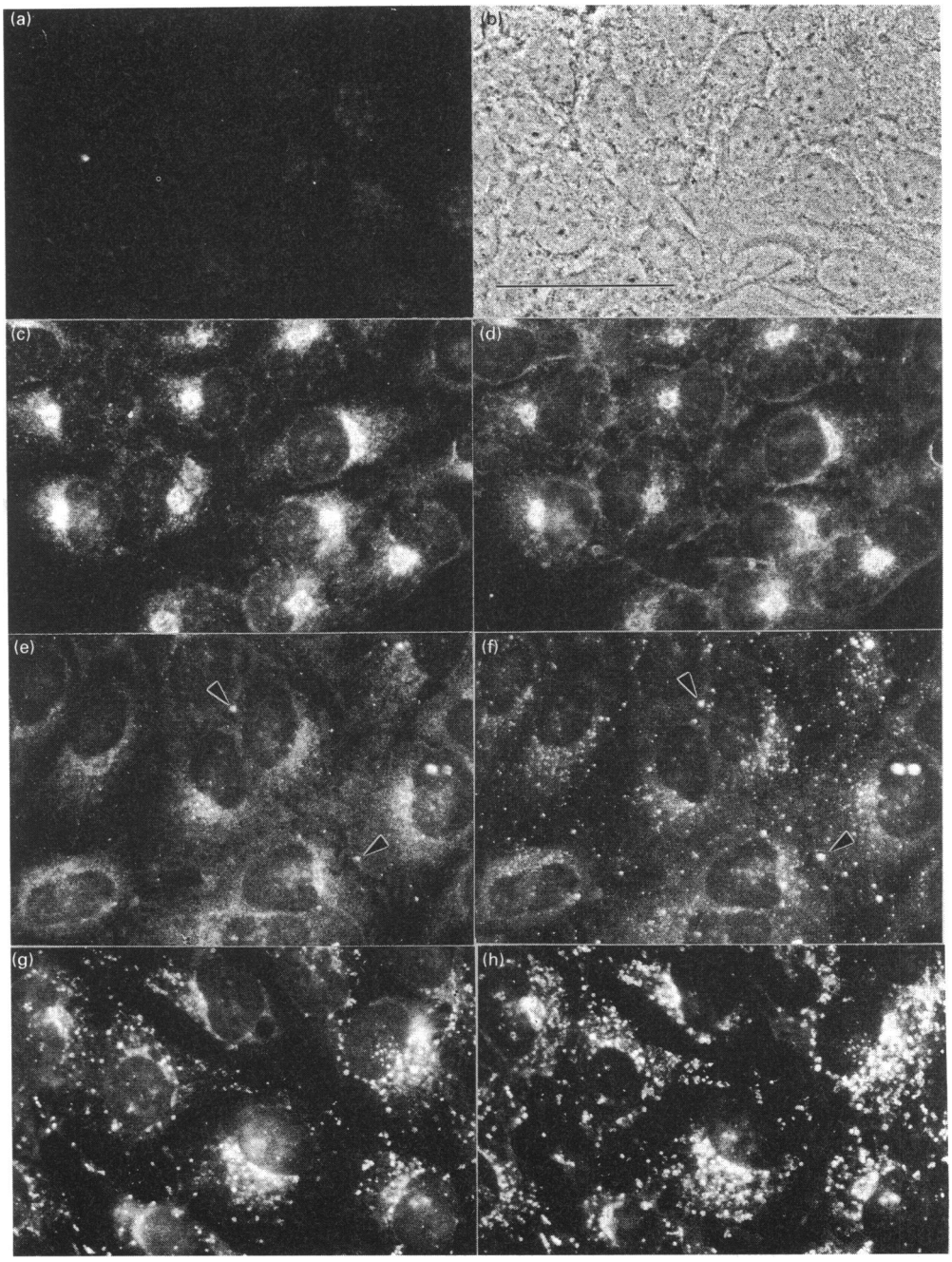

Figure 12 Immunofluorescent localization of βAPP in detergent-permeabilized H4 cells

Cells incubated with preimmune Ab188 (**a**) (corresponding phase-contrast image (**b**)) confirms the specificity of this antibody. In the subsequent panels, βAPP is localized with Ab188 (**c, e** and **g**) and the distribution is co-localized with TRITC–WGA (**d, h**) and cathepsin B (**f**) followed by TRITC-conjugated anti-IgG. The arrowheads mark vesicles that contain both βAPP and cathepsin B. Chloroquine (150 μM for 2 h) resulted in redistribution of Golgi-localized βAPP (**g**) identified as a subset of vesicles that are stained with TRITC–WGA (**h**). The bar equals 50 μM.

1982; Tartakoff, 1983) were used to determine their effects on cleavage of βAPP by the secretase. The weakly basic amines chloroquine and NH_4Cl were used at 150 μM and 25 mM respectively, and the ionophore monensin was used at 1 μM, as protein synthesis was inhibited (> 20%) in H4 cells after 2 h at higher concentrations. None of these agents had any effect on βAPP secretase activity at these concentrations as cleaved βAPP was found in the medium (results not shown). The same result was obtained with the serine protease inhibitor leupeptin (100 μg/ml).

Cleaved N-terminal βAPP and the C-terminal fragment are transported differently

Subcellular fractionation in isotonic Percoll gradients was used to further identify the intracellular distribution of βAPP and to

determine whether the cleaved βAPP and C-terminal fragments are sorted separately after secretase cleavage. Plasma-membrane fractions were identified by both the presence of alkaline phosphodiesterase activity and the distribution of trichloroacetate-insoluble ^{125}I radioactivity associated with total membrane proteins from intact cells that had been radioiodinated (Figure 11a). Cells were labelled with [^{35}S]methionine, broken by homogenization and the post-nuclear fraction equilibrated in a 27% Percoll gradient. The gradient was fractionated and the membranes from each fraction were recovered, solubilized in lysis buffer and subjected to immunoprecipitation with either Ab188 or Ab369 followed by SDS/PAGE and autoradiography. The full-length βAPP forms associated with the ER and Golgi were broadly distributed throughout the more buoyant gradient fractions (Figure 11b), with more than 80% recovered from fractions 11–15. Less than 20% of the total radioactivity of the mature βAPP recovered in the upper third of the gradient co-sedimented with the plasma-membrane fractions 16–18.

Whereas immunoprecipitation with Ab369 revealed that the C-terminal 15 kDa fragment was distributed to the buoyant membrane fractions where intracellular cleaved βAPP equilibrated, another more truncated C-terminal species of 13–14 kDa was immunoprecipitated from the lysosomal fractions (Figure 11b). Addition of the peptide antigen to the antiserum abolished Ab369 immunoprecipitation of these fragments (Figure 6). When cells were treated with 25 mM NH_4Cl before fractionation on Percoll gradients, the 15 kDa fragment was found in the lysosomal fractions and the lower-molecular-mass species was not (Figure 11c). Under these conditions, we did not detect accumulation of either the immature or the mature full-length βAPPs within lysosomes. The decreased amount of 15 kDa fragment in the lysosomal fraction of NH_4Cl-treated cells suggested that delivery to lysosomes from the site of cleavage was affected or that the rate of secretase cleavage was lower. The C-terminal fragments were not detected in the culture supernatant and were tightly associated with the membrane in both lysosomes and the buoyant membrane fractions as shown by resistance to Na_2CO_3 extraction and partitioning into Triton X-114 (Figure 8).

Distribution of full-length βAPP within H4 cells

It has been suggested that significant amounts of mature full-length βAPPs are delivered to the cell surface of a variety of different cell types (Weidemann et al., 1989; Refolo et al., 1991; Haass et al., 1992; Knops et al., 1992; Sisodia, 1992). To examine this quantitatively in H4 cells, we measured the fraction of total [^{35}S]methionine-labelled βAPP that was biotinylated by the membrane-impermeant sulpho-NHS-SS-biotin. Only 2.8 ± 0.7% ($n = 3$) of the total ^{35}S-labelled βAPP was biotinylated at steady state. The value of 2.8% is an upper estimate for plasma-membrane-associated βAPP, as 1.3 ± 0.5% ($n = 3$) of the immature βAPP was also biotinylated under even milder conditions (30 min at 0.5 mg/ml). That this represents biotinylation of intracellular forms that were there because of leakiness of the plasma membrane was confirmed by the observation that actin was also biotinylated under these conditions. It is clear therefore that 1.5% or less of steady-state mature full-length βAPP resided at the cell surface of H4 cells. In contrast, wild-type HeLa cells expressed between 3.5 and 5% ($n = 2$) of total mature full-length βAPP at their cell surface (results not shown). Given the relatively large fraction of intracellular immature βAPP that was biotinylated, we could not distinguish whether the biotinylated βAPP actually resided at the plasma membrane or within an intracellular compartment. Owing to the difficulties in interpretation caused by these methodological problems associated with cell-surface biotinylation we did not attempt to isolate an intracellular endocytic compartment that contained biotinylated βAPP because of the small fraction of βAPP expressed at the cell surface of H4 cells.

A predominantly intracellular distribution of βAPP was confirmed by immunolocalization using fluorescence microscopy (Figure 12). Paraformaldehyde-fixed cells were permeabilized with detergent and incubated with either Ab188 or Ab369 followed by an appropriate fluorophore-labelled anti-rabbit IgG antibody. The two antibodies labelled the same structures, and staining was not observed in non-permeabilized cells or in control cells incubated with preimmune serum (Figure 12a). βAPP was localized mostly to vesicles with a perinuclear distribution (Figure 12c). Prolonged incubation in serum-free medium for 24 h or overgrowth of cell cultures had no apparent effect on βAPP distribution, suggesting that processing was not affected by culture conditions. The βAPP-positive vesicles were also stained with TRITC–WGA (Figure 12d) which identifies *trans*-Golgi compartments (Tartakoff and Vassali, 1983). The majority of the βAPP-positive vesicles did not contain cathepsin B (Figure 12f), suggesting that they were not lysosomes. As an adjunct to the studies described above to perturb βAPP processing, βAPP was immunolocalized by fluorescence microscopy after treatment with chloroquine. Chloroquine caused a marked redistribution of βAPP within large vesicles that were derived from the Golgi region (compare Figure 12g with Figure 12c). These vesicles clearly represented a subset of the compartment that was labelled with TRITC–WGA (Figure 12h). In contrast, redistribution of most of the transferrin receptor in the perinuclear region was not observed and only rarely was it co-localized with βAPP (results not shown). These results suggest that most of these compartments are not continuous with the endocytic pathway including lysosomes. This interpretation is consistent with the observation that most βAPP was not co-localized with Lucifer Yellow, a molecule that is accumulated by endocytosis (Swanson et al., 1985), after a 2 h incubation with 2 mg/ml and a chase of 1 h (results not shown).

DISCUSSION

The present study utilized subcellular fractionation and pulse–chase radioimmunochemistry kinetics, along with temperature and drug perturbation, to examine maturation of Kunitz-containing βAPP in H4 cells, a spontaneously transformed human cell type of central nervous system origin. The data indicate that almost all of the mature βAPP is constitutively cleaved by secretase within a post-Golgi compartment that is discrete from the compartment(s) where sulphation and terminal glycosylation occur. The presence of soluble βAPP within an intracellular compartment and its maximal accumulation before appreciable appearance in the extracellular medium suggests that secretase acts in an intracellular compartment and not at the cell surface as proposed by others (see Sisodia, 1992). The data provide evidence for an alternative interpretation of βAPP trafficking within cells, depending upon the cell type. This processing is not exclusive of the delivery of full-length βAPP to lysosomes in some cell types resulting in the generation of amyloidogenic fragments (Estus et al., 1992; Gandy et al., 1992; Golde et al., 1992; Haass et al., 1992). Although proteolysis of mature full-length βAPP by secretase in H4 cells is dominant, we also find that lysosomal processing of βAPP occurs because the C-terminal fragment, which remains membrane-associated after proteolysis

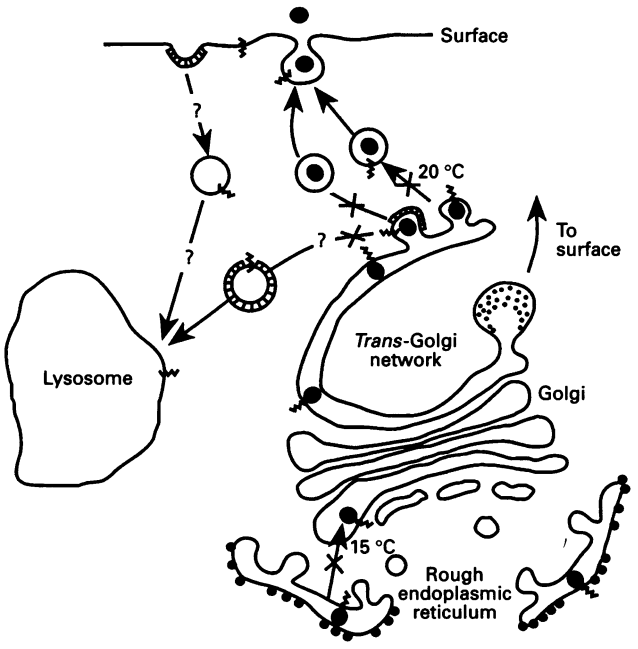

Figure 13 Proposed intracellular transport of βAPP in H4 cells after synthesis

The intracellular site of secretase cleavage is in the TGN. It is unclear if the C-terminal fragment is sorted there and delivered directly to the lysosome, possibly via clathrin-coated vesicles, or delivered to the cell surface where it is internalized again by clathrin-mediated endocytosis. ~●, Pre-APP; ~, C-terminal/transmembrane; ●, N-terminal/secreted APP.

of the mature βAPP, is transported to lysosomes. A summary of this processing is shown in Figure 13.

The maturation and overall kinetics of processing of βAPP in H4 cells were similar to those described by others for a variety of non-transfected cells and cells transfected with βAPP cDNA (Weidemann et al., 1989; Oltersdorf et al., 1990; Buxbaum et al., 1990; Luo et al., 1990; Sambamurti et al., 1992). As immature βAPP moved from ER through the Golgi apparatus, glycosylation with predominantly O-linked oligosaccharides, sulphation, sialylation and phosphorylation increased its molecular mass by 20–30 kDa (Weidemann et al., 1989; Oltersdorf et al., 1990). From the pulse–chase experiments, it was obvious that only a fraction (20–30%) of the newly synthesized βAPP was transported to the Golgi for maturation and subsequent cleavage in H4 cells. Similarly, 70% of βAPP expressed in 293 cells was degraded (Knops et al., 1992) and 15–30% of newly synthesized βAPP appeared in the medium of transfected and non-transfected cells (Weidemann et al., 1989; Caporaso et al., 1992; Sahasrabudhe et al., 1992). Because processing of the immature βAPP in H4 cells was not affected by weak bases, degradation does not occur in an acidic compartment or lysosomes. The absence of lysosomal involvement is consistent with our cell fractionation and immunofluorescence results. Degradation of the immature βAPP probably occurs within the ER as described for other proteins (Klausner and Sitia, 1990). This conclusion contradicts that of Knops et al. (1992) showing that degradation of βAPP was probably lysosomal and may be attributed to a difference in overexpression of βAPP in a transfected cell versus endogenous expression in H4 cells or simply a difference in cell type.

What is less well understood are the events subsequent to transport to the Golgi. One possibility is that mature full-length βAPP is cleaved within the βA4 region by an unknown protease resulting in secretion of an N-terminal fragment (Esch et al., 1990; Sisodia et al., 1990). Another possibility is that full-length βAPP is processed within alternative proteolytic pathways which have been shown to result in the generation of amyloidogenic fragments (Estus et al., 1992; Gandy et al., 1992; Golde et al., 1992; Haass et al., 1992). For instance, it has been shown that mature full-length βAPP is expressed at the cell surface, then endocytosed and delivered to lysosomes (Haass et al., 1992). Which pathway is emphasized apparently depends upon the cell type and even the cell line studied (Wolozin et al., 1992).

In H4 cells, the majority of the fully matured βAPP is cleaved by secretase. Full-length βAPP is cleaved constitutively between Gln^{611}–Lys^{612}, thus leaving the cytoplasmic tail and membrane-spanning domain within the cell membrane (Esch et al., 1990; Sisodia et al., 1990; Overly et al., 1991). What is not clearly established is the precise subcellular site at which this proteolysis occurs. Using a variety of pharmacological agents, Caporaso et al. (1992) concluded that secretase cleaves βAPP in a non-acidic post Golgi location. Our results and those recently described by Sambamurti et al. (1992) confirm this and define the site in more detail. Sulphation of mature full-length βAPP at 20 °C indicated that it had been transported into the TGN (Baeurle and Huttner, 1987; Rosa et al., 1992). At this temperature, mature full-length βAPP was not cleaved, as determined by the absence of intracellular 135 kDa protein (see Figure 10). This suggested that the secretase is located in a compartment that is temporally separate from these late Golgi events. As pulse–chase kinetics showed that the N-terminal fragment is immediately released after secretase cleavage of mature full-length βAPP, the secretase must either be inhibited at 20 °C, or, more likely, sorting of mature βAPP into a compartment that contains the secretase activity is inhibited at this reduced temperature. The increase in molecular mass of the mature βAPP at 20 °C is consistent with an increase in net charge as a result of the βAPP being accumulated within a compartment containing terminal glycosyltransferases. For instance, vesicular stomatitis virus G protein, which is transported from Golgi to the cell surface as a membrane glycoprotein, is localized within the TGN at 20 °C (de Curtis et al., 1988) and acquires additional sialic acids and galactosyl residues as a result of the increased residence time (Griffiths et al., 1985; Fuller et al., 1985). As the TGN is the site at which sorting of membrane and secreted proteins occurs (Griffiths and Simons, 1986), it seems likely that βAPP fragments are also sorted here. Furthermore, the localization of most of the cellular βAPP at steady state within a perinuclear compartment rich in sialic acids, as shown by staining with WGA (Tartakoff and Vassali, 1983), is consistent with a TGN distribution. A predominantly intracellular Golgi-like distribution has been described for many cultured cell types (Benowitz et al., 1989; Berkenbosch et al., 1990; Catteruccia et al., 1990; Luo et al., 1990; Shelton et al., 1990; Haass et al., 1991, 1992); however, the only evidence confirming that βAPP is localized mostly in the Golgi was obtained by Palacios et al. (1992) in rat brain neurons. Several studies have interpreted the immunolocalization of βAPP within intracellular vesicles after treatment with chloroquine as indicative of lysosomal accumulation (Hayashi et al., 1992; Wolozin et al., 1992). We show here by co-localization with WGA that chloroquine results in marked redistribution of Golgi-associated βAPP and does not cause an appreciable accumulation of βAPP within lysosomes. This is consistent with the conclusion that most of the synthesized βAPP is degraded via a non-lysosomal pathway and with the absence of full-length βAPP in lysosomal fractions on Percoll density gradients.

It has been proposed that the βAPP secretase is a plasma-membrane endoprotease which cleaves βAPP at the cell surface

of all cells (Sisodia, 1992). In contrast, our study with H4 cells and a recent study with PC12 cells (Sambamurti et al., 1992) provide evidence for intracellular secretase cleavage. A similar conclusion was reached in a study employing various protease inhibitors (De Strooper et al., 1992). Evidence for cleavage at the cell surface involves the presence of mature full-length βAPP at the plasma membrane and its subsequent release into the extracellular medium. Evidence for an intracellular site of secretase cleavage is provided by the presence of a membrane vesicle fraction that contains cleaved βAPP and, importantly, kinetic analyses showing that the accumulation of the cleaved N-terminal βAPP fragment within cellular compartment precedes and is proportional to its appearance in the extracellular medium. These kinetic results argue against the intracellular soluble βAPP arising from mature full-length βAPP that had been endocytosed from the cell surface or from soluble βAPP that was reinternalized from the extracellular medium by fluid-phase endocytosis. Because cleaved βAPP is rapidly secreted, only about 30% of the mature full-length βAPP (about 8% of the total synthesized βAPP) was associated with this fraction at steady state. Consequently, the intracellular soluble βAPP fraction is frequently missed in whole cell lysates and can only be observed by enrichment through isolation of a membrane vesicle fraction.

The presence of mature full-length βAPP at the cell surface of H4 cells is not inconsistent with an intracellular cleavage site, as it is conceivable that βAPP trafficking may follow more than one pathway. It has been argued that overexpression of βAPP in transfected cells has the same result where the levels of βAPP overcome the endogenous levels of secretase (Wolozin et al., 1992). A similar scenario has been described for lysosomal proteins (Carlsson and Fukuda, 1992; Harter and Mellman, 1992). The fraction of total βAPP that is at the cell surface of H4 cells is very small and appears to contribute minimally to the fraction appearing in the medium. Others have observed expression of full-length βAPP at the cell surface of different cell types; however, the size of this fraction relative to total cellular βAPP was not quantified (Weidemann et al., 1989; Refolo et al., 1991; Haass et al., 1992; Knops et al., 1992; Sisodia, 1992). Our results do not eliminate the possibility that some secretase activity exists at the cell surface. Rather, they show that the majority of secretase activity is located intracellularly. We also realize that immediate proteolysis and/or constitutive and rapid endocytosis of cell surface βAPP could result in a short residence time represented as a small fraction of total cellular βAPP. The absence of βAPP immunofluorescence at the plasma membrane and the lack of mature βAPP in plasma-membrane fractions would also be consistent with this. However, it is unlikely that this fraction contributes significantly to processing of full-length βAPP in H4 cells given the kinetic evidence for secretase cleavage.

Rapid delivery of the 15 kDa C-terminal fragment to lysosomes in H4 cells indicated that a lysosomal degradative pathway exists in these cells. What is unclear is whether transport to the lysosome requires passage through the plasma membrane (see Figure 13). The fact that the majority of the C-terminal fragment co-migrated with a membrane fraction that was slightly more dense than plasma membrane on Percoll gradients argues against this; however, it is not unequivocal given the arguments above for surface expression of mature full-length βAPP. It has been suggested that βAPP is transported through the plasma membrane to the lysosomes, where it is subsequently degraded (Haass et al., 1992). This pathway is further supported by (1) the presence of the N-P-T-Y sequence (residues 682–687 of APP-695) in the C-terminal tail of βAPP which is implicated in the binding of adaptins which mediate clathrin-dependent endocytosis (Chen et al., 1990; Trowbridge, 1991) and (2) the recovery of mature βAPP and the C-terminal fragment with immunoisolated clathrin-coated vesicles (Nordstedt et al., 1993). However, the potential involvement of Golgi-associated clathrin-coated domains in the sorting of βAPP before it reaches the plasma membrane has not received adequate consideration. It has not been established whether the N-P-T-Y motif plays a role in such sorting, which also involves clathrin-coated vesicles (Griffiths et al., 1985). Recently, it has been shown that a second cytoplasmic tail sequence is important for transport of lysosomal proteins directly from TGN to late endosomes and, then, lysosomes (Johnson and Kornfeld, 1992; Letourneur and Klausner, 1992). Specifically, Johnson and Kornfeld (1992) implicated a H-L-L-P-M sequence in sorting of mannose 6-phosphate receptor from TGN to late endosomes. A search of the Swissport protein sequence database showed that βAPP contains a homologous H-L-S-K-M sequence (residues 673–677) in its cytoplasmic tail. Although the βAPP sequence lacks the proposed critical L-L motif, it is still tempting to speculate on the importance of this βAPP region in Golgi sorting. The presence of the serine does not completely discount this possibility, as Letourner and Klausner (1992) showed that replacing the second leucine with an alanine did not result in complete loss of sorting. The importance of a direct TGN-to-lysosome pathway for βAPP processing has not been ruled out (Haass et al., 1992; Nordstedt et al., 1993) and the involvement of Golgi clathrin-coated membrane in sorting of βAPP to lysosomes, a degradative pathway shown to generate amyloidogenic fragments, must still be addressed.

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REFERENCES

Alcaraz, G., Kinet, J. P., Kumar, N., Wank, S. A. and Metzger, H. (1984) J. Biol. Chem. **259**, 14922–14927

Anderson, J. P., Esch, E. R., Keim, P. S., Sambaurti, K., Lieberburg, I. and Robakis, N. (1991) Neurosci. Lett. **128**, 126–128

Aronson, Jr., N. N. and Touster, O. (1974) Methods Enzymol. **31**, 90–102

Baeurle, P. A. and Huttner, W. B. (1987) J. Cell Biol. **105**, 2655–2664

Benowitz, L. I., Rodriguez, W., Paskevich, P., Mufson, E. J., Schenk, D. and Neve, R. L. (1989) Exp. Neurol. **106**, 237–250

Berkenbosch, F., Refolo, L. M., Friedrich, V. L., Jr., Casper, D., Blum, M. and Robakis, N. K. (1990) J. Neurosci. Res. **25**, 431–440

Bourdier, C. (1981) J. Biol. Chem. **256**, 1604–1607

Buxbaum, J. D., Gandy, S. E., Cicchetti, P., Ehrlich, M. E., Czernik, A. J., Fracasso, R. P., Ramabhadran, T. V., Unterbeck, A. J. and Greengard, P. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 6003–6006

Caporaso, G. L., Gandy, S. E., Buxbaum, J. D. and Greengard, P. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 2252–2256

Carlsson, S. R. and Fukuda, M. (1992) Arch. Biochem. Biophys. **296**, 630–649

Catteruccia, N., Willingale-Theune, J., Bunke, D., Prior, R., Masters, C. L., Crisanti, A. and Beyreuther, K. (1990) Am. J. Pathol. **137**, 19–26

Chen, W.-J., Goldstein, J. L. and Brown, M. S. (1990) J. Biol. Chem. **265**, 3116–3123

Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159

de Curtis, I., Howell, K. E. and Simons, K. (1988) Exp. Cell Res. **175**, 248–265

De Strooper, B., Van Leuven, F. and Van Den Berghe, H. (1992) FEBS Lett. **308**, 50–53

Esch, F. S., Keim, P. S., Beattie, E. C., Blacher, R. W., Culwell, A. R., Oltersdorf, T., McClure, D. and Ward, P. J. (1990) Science **248**, 1122–1124

Estus, S., Golde, T. E., Kunishita, T., Blades, D., Lowery, D., Eisen, M., Usiak, M., Qu, X., Tabira, T., Greenberg, B. D. and Younkin, S. G. (1992) Science **255**, 726–728

Fujiki, Y., Hubbard, A. L., Fowler, S. and Lazarow, P. B. (1982) J. Cell Biol. **93**, 97–102

Fukuchi, K.-I., Kamino, K., Deeb, S. S., Smith, A. C., Dang, T. and Martin, G. M. (1992) Biochem. Biophys. Res. Commun. **182**, 165–173

Fuller, S. D., Bravo, R. and Simons, K. (1985) EMBO J. **4**, 297–307

Gandy, S. E., Bhasin, R., Ramabhadran, T. V., Koo, E. H., Price, D. L., Goldgaber, D. and Greengard, P. (1992) J. Neurochem. **58**, 383–386

Golde, T. E., Estus, S., Younkin, L. H., Selkoe, D. J. and Younkin, S. G. (1992) Science **255**, 728–730
Green, S. A., Zimmer, K.-P., Griffiths, G. and Mellman, I. (1987) J. Cell Biol. **105**, 1227–1240
Greenberg, B. D., Kezdy, F. J. and Kisilevsky, R. (1991) Annu. Rep. Med. Chem. **26**, 229–238
Griffiths, G. and Simons, K. (1986) Science **234**, 438–443
Griffiths, G., Pfeiffer, S., Simons, K. and Matlin, K. (1985) J. Cell Biol. **101**, 949–964
Haass, C., Hung, A. Y. and Selkoe, D. J. (1991) J. Neurosci. **11**, 3783–3793
Haass, C., Koo, E. H., Mellon, A., Hung, A. Y. and Selkoe, D. J. (1992) Nature (London) **357**, 500–503
Hall, W. H., Liebaers, I., Di Natale, P. and Neufeld, E. F. (1978) Methods Enzymol. **50**, 439–456
Harter, C. and Mellman, I. (1992) J. Cell Biol. **117**, 311–325
Hayashi, Y., Kashiwagi, K. and Yoshikawa, K. (1992) Biochem. Biophys. Res. Commun. **187**, 1249–1255
Hubbard, A. L. and Cohn, Z. A. (1975) J. Cell Biol. **64**, 438–460
Joachim, C. L. and Selkoe, D. J. (1992) Alzheimer Dis. Assoc. Disorders **6**, 7–34
Johnson, K. F. and Kornfeld, S. (1992) J. Biol. Chem. **267**, 17110–17115
Kang, J., Lemaire, H.-G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K.-H., Multhaup, G., Beyreuther, K. and Muller-Hill, B. (1987) Nature (London) **325**, 733–736
Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shiojiri, S. and Ito, H. (1988) Nature (London) **331**, 530–532
Klausner, R. D. and Sitia, R. (1990) Cell **62**, 611–614
Knops, J., Lieberburg, I. and Sinha, S. (1992) J. Biol. Chem. **267**, 16022–16024
Kuismanen, E. and Saraste, J. (1989) Methods Cell Biol. **32**, 257–274
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Letourneur, F. and Klausner, R. D. (1992) Cell **69**, 1143–1157
Lisanti, M. P., Sargiacomo, M., Graeve, L., Saltiel, A. R. and Rodriguez-Boulan, E. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 9557–9561
Lowery, D. E., Pasternack, J. M., Gonzalez-DeWhitt, P. A., Zurcher-Neely, H., Tomich, C.-S., Altman, R. A., Fairbanks, M. B., Heinrickson, R. L., Younkin, S. G. and Greenberg, B. D. (1991) J. Biol. Chem. **266**, 19842–19850
Luo, L., Martin-Morris, L. E. and White, K. (1990) J. Neurosci. **10**, 3849–3861
Maxfield, F. R. (1982) J. Cell Biol. **95**, 676–681
Nordstedt, C., Gandy, S. E., Alafuzoff, I., Caporaso, G. L., Iverfeldt, K., Grebb, J. A., Winblad, B. and Greengard, P. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 8910–8914
Nordstedt, C., Caporaso, G. L., Thyberg, J., Gandy, S. E. and Greengard, P. (1993) J. Biol. Chem. **268**, 608–612
Oltersdorf, T., Fritz, L. C., Schenk, D. B., Lieberburg, I., Johnson-Wood, K. L., Beattie, E. C., Ward, P. J., Blacher, R. W., Dovey, H. F. and Sinha, S. (1989) Nature (London) **341**, 144–147
Oltersdorf, T., Ward, P. J., Henriksson, T., Beattie, E. C., Neve, R., Lieberburg, I. and Fritz, L. C. (1990) J. Biol. Chem. **265**, 4492–4497
Overly, C. C., Fritz, L. C., Lieberburg, I. and McConlogue, L. (1991) Biochem. Biophys. Res. Commun. **181**, 513–519
Palacios, G., Palacios, J. M., Mengod, G. and Frey, P. (1992) Mol. Brain Res. **15**, 195–206
Palmert, M. R., Podlisny, M. B., Witker, D. S., Oltersdorf, T., Younkin, L. H., Selkoe, D. J. and Younkin, S. G. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 6338–6342
Ponte, P., Gonzalez-DeWhitt, P., Schilling, J., Miller, J., Hsu, D., Greenberg, B., Davis, K., Wallace, W., Lieberburg, I., Fuller, F. and Cordell, B. (1988) Nature (London) **331**, 525–527
Refolo, L. M., Wittenberg, I. S., Friedrich, Jr., V. L. and Robakis, N. K. (1991) J. Neurosci. **11**, 3888–3897
Rosa, P., Mantovani, S., Rosboch, R. and Huttner, W. B. (1992) J. Biol. Chem. **267**, 12227–12232
Sahasrabudhe, S. R., Spruyt, M. A., Muenkel, H. A., Blume, A. J., Vitek, M. P. and Jacobsen, J. S. (1992) J. Biol. Chem. **267**, 25602–25608
Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. (1988) Science **239**, 487–491
Sambamurti, K., Shioi, J., Anderson, J. P., Pappolla, M. A. and Robakis, N. K. (1992) J. Neurosci. Res. **33**, 319–329
Selkoe, D. J. (1989) Cell **58**, 611–612
Selkoe, D. J., Podlinsky, M. B., Joachim, C. L., Vickers, E. A., Lee, G., Fritz, L. C. and Oltersdorf, T. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 7341–7345
Shelton, E. R., Cohn, R., Fish, L., Obernolte, R., Tahilramani, R., Nestor, J. J. and Chan, H. W. (1990) J. Neurochem. **55**, 60–69
Shivers, B. D., Hilbich, C., Multhaup, G., Salbaum, M., Beyreuther, K. and Seeburg, P. H. (1988) EMBO J. **7**, 1365–1370
Sisodia, S. S. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 6075–6079
Sisodia, S. S., Koo, E. H., Beyreuther, K., Unterbeck, A. and Price, D. L. (1990) Science **248**, 492–495
Swanson, J. A., Yirinec, B. D. and Silverstein, S. C. (1985) J. Cell Biol. **100**, 851–859
Tamaoka, A., Kalaria, R. N., Lieberburg, I. and Selkoe, D. J. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 1345–1349
Tanzi, R. E., McClatchey, A. I., Lamberti, E. D., Villa-Komaroff, L., Gusella, J. F. and Neve, R. L. (1988) Nature (London) **331**, 528–530
Tartakoff, A. M. (1983) Cell **32**, 1026–1028
Tartakoff, A. M. and Vassali, P. (1983) J. Cell Biol. **97**, 1243–1248
Trowbridge, I. S. (1991) Curr. Opin. Cell Biol. **3**, 634–641
Van Nostrand, W. E., Schmaier, A. H., Farrow, J. S. and Cunningham, D. D. (1990) Science **248**, 745–748
Weidemann, A., Konig, G., Bunke, D., Fischer, P., Salbaum, J. M., Masters, C. L. and Beyreuther, K. (1989) Cell **57**, 115–126
Wolozin, B., Bacic, M., Merrill, M. J., Lesch, K. P., Chen, C., Lebovics, R. S. and Sunderland, T. (1992) J. Neurosci. Res. **33**, 163–169
Yamaoka, K., Tangigawara, Y., Nakagawa, T. and Uno, T. (1981) J. Pharm. Dyn. **4**, 879–885

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