ported by two findings. First, Ig-α–CD8 chimeric heterodimers but not Ig-β–containing chimeras are able to transduce signals leading to interleukin-2 production in the B cell lymphoma, A20 (*24*). Second, stimulation through isolated CD3 ε or TCR ζ chains results in distinct patterns of phosphorylation (*9*) and Ca^{2+} mobilization responses (*25*).

The observation that Ig-α and Ig-β can bind to different effector molecules may have implications for the function of other oligomeric receptors that can utilize alternate ARH1-containing chains to assemble different signal transduction complexes. For example, TCRs that contain δ-ε heterodimers may use different signal transduction pathways than those that contain γ-ε heterodimers (*26*). Differences in lymphocyte responses to antigen, which occur as a function of maturation, could be determined by such expression of alternative signal transduction complexes (*27*).

The Src family tyrosine kinases and pp40 and pp42 have different patterns of association with the ARH1 motifs of Ig-α and Ig-β, which suggests that they may interact with distinct sites within the cytoplasmic domains. These interactions, which are stable in NP-40, are reminiscent of other kinase-linked receptors such as the PDGFR, in which discrete regions mediate the receptor's association with distinct effector molecules (*21, 28*). Presumably, chain-specific residues, in the context of the antigen receptor's ARH1 motifs, determine the specificity of these interactions.

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Production of the Alzheimer Amyloid β Protein by Normal Proteolytic Processing

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The 4-kilodalton (39 to 43 amino acids) amyloid β protein (βAP), which is deposited as amyloid in the brains of patients with Alzheimer's disease, is derived from a large protein, the amyloid β protein precursor (βAPP). Human mononuclear leukemic (K562) cells expressing a βAP-bearing, carboxyl-terminal βAPP derivative released significant amounts of a soluble 4-kilodalton βAPP derivative essentially identical to the βAP deposited in Alzheimer's disease. Human neuroblastoma (M17) cells transfected with constructs expressing full-length βAPP and M17 cells expressing only endogenous βAPP also released soluble 4-kilodalton βAP, and a similar, if not identical, fragment was readily detected in cerebrospinal fluid from individuals with Alzheimer's disease and normal individuals. Thus cells normally produce and release soluble 4-kilodalton βAP that is essentially identical to the 4-kilodalton βAP deposited as insoluble amyloid fibrils in Alzheimer's disease.

In the brains of patients with Alzheimer's disease (AD), amyloid is deposited in senile plaques (*1, 2*) and, in many cases, in the walls of cerebral and meningeal blood vessels (*3*). The amyloid fibrils in the brains of patients with AD are composed of a 39- to 43–amino acid amyloid β protein (βAP) (*4–8*) that is derived from a set of 695- to 770–amino acid precursor proteins (*7, 9–11*) collectively referred to as the amyloid β protein precursor (βAPP). In each of the 695- to 770-residue βAPPs, the βAP is an internal peptide that begins 99 residues from the COOH-terminal end of the βAPP and extends from the extracellular-intraluminal region into the middle of the hydrophobic, membrane-spanning domain. The βAPP is normally processed in constitutive secretory (*12–15*) and endosomal-lysosomal (*16–20*) pathways. In the secretory pathway, the βAPP is cleaved within the βAP (*21, 22*) to produce a large secreted derivative and a small membrane-associated fragment, neither of which can produce amyloid because they do not contain the entire

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βAP. The endosomal-lysosomal system processes a complex set of COOH-terminal derivatives that includes potentially amyloidogenic forms with the entire βAP at or near their NH_2-terminus (*18, 20, 23*).

To determine whether βAP can be produced from βAP-bearing COOH-terminal βAPP derivatives (*18*), human mononuclear leukemic (K562) cells were stably transfected with LC_{99}, a construct (Fig. 1A) that begins with the 17–amino acid βAPP signal peptide, continues with the leucine that normally follows the βAPP signal peptide, and ends with the 99 amino acids at the COOH-terminal end of the βAPP ($βAP_{1-43}$ followed by C_{56}). As expected (Fig. 1B), lysates of these cells have large amounts of an 11.4-kD protein that is immunoprecipitated both by an antiserum to the COOH-terminal end of the βAPP and by SGY2134, an antiserum raised to $βAP_{1-40}$ that contains antibodies directed primarily at $βAP_{1-16}$ (*18*). The size of this 11.4-kD protein is precisely that expected after the signal peptide has been removed to produce LC_{99}, and this 11.4-kD protein is rapidly metabolized after metabolic labeling for 1 hour (Fig. 1B).

In an effort to identify βAP released from K562-LC_{99} cells, we used SGY2134 to immunoprecipitate the protein in medium conditioned either with K562-LC_{99} cells or with cells (K562-CEP4β) transfected with vector alone. The immunoprecipitated protein was separated on 10 to 16% tris-tricine gels (*18*), transferred to immobilon P, and labeled with 4G8, a mouse monoclonal antibody to $βAP_{17-24}$ (*24*). As a control, synthetic $βAP_{1-40}$ (1, 2, 5, 10, 25, or 50 pmol) was added to the culture medium and analyzed identically. A 4-kD protein that comigrated with synthetic $βAP_{1-40}$ was identified in medium conditioned with K562-LC_{99} cells but not in medium conditioned with K562 cells transfected with vector alone (Fig. 1C).

Virtually all K562-LC_{99} cells (>95%) were viable at the end of conditioning as judged by trypan blue exclusion. Nonetheless, to exclude the possibility that the externalized 4-kD protein was associated with sloughed membranes (possibly from dying cells), we compared the immunoprecipitates obtained from medium before and after centrifugation at 100,000*g* for 90 min. Neither synthetic $βAP_{1-40}$ nor the 4-kD protein released from K562-LC_{99} cells was removed appreciably by high speed centrifugation (Fig. 1C).

For definitive identification, the 4-kD protein in 400 ml of medium conditioned for 24 hours with 2×10^8 K562-LC_{99} cells was concentrated, immunoprecipitated with SGY2134, transferred to immobilon, excised after visualization with Coomassie blue, and sequenced. The sequence (Fig. 1D), which could be read as far as cycle 31, showed the 4-kD protein to be βAP with a leucine at its NH_2-terminus. Thus our data show that cells transfected with a model βAP-bearing, COOH-terminal derivative process that derivative to release βAP into the medium.

Because our previous data indicated that βAP-bearing COOH-terminal derivatives are produced in the endosomal-lysosomal system (*23*), we investigated the effect of inhibitors of this system (leupeptin, E-64, ammonium chloride, and chloroquine) on the production of βAP from LC_{99} (Fig. 1E). Control cells showed a slight progressive increase in the βAP released in the 0- to 6-, 6- to 12-, and 12- to 18-hour intervals, most likely due to continuing cell division. Cells treated with leupeptin and E-64 behaved similarly. Thus proteases inhibited by these agents are not involved in the production of βAP from LC_{99}. However, ammonium chloride, added when the medium was changed after a control conditioning period of 6 hours, caused a marked reduction in the production of βAP in the 6- to 12-hour interval, and it virtually abolished production in the 12- to 18-hour interval. Chloroquine had a similar but less dramatic effect. Thus the two lysosomotropic agents, which block proteolysis by alkalinizing acidic compartments, reduce the production of βAP. These data suggest that the βAP may be produced, at least in part, by lysosomal processing of βAP-bearing, COOH-terminal derivatives and that the

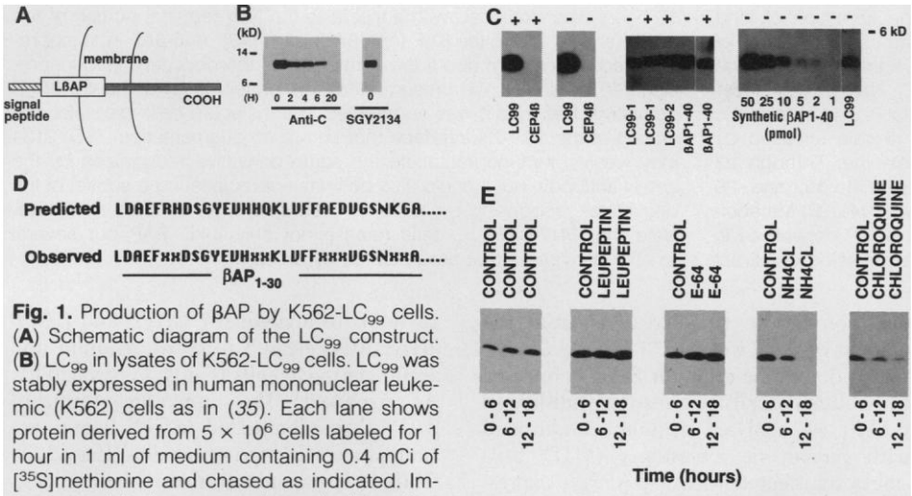

Fig. 1. Production of βAP by K562-LC_{99} cells. (**A**) Schematic diagram of the LC_{99} construct. (**B**) LC_{99} in lysates of K562-LC_{99} cells. LC_{99} was stably expressed in human mononuclear leukemic (K562) cells as in (*35*). Each lane shows protein derived from 5×10^6 cells labeled for 1 hour in 1 ml of medium containing 0.4 mCi of [^{35}S]methionine and chased as indicated. Immunoprecipitation was performed with antiserum to the COOH-terminus of the βAPP (4 μl) or SGY2134 (5 μl) and protein A–agarose slurry (30 μl) (Boehringer Mannheim). Immunoprecipitates were separated by 10/16% tris-tricine SDS-PAGE. Methods were as described (*18, 23*). (**C**) βAP in medium conditioned with K562 cells expressing LC_{99} or CEP4β vector alone. Medium (Ultraculture, BioWhittaker) from cultures seeded at 2×10^5 cells per milliliter and grown for 4 to 7 days was examined before (−) or after (+) centrifugation at 100,000*g* for 90 min. Immunoprecipitation and immunoblotting were as in (*35*). Media from five separate cultures were analyzed in the three experiments shown in lanes 1 to 4 (two cultures), lanes 5 to 9 (two cultures), and lanes 10 to 16 (one culture). βAP was added to culture medium and analyzed identically [lanes 8 and 9 (10 pmol) and lanes 10 to 15 (50, 25, 10, 5, 2, and 1 pmol)]. (**D**) Sequence of 4-kD protein in conditioned medium. The 4-kD protein in 400 ml of medium conditioned for 24 hours with K562-LC_{99} cells seeded at 5×10^5 cells per milliliter was isolated and sequenced as in (*38*). The sequence assignment was definite for all residues except those shown as X. (**E**) Effect of inhibitors on the release of βAP. Medium was conditioned with K562-LC_{99} cells in the presence or absence of inhibitors as described (*39*). Analysis of the βAP in these 20-ml samples of conditioned medium was as in (*37*).

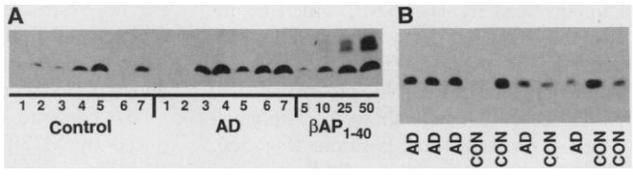

Fig. 2. βAP in CSF. Analysis of 3-ml samples was as in (*37*). (**A**) Autopsy CSF. The age and postmortem interval (mean ± SEM) of the histopathologically confirmed AD patients were 74.6 ± 1.8 years and 7.5 ± 2.5 hours, respectively. The age and postmortem interval of patients shown histopathologically to be without central nervous system disease were 68.7 ± 4.3 years and 8.9 ± 1.6 hours, respectively. (**B**) CSF from living patients. The diagnosis of AD in the living patients was established clinically by reviewing established criteria (*40–42*). All patients included were classified as having probable AD. Patients classified in the non-AD category were evaluated similarly. The ages (mean ± SEM) of the AD and non-AD patients were 73.2 ± 1.3 and 73.6 ± 0.7 years, respectively.

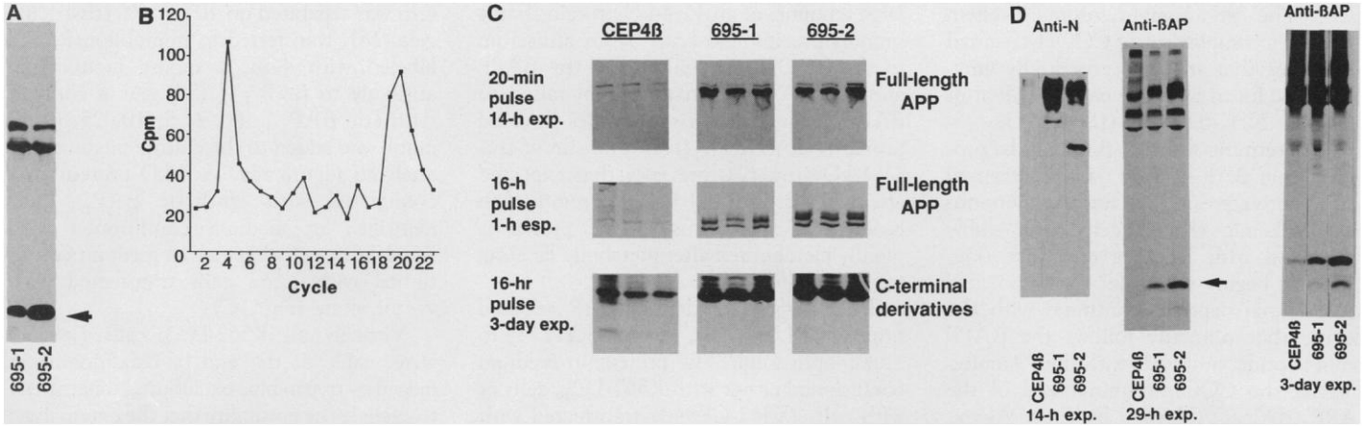

Fig. 3. Production of βAP by M17 cells. (**A**) Immunoprecipitation and immunoblotting of the 4-kD protein released by M17-βAPP$_{695}$ cells. Two M17 lines stably transfected to express βAPP$_{695}$ as in (*18*) were treated with retinoic acid (1 μM) for 7 days to induce the formation of long neurites. Confluent (10-cm) dishes were then used to condition 10 ml of medium containing 1 μM retinoic acid for 3 days. Immunoprecipitation with SGY2134 and labeling with 4G8 were as in (*37*). Note the antibody-related artifact at the 10/16% interface of the gels that is also seen in (D) (lanes 3 to 5). (**B**) Radiosequencing of the 4-kD protein released by M17-βAPP$_{695}$ cells after labeling with [^{3}H]phenylalanine. Through 23 cycles, [^{3}H]phenylalanine was detected at positions 4, 19, and 20 indicating that the 4-kD protein is βAP. For details see (*44*). (**C**) Metabolically labeled full-length βAPP and COOH-terminal βAPP derivatives in M17 cell lines stably transfected with a βAPP$_{695}$ expression construct (695-1, 695-2) or vector alone (CEP4β). For details see (*43*). (**D**) Immunoprecipitation of metabolically labeled derivatives released into the medium. Medium from the cells in (C) that were metabolically labeled for 16 hours was examined with antisera to the NH_2-terminal portion of the βAPP (anti-N) and to the βAP (anti-βAP) as in (*43*). Anti-βAP immunoprecipitated a protein of about the same size as the derivative immunoprecipitated by anti-N. We presume that this protein is an authentic βAPP derivative, because it was augmented in transfected cells (see also the artifact at the 10/16% interface that shows no augmentation). SGY2134 may weakly immunoprecipitate the same derivative recognized by the anti-N antibody, but it could also be immunoprecipitating a subset of the derivatives recognized by anti-N or even an entirely different derivative. Note that M17-βAPP$_{695}$ cells release not only 4-kD βAP but several smaller derivatives that are detected by anti-βAP.

βAP may be a relatively indigestible βAPP fragment that is then externalized (*25, 26*). Additional data are clearly needed, however, to establish the role of lysosomal processing in βAP production and to rule out the possibility that chloroquine and ammonium chloride are interfering with a different processing pathway.

To determine whether βAP is also released into human cerebrospinal fluid (CSF) and by cultured cells expressing full-length βAPP, we used the same criteria that were successful in assaying synthetic βAP$_{1-40}$ and the βAP released from K562-LC$_{99}$ cells: immunoprecipitation by SGY2134 of a 4-kD protein that can be detected on 4G8 immunoblots. In our initial experiment on CSF, we analyzed 3-ml samples obtained at autopsy from seven patients with AD and seven control subjects (Fig. 2A). Strong signals were obtained in CSF from five of the seven autopsy-confirmed AD patients, but considerable βAP was also present in samples from three of the seven control subjects. We also examined 3-ml samples of CSF from living patients, five from patients with probable AD and five from age-matched patients without AD (Fig. 2B). Again, there was considerable overlap in the amount of βAP observed in the AD and non-AD group. Thus our initial survey indicates that there is (i) readily detectable βAP in the CSF of AD and control patients, (ii) considerable apparent interindividual variation in the amount of βAP in CSF, and (iii) no obvious correlation between AD and the amount of βAP in CSF.

To determine whether βAP is released by cultured cells expressing full-length βAPP, we analyzed medium conditioned with human neuroblastoma (M17) cells stably transfected with a βAPP$_{695}$ expression construct. In these experiments, the transfected M17 cells were differentiated for 7 days with retinoic acid to induce the formation of long neurites. M17-βAPP$_{695}$ cells (Fig. 3A) released readily detectable amounts of a 4-kD protein that was immunoprecipitated by SGY2134 (anti-βAP$_{1-16}$) and labeled by 4G8 (anti-βAP$_{17-24}$). On immunoblots this protein was indistinguishable from synthetic βAP$_{1-40}$ or the βAP released by K562-LC$_{99}$ cells (Fig. 1).

To definitively identify the 4-kD protein released from M17-βAPP$_{695}$ cells, we metabolically labeled these cells with [^{3}H]phenylalanine for 16 hours, isolated the 4-kD protein released into the culture medium, and radiosequenced it. The βAP, which begins with the sequence Asp-Ala-Glu-Phe, has phenylalanine at positions 4, 19, and 20. Radiosequencing of the 4-kD protein released by M17-βAPP$_{695}$ cells showed [^{3}H]phenylalanine at precisely those positions (Fig. 3B), indicating that this protein is βAP.

In order to better quantitate the βAP released from these cells, M17-βAPP$_{695}$ cells and M17 cells transfected with vector alone (M17-CEP4β) were metabolically labeled with [^{35}S]methionine for 20 min to analyze the rate of βAPP synthesis and for 16 hours to analyze the βAPP and βAPP derivatives released into the medium and accumulating in cells (Fig. 3, C through E). As expected, the immature form of βAPP$_{695}$ predominated in cell lysates examined after 20 min of labeling (Fig. 3C). With 16 hours of continuous labeling, cells accumulated considerable full-length βAPP, particularly the mature form (Fig. 3C); they accumulated readily detectable amounts of the 8- to 12-kD COOH-terminal derivatives (Fig. 3C); and they secreted large amounts of the ~105-kD NH_2-terminal derivative produced by βAPP secretase (Fig. 3D, lanes 2 and 3).

Significantly, M17-βAPP$_{695}$ cells continuously labeled for 16 hours released the 4-kD βAP immunoprecipitated by SGY2134 in amounts that permitted detection after exposing the fluorogram for only 29 hours (Fig. 3D). After a 3-day exposure of the fluorograms (Fig. 3D), it was evident that M17 cells transfected with vector alone (which endogenously express much more βAPP than K562-CEP4β cells) also release the 4-kD βAP. Thus even M17 cells expressing only endogenous βAPP release the 4-kD βAP.

To compare the various derivatives produced by M17-βAPP$_{695}$ cells, we analyzed them with phosphorimaging technology. Since good estimates of the number of radiolabeled amino acids in each derivative can be made, the relative number of moles of derivative present in each dish can be accurately assessed by dividing the per dish signal obtained for each derivative by the number of

radiolabeled amino acids that it contains. This analysis showed that, in M17-βAPP$_{695}$ cells labeled continuously for 16 hours, the 4-kD βAP accumulates in the medium at about 40% of the amount of the major ~105-kD derivative. Thus, M17 cells externalize a significant amount of the ~4-kD βAP.

Our analysis of human CSF and transfected cells indicates that the βAPP is normally cleaved both in its membrane-spanning domain on the COOH side of the βAP, an unusual site for proteolytic cleavage, and in its extracellular-intraluminal region on the NH_2 side of the βAP to release a soluble 4-kD derivative essentially identical to the βAP that forms amyloid in AD (*27*). Thus it is likely that amyloid deposition in AD involves pathways that normally produce extracellular βAP and that the amount of amyloid deposited depends on the rate of βAP production, its rate of removal, and the rate at which soluble βAP forms insoluble amyloid fibrils. It will, therefore, be important to exploit cultured cells to identify (i) the specific pathway and proteases that produce and release βAP, (ii) factors that foster βAP production, and (iii) potential therapeutic agents that can prevent βAP production. In addition, it will be important to identify the mechanism or mechanisms that remove soluble extracellular βAP and to clarify the factors (*28–32*) that cause soluble, extracellular βAP to form amyloid fibrils.

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27. On the basis of its size and solubility, the 4-kD βAP identified here appears to be most closely related to the βAP$_{1-39}$ that forms cerebrovascular amyloid fibrils (*8*) and the 4-kD βAP recently identified (*33, 34*) as the major constituent of diffuse plaques (preamyloid deposits) found in the AD brain and to a lesser extent in the normal aged brain. The amyloid in classic senile plaques, which is less soluble than preamyloid or vascular amyloid, consists primarily of βAP$_{1-42\ or\ 43}$. The βAPP processing that produces the soluble 4-kD βAP identified in this report may produce βAP$_{1-42\ or\ 43}$, but this peptide would be less soluble and thus more difficult to detect than the slightly shorter βAP that forms cerebrovascular amyloid fibrils or preamyloid.
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35. LC$_{99}$ was expressed by using an episomal replicon (CEP4β) similar to that described (*36*) except that expression was driven by a CMV promoter. To assemble CEP4β-LC$_{99}$, we used a βAPP$_{695}$ template and appropriate oligonucleotides to amplify by the polymerase chain reaction (PCR) two βAPP cDNAs. Using oligos 1 and 2, we amplified a cDNA with a Hind III restriction site followed by bases −30 to 54 and bases 1789 to 1809; with oligos 3 and 4, we amplified a cDNA with bases 39 to 54 followed by bases 1789 to 2119 and a Bam HI restriction site [numbering is according to (*7*)]. The two amplified cDNAs were gel-purified and mixed in equimolar amounts, and the hybrid was amplified in a second PCR. The amplified cDNA was cut with Hind III and Bam HI and cloned into CEP4β to produce a vector containing untranslated 5′ sequence (−30 to −1) and the adjacent sequence (1 to 54) encoding βAPP$_{1-18}$ (signal peptide plus leucine) followed by sequence encoding C$_{99}$ (1789 to 2085) and 3′ untranslated sequence (2086 to 2119). The oligos employed were (1) CCCAAGCTTCACAGCTAGCGCACTCGGTGCCCCGCGCAGGGTCG, (2) GTCATGTCGGAATTCTGCATCCAGCGCCCGAGCCGT, (3) TGGACGGCTCGGGCGCTGGATGCAGAATTCCGACAT, and (4) GACTCGAGTCGACGGATCCTGTCCAACTTCAGAGGCTGC. Stable lines expressing CEP4β or CEP4β-LC$_{99}$ were established by transfection with lipofectin (BRL).
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37. For immunoprecipitation of βAP, 5 to 20 ml of medium was made 1× in RIPA (150 mM NaCl, 10 mM tris, pH 8.0, 1% Nonidet P-40, 0.5% cholic acid, 0.1% SDS, and 5 mM EDTA)–LPT (1 μg/ml leupeptin, 0.1 μg/ml pepstatin, 2 mM phenylmethyl sulfonylfluoride, and 1 μg/ml 7-amino-1-chloro-3-tosylamido-2-heptanone) buffer; SGY2134 (20 μl) and RIPA-LPT–washed protein A–agarose slurry (40 μl) were added; and the medium was rocked at 4°C for 24 hours. After washing three times with RIPA-LPT and once with TBS (10 mM tris, pH 8, 150 mM NaCl), the immunoprecipitated protein was separated by 10/16% tris-tricine SDS–polyacrylamide gel electrophoresis (PAGE), transblotted to immobilon P (Millipore) at 100 V for 2 hours, incubated with 5% dried milk for 1 hour, and labeled with 4G8 (1:500) for 3 hours. Fluorograms were then prepared with an ECL Western Blotting Kit (Amersham) according to the manufacturer's specifications.
38. Medium (400 ml) conditioned with K562-LC$_{99}$ cells was concentrated to 11 ml (Amicon concentrator, 3-kD cutoff filter). The 4-kD βAP was then immunoprecipitated with anti-βAP$_{1-40}$ antibody (SGY2134), separated by tris-tricine SDS-PAGE, and transblotted to Immobilon P as in (*37*). After visualization with Coomassie blue R-250, the 4-kD protein was excised and thoroughly washed with distilled H_2O, and the sequence shown in Fig. 1D was obtained on a 477A Sequenator with 120A-PTH analyzer (Applied Biosystems).
39. Each of five 20-ml aliquots of medium were conditioned with K562-LC$_{99}$ cells at 5 × 10^5 cells per milliliter for 6 hours, and the control medium (0 to 6 hours) from each set of cells was removed. Fresh control medium was added to the first set of cells; medium containing leupeptin (100 μg/ml), E-64 (100 μM), and ammonium chloride (20 mM) or chloroquine (100 μM) was added to the other sets of cells. After 6 hours of conditioning, this medium (6 to 12 hours) was removed. A fresh 20-ml aliquot of the identical medium was then added for six additional hours of conditioning (12 to 18 hours).
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43. Two M17 lines stably transfected with βAPP$_{695}$ (different from those in Fig. 3A) and a line stably transfected with CEP4β alone were generated, selected, and maintained with Hygromycin (500 μg/ml) as in (*18*). Triplicate 10-cm dishes containing each of these lines were treated with retinoic acid (1 μM) for 7 days. Confluent dishes were then labeled for 20 min (Fig. 3C) or 16 hours (Fig. 3, C and D) in 5 ml of methionine-deficient medium (Gibco) containing 0.8 mCi of [^{35}S]methionine. For Fig. 3C, the cells in each 10-cm dish were lysed and immunoprecipitated for 3 hours at 4°C with antiserum to the COOH-terminus of the βAPP (5 μl) and protein A–agarose slurry (25 μl) as in (*18*). For Fig. 3D, the medium covering the triplicate dishes in Fig. 3C (16-hour pulse) was pooled to give 15 ml of conditioned medium. Immunoprecipitation was performed as in (*37*). From each pool (CEP4β, 695-1, and 695-2), 1 ml was immunoprecipitated for 3 hours at 4°C with 10 μl of anti-N and 25 μl of protein G–agarose slurry (lanes 1 to 3), and 6 ml were immunoprecipitated for 24 hours at 4°C with 20 μl of anti-βAP and 40 μl of protein A–agarose (lanes 4 to 6). Anti-N is a goat polyclonal antiserum (207) raised to a truncated form of βAPP$_{770}$ (βAPP$_{18-686}$). Anti-βAP is a rabbit polyclonal antiserum (SGY2134) raised to synthetic βAP$_{1-40}$ that contains antibodies directed primarily to βAP$_{1-16}$. Immunoprecipitates in Fig. 3, C and D, were separated by 10/16% tris-tricine SDS-PAGE.
44. For radiosequencing, each of four confluent 10-cm dishes of M17-βAPP$_{695}$ cells was labeled for 16 hours in 5 ml of phenylalanine-deficient medium (Gibco) containing 1.0 mCi of [^{3}H]phenylalanine. The labeled 4-kD protein in each dish was immunoprecipitated, separated, and transferred to Immobilon P as in (*37*). After autoradiographic visualization, the 4-kD protein excised from each of four lanes was pooled and sequenced as in (*38*), and the ^{3}H signal released in each cycle was analyzed.

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