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# Cell-Surface β-Amyloid Precursor Protein Stimulates Neurite Outgrowth of Hippocampal Neurons in an Isoform-Dependent Manner

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**β-Amyloid precursor protein (βAPP) is an integral membrane polypeptide expressed in many neural and non-neural cells. βAPP occurs in part at the cell surface and undergoes proteolytic processing to release the large soluble ectodomain ($APP_s$) and the amyloid β-peptide (Aβ), both of which have apparent trophic activity *in vitro*. Despite intense interest in βAPP expression and metabolism, there is limited knowledge about the function mediated by βAPP inserted at the cell-surface. We established a coculture system in which βAPP-transfected CHO cells serve as a substrate for the growth of primary rat hippocampal neurons. Compared to nontransfected CHO cells, the increased surface βAPP of the transfectants stimulated short-term neuronal adhesion and longer-term neurite outgrowth, whereas the increased amount of secreted $APP_s$ and Aβ in conditioned medium produced no such effects when neurons were grown either on untransfected CHO cells or on a polylysine substrate. Moreover, a peptide which has been shown to block the trophic effects of secreted $APP_s$ (Ninomiya et al., 1993) failed to interrupt the neurite promoting activity mediated by the surface-expressed βAPP. Surface-expressed $βAPP_{751}$ or $βAPP_{770}$ isoforms mediated more neurite outgrowth than did the $βAPP_{695}$ isoform. Antibody blocking and regional deletion experiments indicated that the mid-region of the βAPP ectodomain (residues 361–648) is involved in promoting neurite outgrowth. We conclude that surface-expressed cellular βAPP has a neurite-promoting function which is distinct from the trophic function of the secreted βAPP derivatives and may have special significance during brain development.**

***[Key words: surface-expressed β-amyloid precursor protein (βAPP), CHO cells, hippocampal neurons, adhesion, neurite outgrowth]***

Like many adhesion molecules of the cell surface, the β-amyloid precursor protein (βAPP) is an integral membrane polypeptide containing a large extracellular domain, a single transmembrane region and a short cytoplasmic tail (Kang et al., 1987). Because proteolytic processing of βAPP generates the amyloid β-peptide (Aβ), the major component of the cerebral amyloid progressively deposited in Alzheimer's disease (Glenner and Wong, 1984), much effort is being directed at understanding the role of βAPP metabolism in the pathogenesis of this complex disorder. Two βAPP processing pathways—secretory cleavage at or near the cell surface (Esch et al., 1990; Sisodia et al., 1990) and reinternalization and lysosomal targeting (Cole et al., 1989; Golde et al., 1992; Haass et al., 1992a)—have been identified to date. Although both of these pathways regulate the amount of intact βAPP at the cell surface, little is known about the normal function of surface βAPP, particularly in the CNS where βAPP is expressed at highest abundance.

The βAPP pre-mRNA undergoes alternative exon splicing to yield three major isoforms of 695, 751, or 770 amino acids (Kang et al., 1987; Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988). Two of these ($βAPP_{751}$ and $βAPP_{770}$) contain a 56 residue insert which is highly homologous to the Kunitz family of serine protease inhibitors (KPI) and are expressed ubiquitously. In contrast, the shorter isoform lacking the KPI motif, $βAPP_{695}$, is almost solely expressed in the nervous system, both in neurons (Tanzi et al., 1987; Neve et al., 1988) and glial cells (Haass et al., 1991). Although the functional role of $βAPP_{695}$ is not clear, its expression is developmentally regulated during the differentiation of hippocampal neurons and neuronal-like cell lines *in vitro* (Fukuchi et al., 1992; Hung et al., 1992). In addition, the expression of βAPP is increased during cerebral genesis *in vivo* (Loffler and Huber, 1992; Masliah et al., 1992). Despite their high expression of βAPP, fetal neurons, astrocytes, and microglia show little secretory processing of the precursor into its soluble derivative ($APP_s$) (Haass et al., 1991; Hung et al., 1992), implying that the cellular holoprotein, including that at the cell surface, plays the principal functional role during CNS development.

Secreted $APP_s$ has been shown to have trophic effects on cultured fibroblasts and to promote process outgrowth from neuronal-like cells (Saitoh et al., 1989; Schubert et al., 1989; Chen and Yankner, 1991; Milward et al., 1992). Biochemical studies indicate that $APP_s$ can bind to sulfated proteoglycans, suggesting that the trophic effects of secreted $APP_s$ may be mediated by its adhesion to the extracellular matrix (Schubert et al., 1989; Narindrasorasak et al., 1991; Shioi et al., 1992; Small et al., 1994).

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Both soluble and membrane-associated human brain βAPP have been reported to increase neurite length and branching in PC12 cells (Milward et al., 1992). Furthermore, neurite outgrowth from neuroblastoma cells grown on collagen could be blocked by an antibody to βAPP, and cell-cell contact was also impaired by this antibody (Breen et al., 1991).

These various results raise the question of whether surface-expressed βAPP has a specific functional activity for developing neurons in addition to its role in generating APPs and other secreted products. If so, do secreted $APP_s$ and surface-expressed βAPP promote neurite outgrowth through the same or different mechanisms? To investigate the function of cell-surface βAPP in the nervous system and examine differences between its mode of action and that of $APP_s$, we established a coculture system in which primary rat fetal hippocampal neurons are grown on monolayers of transfected CHO cells expressing different isoforms of βAPP on their surfaces. To compare the effects of surface βAPP to those of the secreted derivatives, neurons were also grown on either untransfected CHO cells or a poly-D-lysine substrate, each of which was supplemented with conditioned medium from βAPP-transfected cells. The results demonstrate that neurite outgrowth from hippocampal neurons in this culture system is promoted by increased surface-expressed βAPP but not by increased $APP_s$ and other secreted derivatives in the medium. In addition, surface-expressed βAPP isoforms containing the KPI domain (but not their $APP_s$ derivatives) promote neurite outgrowth more vigorously than does $βAPP_{695}$ in this system. Further, we define a region of the βAPP ectodomain which mediates in part the cell adhesion/neurite-promoting function of surface βAPP.

## Materials and Methods

*CHO cell culture and DNA transfection.* Chinese hamster ovary (CHO) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM glutamine, and antibiotics (complete medium). βAPP cDNAs were constructed into the pRC/CMV or pSV vectors, in which expression is under the control of cytomegalovirus promoter or SV40 enhancer/promoter, respectively. Single colonies of stable transfectants obtained by using lipofectin (GIBCO, Bethesda Research Labs) as described by the manufacturer were selected in G418 medium (400 μg/ml) and cloned.

*Metabolic labeling, immunoprecipitation, and surface βAPP assay.* For metabolic labeling, confluent monolayers of CHO cells were preincubated for 15 min in medium without methionine (GIBCO) containing 1% FBS. The cells were then labeled for 2 hr in the presence of 300 μCi of $^{35}$S-methionine, washed, and harvested. NP-40 detergent extracts of the labeled cells and culture supernatants were then analyzed by immunoprecipitation with various antibodies to βAPP, as previously described (Haass et al., 1991).

For the surface βAPP binding assay, a βAPP monoclonal antibody, 5A3, which recognizes the mid-region of the βAPP ectodomain, was used to quantitate the amount of surface-expressed precursor (Koo et al., 1993). Briefly, $1 \times 10^5$ CHO cells were seeded into 12 well plates. After 48 hr, the cells were extensively washed to remove all but trace amount of $APP_s$ that might be associated with the cell surface. Then the cells were chilled on ice and incubated with binding medium consisting of RPMI 1640 medium supplemented with 0.2% BSA. 5A3 was radioiodinated with Iodobeads (Pierce) to approximately 2–3 μCi/μg protein and used at 10 nM per well. The cells were incubated with the labeled antibody for 1 hr at 4°C and then washed five times with cold PBS over 30 min. The cells were subsequently lysed with 0.2 M NaOH, and radioactivity (cpm) was determined with a gamma counter. All samples were assayed in triplicate and the results expressed as average ± SEM.

*Adhesion assays.* Primary neurons were prepared from the dissected hippocampi of embryonic day 19 rats as previously described (Bartlett and Banker, 1984; Goslin and Banker, 1991). To assay CHO and hippocampal neuron cell-cell adhesion, $1.5 \times 10^5$ dissociated rat fetal hippocampal neurons in 3 ml of complete medium with 10% FBS were plated in 60 mm tissue culture dishes onto confluent monolayers of βAPP-transfected and untransfected CHO cells which had been washed three times with complete medium. After incubation at 37°C for the indicated times, the plates were swirled and washed three times. The unattached neurons were collected and counted by hemocytometer; the same procedure performed on the CHO cultures alone (without neurons) yielded virtually no unattached cells. This result was confirmed by directly counting the attached neurons in 10 randomly chosen fields, based on the morphological differences between neurons and CHO cells. At each time point, each cell type had triplet samples assayed, and each experiment was repeated three times.

*Neurite outgrowth assay.* Approximately $6 \times 10^5$ untransfected or βAPP-transfected CHO cells were seeded in 60 mm tissue culture plates and grown for about 36 hr in complete DMEM medium until reaching approximately 80% confluence. For coculture experiments, the hippocampal neurons were plated at a density of 100,000 cells per 60 mm petri dish on glass coverslips that were covered with monolayers of CHO cells. In the experiments analyzing different conditioned media, the same number of neurons was plated on glass coverslips coated with poly-D-lysine (1 mg/ml). The neurons were grown in a 1:1 mixture of DMEM and Ham's F12 medium supplemented with 10% FBS, 600 mg% glucose, 1 mM pyruvate, 2 mM glutamine, and the N2 supplements (complete medium) (Bottenstein and Sato, 1979) and maintained at 37°C with 5% $CO_2$. Two to four hours later, the medium was changed to medium without FBS.

After incubation at 37°C for 26–28 hr, the coverslips were gently rinsed in PBS and fixed with 4% paraformaldehyde. The fixed cells were reacted with polyclonal antiserum DJ (Kosik et al., 1988) to human microtubule-associated proteins (principally tau and MAP2), followed by biotin-conjugated goat anti-rabbit antibodies, ExtrAvidin peroxidase and reaction with DAB, as described by the manufacturer (Sigma). These proteins are specifically expressed in neurons (Kosik et al., 1988) and absent from CHO cells, and DJ was therefore used to visualize and quantitate neurites. Fixed cells were viewed through an inverted phase microscope using a video camera and images were projected onto the screen. Neurons were selected at random, traced from the screen, and the lengths of their processes measured using a digitizing tablet. The data from multiple neurons in a culture were pooled, and an estimate of the total neuritic length per cell was calculated as the ratio of the sum of the lengths of neurites over the number of cell bodies counted.

## Results

### *Increased surface expression of βAPP in CHO cells enhances cell-cell adhesion*

Four constructs encoding human $βAPP_{695}$, $βAPP_{751}$, $βAPP_{770}$, or $βAPP_{770}$ containing a deletion of residues 380–666 [$βAPP_{770del}$ (Wang et al., 1991)] (see Fig. 1*A*) were cloned into either the pRC/CMV vector or pSV vector, in which expression is under the control of the cytomegalovirus promoter or the SV40 promoter, respectively. Following stable transfection into CHO cells, clones derived from single cells were obtained by selection in G418 medium.

Biochemical characterization of βAPP and its secreted derivatives in the parent CHO cells and representative clones transfected with $βAPP_{695}$, $βAPP_{770}$, or $βAPP_{770}$del are shown in Figure 1. To analyze the polypeptide forms of βAPP expressed and metabolized by the CHO transfectants, detergent extracts of metabolically labeled cells and their conditioned media were analyzed by immunoprecipitation with four antibodies recognizing different regions of βAPP. An antiserum (C7) (Haass et al., 1992a) to the conserved cytoplasmic domain of βAPP detected a doublet in all the lines which had the expected migration of the N- and N+O-glycosylated full-length proteins (Weidemann et al., 1989; Oltersdorf et al., 1990) (Fig. 1*B*). The parental CHO cells expressed endogenous $βAPP_{751/770}$, and the $βAPP_{770}$del transfectants expressed the expected 65 kDa and 70 kDa truncated proteins (Fig. 1*B*). To characterize APPs, the principal se-

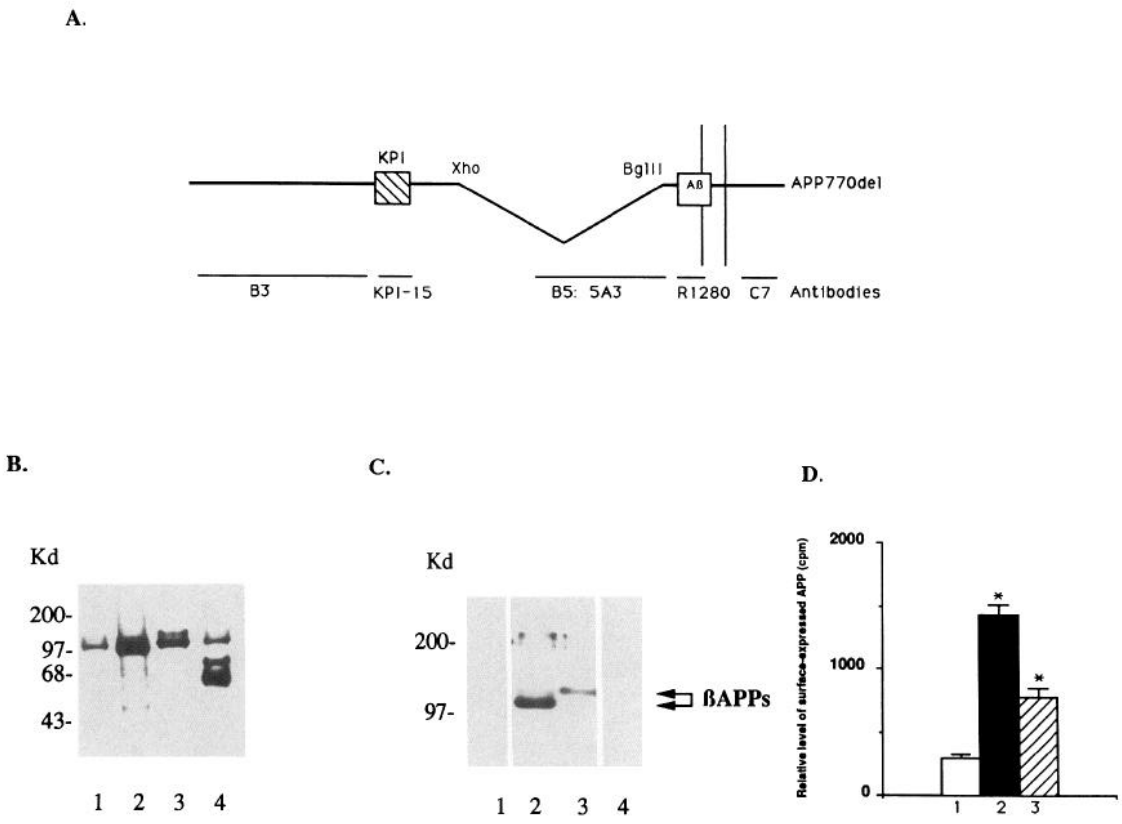


*Figure 1.* Biochemical analysis of the β-amyloid precursor protein and its surface expression by CHO transfectants. *A*, The structure of $APP_{770}$del is schematically presented. *Vertical lines* designate the single transmembrane domain of βAPP. The Kunitz protease inhibitor (*KPI*) motif and the Aβ peptides are indicated. The regions of βAPP which were the immunogens for the antibodies used in this study are shown as *horizontal lines under the schematic. B,* Immunoprecipitates of βAPP holoproteins from cell lysates with an antiserum (*C7*) to the carboxyl terminus of the cytoplasmic domain. Monolayers of parental CHO cells (*lane 1*) or CHO transfected with $βAPP_{695}$ (*lane 2*), $βAPP_{770}$ (*lane 3*) or $βAPP_{770}$del (*lane 4*) were biosynthetically labeled for 2 hr with $^{35}$S-methionine and their lysates were precipitated with C7 (1:250). *Lane 1* demonstrates the predominant $βAPP_{751}$ isoform expressed endogenously by CHO cells. This form is again seen in *lane 4*, together with the expected truncated forms of the precursor arising from the $βAPP_{770del}$ construct. The characteristic N- and N+O-glycosylated species of βAPP (Weidemann et al., 1989; Oltersdorf et al., 1990) are only partially resolved in this 10–30% tricine gel. *C*, Immunoprecipitation of $APP_s$ in the conditioned media with a human-specific βAPP antibody (*B5*) to recombinant $βAPP_{519-666}$ ($βAPP_{770}$ numbering). Aliquots of the conditioned media from the same cells shown in (*B*) were immunoprecipitated from plain CHO (*lane 1*) or $βAPP_{695}$ (*lane 2*), $βAPP_{770}$ (*lane 3*) or $βAPP_{770}$del (*lane 4*) CHO transfectants. Only $βAPP_{695}$ and $βAPP_{770}$ produce $APP_s$ immunoprecipitable with this antibody, as expected. *D*, Quantitative analysis of surface-expressed βAPP using the binding of $^{125}$I-labeled monoclonal antibody 5A3, directed at an epitope of the mid-region ectodomain (see *A*). Plates of untransfected CHO (*bar 1*), or $βAPP_{695}$ (*bar 2*) or $βAPP_{770}$ (*bar 3*) transfectants were seeded with equal numbers of cells, grown to confluence and incubated with the same amount of $^{125}$I-labeled 5A3. The cells were washed three times and measured for bound 5A3. Bars represent means ± SD of binding calculated from triplicate dishes in each of three independent experiments. *Asterisks* indicate significant increase ($p < 0.05$) of 5A3 binding compared to that for untransfected CHO cells.

creted derivative of βAPP, a human-specific βAPP antibody (B5) (Oltersdorf et al., 1990; Haass et al., 1992a) was used to precipitate conditioned media (Fig. 1*C*). Because the parental CHO is a rodent-derived line and because the region recognized by B5 is deleted in $βAPP_{770del}$, the soluble forms of these proteins are not precipitable by B5 (lanes 1 and 4 of Fig. 1*C*). In addition, an antiserum [(R1280) (Haass et al., 1992b)] directed against the $Aβ_{1-40}$ fragment of βAPP was used to immunoprecipitate the media. Soluble $APP_s$ of the appropriate size expected from each construct as well as Aβ (4 kDa) and a related 3 kDa fragment (p3) were all detected, in agreement with previous studies (Haass et al., 1992b; Seubert et al., 1992b; Shoji et al., 1992) (data not shown). $βAPP_{770del}$ cell line secreted a truncated $APP_s$ protein of the predicted size.

In order to examine how much βAPP holoprotein was expressed at the surface of transfected compared to untransfected CHO cells, an $^{125}$I-labeled monoclonal antibody, 5A3 (Koo et al., 1993), which recognizes an epitope in the extracellular domain of both human and rodent βAPP (Fig. 1*A*), was incubated with confluent monolayers of each cell type. Because both light and electron microscopy has shown that 5A3 reacts primarily with cell-surface βAPP in these CHO cells (with subsequent rapid internalization of the antibody/βAPP complex) while no $APP_s$ is detected on the matrix between cell bodies (T. Yamazaki, E. Koo, and D. J. Selkoe, unpublished data), this experiment reveals the relative expression level of βAPP on the cell surface but not the association of $APP_s$ with extracellular matrix. As shown in Figure 1*D*, the amount of surface-expressed $βAPP_{695}$ is approximately five times that in parental CHO cells, whereas the $βAPP_{770}$ CHO clone has an approximately 2.5-fold increase in surface molecules. Taking all of the above results together, our transfected CHO cells produce variable amounts of surface-inserted βAPP and metabolize the βAPP in the same pattern as described previously in other βAPP-expressing cells (Weidemann et al., 1989; Olterdorf, 1990; Haass et al., 1992b; Shoji et al., 1992).

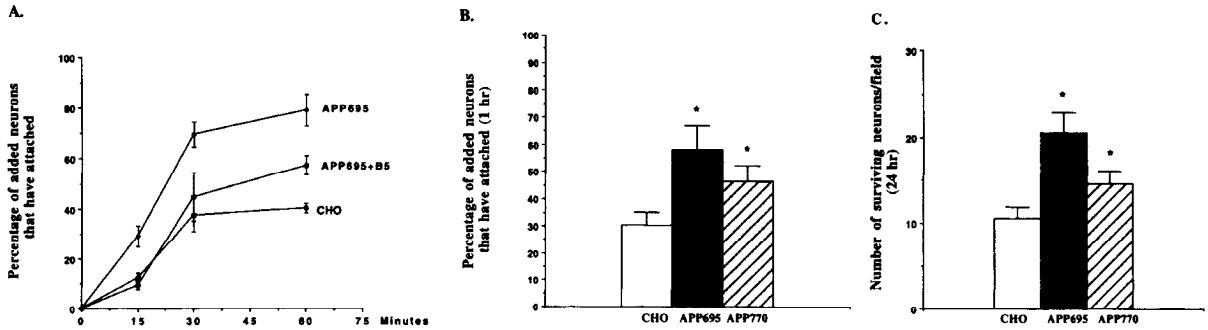


*Figure 2.* Enhanced short term adhesion of hippocampal neurons to βAPP-expressing CHO cells is accompanied by longer term survival. *A*, Kinetics of short-term adhesion. Dissociated rat fetal hippocampal neurons ($1.5 \times 10^5$ cells) in complete medium were plated on monolayers of untransfected or $\beta APP_{695}$-transfected cells and incubated at 37°C in the presence or absence of antibody, B5. At each time point, the unattached neurons were collected and counted; attached neurons were also observed and counted after washing the cultures three times. Results are expressed as the percentage of neurons seeded at time zero that have attached. Values shown are the means ± SEM of three independent experiments. *B*, Adhesion of hippocampal neurons to untransfected and $\beta APP_{695}$- or $\beta APP_{770}$-transfected CHO after incubation at 37°C for 1 hr (means ± SEM for four independent experiments). *Asterisks*, Significantly different at $p < 0.05$ from untransfected CHO cells. *C*, Survival of hippocampal neurons grown on untransfected, $\beta APP_{695}$- or $\beta APP_{770}$-transfected CHO, as quantitated after coculturing for 26–28 hr (means ± SEM of surviving neurons in a total of 30 random microscopic fields obtained from three independent experiments). *Asterisks* indicate significant differences ($p < 0.02$) relative to the effect of untransfected CHO cells.

We next asked whether surface-expressed βAPP could mediate cell-cell adhesion in neural cells. To observe cell-cell contact, monolayers of various βAPP-transfected CHO cell lines were used as a substrate for short-term adhesion assays of dissociated rat fetal hippocampal neurons. Equal numbers of dissociated neurons were plated on untransfected or transfected CHO cells and incubated at 37°C for various times, after which the cells were washed three times with complete medium and the unattached neurons were collected. The number of unattached neurons was counted, and the percentage of neurons that had attached was calculated. At the same time, the attached neurons were observed for their morphological characteristics and were counted from ten randomly chosen fields to confirm the attachment ratios among the different samples. As shown in Figure 2*A*, a reproducible and substantial increase in adhesiveness of hippocampal neurons to $\beta APP_{695}$-transfected versus parental CHO cells was observed at 15, 30, and 60 min. The adhesion mediated by the $\beta APP_{695}$ transfectant was attenuated by incubation with the human βAPP-specific antibody, B5 (Fig. 2*A*). To support this finding, the same neuronal adhesion experiment was performed on a primary rat astrocyte monolayer instead of CHO cells and also demonstrated a significant ($p < 0.025$) inhibition of neuronal adhesion following the addition of B5 (data not shown). Consistent with the levels of surface-expressed βAPP (Fig. 1*D*), the $\beta APP_{695}$ CHO transfectants had the highest number of attached neurons after one hour incubation, followed by $\beta APP_{770}$ transfectants and then control CHO cells (Fig. 2*B*) (differences significant at $p < 0.05$). Furthermore, the increase in short-term cell-cell adhesion on transfectants correlated well with the greater number of neurons present after 26–28 hr coculturing (Fig. 2*C*) (differences significant at $p < 0.02$). Therefore, our data suggest that the increased expression of cell-surface βAPP confers new adhesive properties to the parental CHO cells that mediate enhanced cell-cell contact and cell retention.

### *Cells expressing surface βAPP stimulate neurite extension of hippocampal neurons in an isoform-dependent manner*

To examine the potential function of cell-surface βAPP in neural development, we next investigated whether monolayers of various βAPP-transfected CHO cells would stimulate the extension of neurites when used as a substrate for growing dissociated rat fetal hippocampal neurons. Neurite outgrowth was quantitated after 26–28 hr of cocultivation by fixing the cells and staining with an antiserum [DJ (Kosik et al., 1988)] which sensitively detects the neuron-specific microtubule-associated proteins, tau and MAP 2. CHO cells are not recognized by this antiserum.

In general, the morphology of neurons grown on monolayers of both control and transfected CHO cells was relatively simple, with approximately 90% of the cells having fewer than three primary processes (Fig. 3). However, marked differences were observed in the ability of the transfected and untransfected CHO cells to promote neurite outgrowth. As illustrated in Figure 3*A–C*, neurons grown on βAPP-transfected cells characteristically showed long neurites extending from the cell bodies, in contrast to shorter processes that grew on control CHO monolayers. When neurite outgrowth was quantified by computer-assisted image analysis, the total neuritic length per cell on $\beta APP_{695}$- or $\beta APP_{770}$-transfected cells was found to be about twofold higher than on parental CHO cells ($p < 0.05$) (Fig. 4*A*). To exclude the possibility that this augmentation was due to an increase in neuronal density rather than to direct stimulation of neurites by the βAPP-expressing transfectants, we plated double the number of hippocampal neurons on the untransfected CHO cells to obtain roughly equal numbers of surviving neurons at 26–28 hr; no increase of neurite outgrowth was observed (Fig. 4*B*). The number of primary neurites per cell did not differ between neurons grown on plain CHO or on the transfectants (Fig. 4*C*), suggesting that βAPP can stimulate the growth of preexisting small processes but cannot initiate outgrowth of new neurites.

Although the level of surface expression of the $\beta APP_{770}$ transfected clone was reproducibly found to be one-half that of the $\beta APP_{695}$ transfectants (Fig. 1*D*), the ability of these cell lines to stimulate neurite outgrowth was approximately the same (Fig. 4*A*). This finding suggests that the KPI domain in $\beta APP_{770}$ may serve to additionally stimulate neurite growth. To confirm this apparent potentiation by $\beta APP_{751/770}$ isoforms, two $\beta APP_{695}$ clones ($\beta APP_{695}$ and $\beta APP_{695}B4$), two $\beta APP_{751}$ clones ($\beta APP_{751}$-2 and $\beta APP_{751}D4$), and one $\beta APP_{770}$ clone were compared as to level of βAPP surface expression and degree of neurite stimulation. As shown in Table 1, the clones $\beta APP_{770}$,

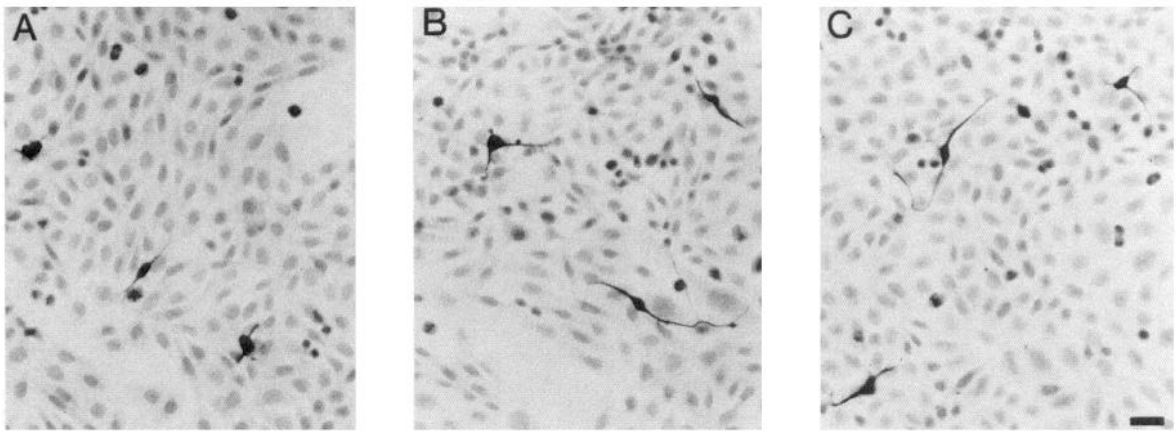


*Figure 3.* Hippocampal neurons cultured on $\beta APP_{695}$- and $\beta APP_{770}$- transfected CHO cells show enhanced neurite outgrowth. Representative photomicrographs of rat fetal day 19 hippocampal neurons were grown on monolayers of untransfected CHO cells (*A*), or $\beta APP_{695}$ (*B*) or $\beta APP_{770}$ (*C*) transfectants. After 26–28 hr, the cultures were fixed and reacted with antiserum DJ to tau and MAP 2 proteins, which are specifically expressed in neurons, followed by sequential reactions with biotin-conjugated goat anti-rabbit antibodies, ExtrAvidin–peroxidase complex and DAB. Scale bar, 20 μm.

$\beta APP_{751}$D4, and $\beta APP_{695}$B4 had highly similar levels of surface-expressed βAPP, but $\beta APP_{770}$ and $\beta APP_{751}$D4 constructs produced greater neurite outgrowth than $\beta APP_{695}$B4 did ($p < 0.05$). Moreover, although clone $\beta APP_{751}$-2 had less surface-expressed βAPP than clone $\beta APP_{695}$β4 did, it stimulated neurite outgrowth to the same or slightly greater degree. Following the addition of an antibody (KPI-15) to the KPI domain in order to block its activity, the ability of clone $\beta APP_{770}$ to stimulate neurite outgrowth decreased significantly ($p < 0.05$) (Table 1). As a control for this experiment, the KPI-15 antibody had no effect on the neurite-promoting activity of $\beta APP_{695}$, as expected (Table 1). This experiment was repeated using a second KPI-specific antibody with similar results (data not shown). These data indicate that the degree of neurite stimulation in our system is dependent in part on the βAPP isoform expressed.

### *Surface-expressed βAPP promotes neurite outgrowth via a mechanism distinct from $APP_s$*

The data presented above do not allow one to determine whether the neuronal adhesion and neurite outgrowth stimulated by the βAPP transfectants are mediated by surface molecules or secreted derivatives of βAPP. To assess whether any trophic effects mediated by the increased amounts of $APP_s$ and Aβ released by the transfected CHO cells contributed to the results shown in Figure 4*A*, we grew the neurons on untransfected CHO cells supplemented with the overnight conditioned medium of the same $\beta APP_{695}$ transfectants. No neurite promotion was observed compared to identical cultures grown without this supplement (Fig. 4*B*). This result argues strongly against a significant role for APPs on neurite outgrowth under our conditions, whether as a diffusible molecule or by an interaction between $APP_s$ and the extracellular matrix. To examine this question further, we used poly-D-lysine as a substrate to grow the hippocampal neurons under otherwise identical conditions in the conditioned media obtained from the same untransfected and transfected CHO cells. After 26–28 hr, no obvious morphological differences were observed among neurons cultured in the conditioned media of untransfected CHO, $\beta APP_{695}$ or $\beta APP_{770}$ cells (Fig. 5*A–C*). Quantitation by image analysis confirmed the absence of detectable effects of the conditioned media of the transfectants on total neurite length per hippocampal neuron (Fig. 6*A*). Because the total neurite length/hippocampal neuron on the same poly-D-lysine substrate extends to 220 μm in the presence of astroglial conditioned medium (Ferreira et al., 1992) but was only 160 μm

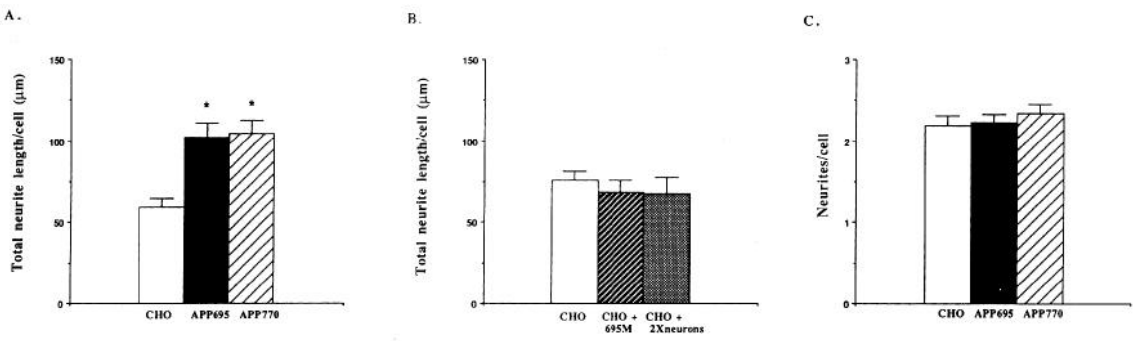


*Figure 4.* Quantitation of neurite length and number in hippocampal neurons cultured on CHO cells. *A* and *C*, Total neurite length per cell (*A*) and neurite number per cell (*C*) under the same conditions described in Figure 3 were quantitated by computer-assisted image analysis. *B*, Total neurite length per cell of neurons which were grown on monolayers of untransfected CHO cells without (*CHO*) or with (*CHO + 695M*) medium conditioned by $\beta APP_{695}$-transfected CHO or which were grown by plating twice the number of neurons as usual (*CHO + 2Xneurons*). Neurite length was defined as the sum of the lengths of all branches extending from a given cell body. Data are expressed as means ± SEM from a total of 70 neurons analyzed. *Asterisks* indicate significant differences ($p < 0.05$) relative to growth on plain CHO cells.

**Table 1. The effect of βAPP-transfected CHO cells on total neurite length/cell of cocultured hippocampal neurons**

| Transfected CHO clones | Surface-expressed βAPP (cpm of $^{125}$I 5A3 binding) | Total neurite length/cell (μm) | Total number of neurons examined | Number of experiments |
|---|---|---|---|---|
| $APP_{695}$ | 1424 ± 82 | 103.7 ± 8.7 | 63 | 3 |
| $APP_{695}$ + KPI-15 | | 109.4 ± 8.4 | 69 | 3 |
| $APP_{695}$B4 | 699 ± 19 | 77.1 ± 6.0* | 65 | 2 |
| $APP_{751}$-2 | 431 ± 24 | 84.0 ± 7.9 | 65 | 2 |
| $APP_{751}$D4 | 800 ± 22 | 113.2 ± 10.5 | 54 | 2 |
| $APP_{770}$ | 774 ± 74 | 95.1 ± 7.9 | 69 | 3 |
| $APP_{770}$ + KPI-15 | | 78.9 ± 5.8** | 79 | 3 |

* $p < 0.005$ when compared with total neurite length/cell on $APP_{751}$D4 and $p < 0.05$ when compared with the one on $APP_{770}$.

** $p < 0.05$ when compared with total neurite length/cell on $APP_{770}$ without KPI-15.

in this experiment, the neurons grown on poly-D-lysine (Figs. 5*A*, 6*A*) had not achieved their maximum possible neurite length. Therefore, because there is considerable endogenous βAPP production and $APP_s$ release in the parental CHO cells (Fig. 1), we propose that the lack of response by neurons to the increased $APP_s$ in the transfectant cocultures is due to the saturation of the neuronal cells by binding of the basal endogenous $APP_s$, rather than a complete lack of neurite-promoting activity of $APP_s$ on hippocampal neurons. As a further control to confirm these results, the conditioned medium from our $\beta APP_{695}$ transfectants was added to cultures of PC12 cells similarly grown on poly-D-lysine. Here, the $\beta APP_{695}$ conditioned medium caused a significant twofold increase in neurite outgrowth of PC12 cells compared to medium from untransfected CHO (Fig. 6*B*), indicating that the secreted $APP_s$ produced by our transfectants can indeed exert the trophic activity of this molecule that has been described previously (Milward et al., 1992).

Because a sequence of five amino acids, RERMS (residues 328–332 of $\beta APP_{695}$) was found to confer this growth-promoting activity of $APP_s$ on fibroblasts and neuroblastoma cells (Ninomiya et al., 1993), an 11-mer synthetic peptide (amino acids 325–335 of $\beta APP_{695}$) or a 17-mer peptide (amino acids 319–335 of $\beta APP_{695}$), each including the RERMS sequence, were added to hippocampal neuronal cultures grown on poly-D-lysine in the conditioned medium of untransfected CHO cells to confirm the apparent saturation effect of the endogenously produced APPs. Both peptide batches had previously been shown to promote growth of fibroblasts and neuroblastoma cells (T. Saitoh, personal communication). Again, no increase in total neurite length per hippocampal neuron was observed in our cultures, using concentrations of these trophic peptides ranging from 10 pM to 100 nM (Fig. 6*C*).

The above data (Figs. 4*B*, 6*A–C*) clearly indicate that the increased amounts of $APP_s$ and Aβ released into the conditioned media of our βAPP-transfected cells do not further stimulate neurite outgrowth from fetal hippocampal neurons when compared to the media of CHO expressing only endogenous βAPP. This finding strongly suggests that the neuronal attachment and neurite-promoting activities observed in our system (Figs. 3, 4) are mediated by surface-expressed βAPP rather than secreted $APP_s$. As a further test, we added the antagonist peptide ERMSQVM, which has been shown to block the growth-promoting activity of secreted $APP_s$ on fibroblasts (Ninomiya et al., 1993), to the cocultures of hippocampal neurons on βAPP-transfected CHO cells. Various concentrations of the antagonist peptide up to 10 μM (Fig. 6*D*) produced no inhibition of the increased total neurite length/cell mediated by the surface-expressed βAPP. Taken together, these various experiments suggest

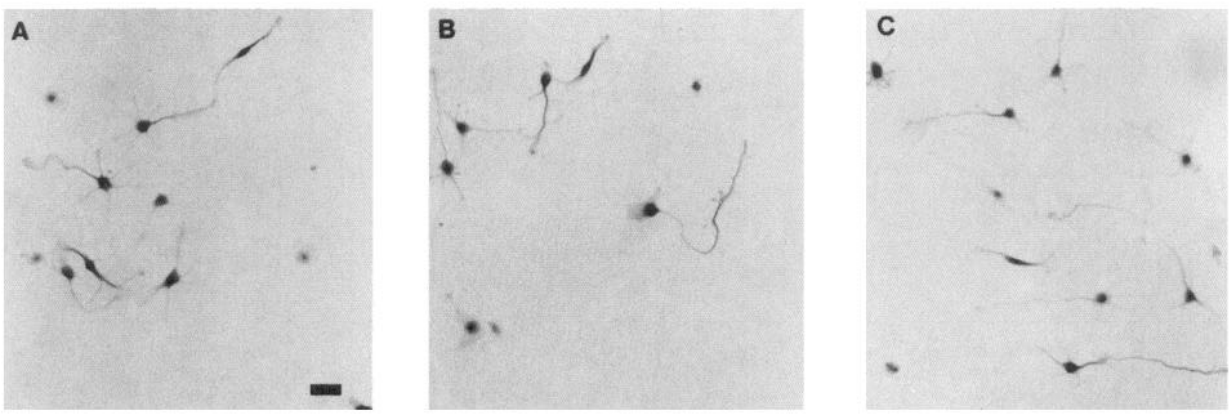


*Figure 5.* Conditioned media from $\beta APP_{695}$- and $\beta APP_{770}$-transfected CHO cells do not enhance neurite outgrowth of hippocampal neurons compared to conditioned medium of untransfected CHO. Rat fetal day 19 hippocampal neurons grown on coverslips coated with poly-D-lysine were incubated in the conditioned medium from either untransfected CHO cells (*A*) or $\beta APP_{695}$ (*B*) or $\beta APP_{770}$ (*C*) transfectants for 26–28 hr. Culture conditions and immunostaining with antiserum DJ were otherwise identical to those described in Figure 3. Scale bar, 20 μm.

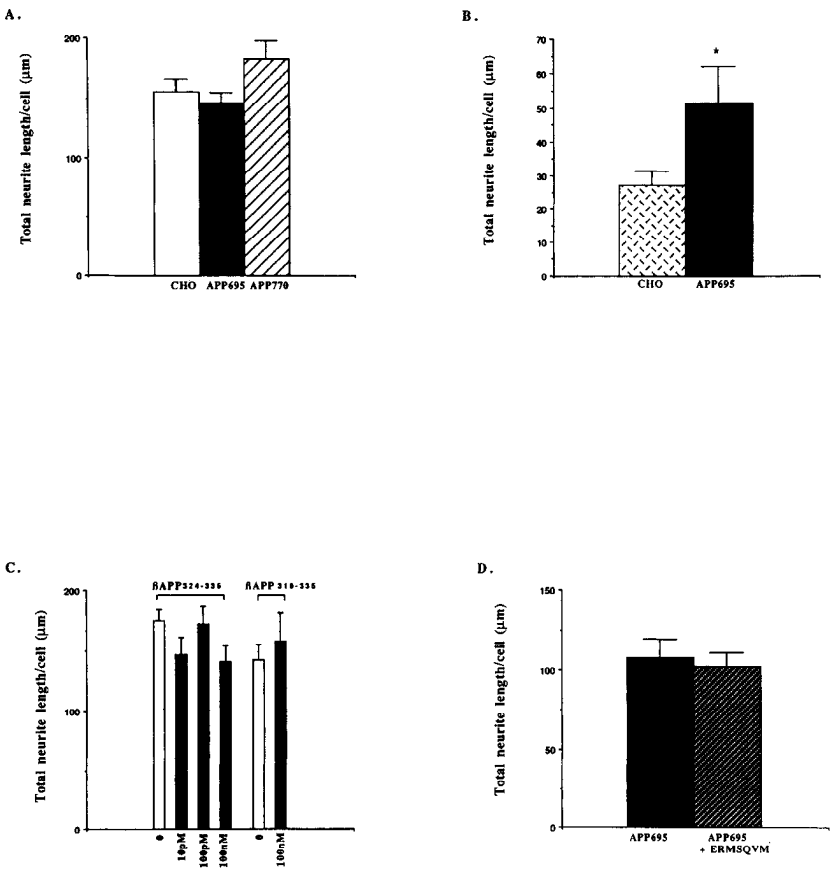


*Figure 6.* Quantitation of neurite length in various conditioned media. *A*, Quantitation of total neurite length/cell of hippocampal neurons grown as exemplified in Figure 5. *B*, As a positive control, PC12 cells cultured overnight in conditioned media from either untransfected CHO or $\beta APP_{695}$-transfectants were analyzed identically. Data in *A* and *B* are expressed as a means ± SEM from 70 counted neurons. *Asterisks* indicate significant differences ($p < 0.05$) relative to the effects of CHO conditioned medium. *C*, Supplementation of conditioned medium of untransfected CHO cells with peptides containing a growth-promoting sequence (RERMS) from secreted $APP_s$ does not increase neurite outgrowth from hippocampal neurons. Rat fetal day 19 hippocampal neurons were cultured on poly-D-lysine coated coverslips in overnight conditioned medium from CHO cells (bar 1 and bar 5) or the same medium supplemented with an 11-mer synthetic peptide (residues 324–335) of $\beta APP_{695}$ (Ninomiya et al., 1993) at concentrations of 10 pM (bar 2), 100 pM (bar 3), or 100 nM (bar 4). A related 17-mer synthetic peptide (residues 319–335) (Ninomiya et al., 1993) was added at a concentration of 100 nM (bar 6). After 26–28 hr, total neurite length/cell was measured and expressed as means ± SEM ($n > 50$ neurons counted). Both batches of peptides had been shown to be trophically active on fibroblasts and neuroblastoma cells (T. Saitoh, personal communication). *D*, A peptide which antagonizes the tropic effects of secreted $APP_s$ does not affect neurite outgrowth mediated by cell-surface $\beta APP_{695}$. Peptide ERMSQVM (10 µM) (Ninomiya et al., 1993) was added to the medium of rat hippocampal neurons cultured on $\beta APP_{695}$-transfected CHO cells. Total neurite length/cell in the absence and the presence of the antagonist peptide was quantitated in three independent experiments (means ± SEM from a total of 50 neurons).

that there are different regions and distinct mechanisms underlying the growth-promoting properties of secreted and surface-expressed βAPP.

### *Delineation of the region in cell-surface βAPP which mediates the neurite outgrowth*

To define which region of cell-surface βAPP confers the observed effect on neurite extension, we conducted blocking experiments using antibodies to different sequences within βAPP. As diagrammed in Figure 1*A*, two antibodies, polyclonal antibody, B5 (to $\beta APP_{519-666}$), and the monoclonal antibody, 5A3 (precise epitope unknown), recognize the mid-region of βAPP. Because the epitope in βAPP which is involved in the neurite promotion is unknown and because B5 can react with the human βAPP molecules transfected into CHO cells but less well with rat βAPP on the surface of the neurons, we chose B5 rather than 5A3 in the blocking experiment. Addition to the culture medium of affinity-purified B5 antibodies completely inhibited the enhanced neurite extension produced by growth on $\beta APP_{695}$-transfected CHO cells (Fig. 7*A*). As a result, total neurite length/cell remained the same as that seen on control CHO cells in the absence of B5 (Fig. 7*A*). Affinity purified polyclonal antibody B3 (Citron et al., 1992) to the N-terminal region $\beta APP_{20-302}$ did not alter the neurite-promoting activity mediated by $\beta APP_{695}$ (Fig. 7*A*). As a further control, a polyclonal antiserum (C7) to the βAPP cytoplasmic domain also had no effect on neurite outgrowth, as expected (Fig. 7*A*). These data suggest that the mid-region of the βAPP ectodomain preceding the Aβ sequence contains an epitope which mediates this function.

To confirm this apparent localization of the adhesion domain of surface-expressed βAPP, CHO cells were transfected with a construct in which $\beta APP_{770}$ is deleted by restriction enzyme digestion with XhoI (position 380) and BglII (position 666), yielding a protein lacking the entire B5 epitope (see Fig. 1*A*). As shown in Figure 7*B*, when this $\beta APP_{770}$del transfectant was used as a substrate for the growth of hippocampal neurons, it pre-

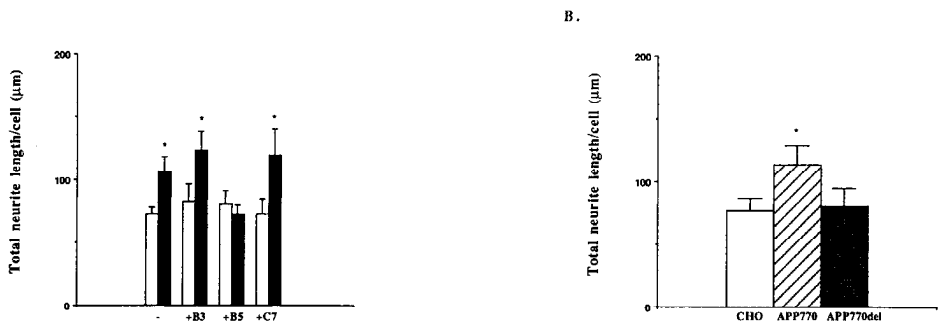


*Figure 7.* The carboxyl terminal portion of the βAPP ectodomain mediates the function of cell-surface βAPP in promoting neurite outgrowth. *A*, Rat fetal day 19 hippocampal neurons were cultured on monolayers of untransfected (*open bars*) or $\beta APP_{695}$-transfected (*solid bars*) CHO cells. In parallel, three different polyclonal antibodies were added at the initial time of plating at concentrations of equivalent antibody activity (1.5 μg of B3 per ml, 2.4 μg of B5 per ml, or 1.8 μg of C7 per ml). After culturing for 26–28 hr, the cultures were fixed and stained with the DJ antiserum. Total neurite length/cell was measured and expressed as mean ± SEM ($n > 70$ neurons counted). *B*, Hippocampal neurons were plated and grown on monolayers of untransfected CHO cells, or on $\beta APP_{770}$ or $\beta APP_{770}$del transfectants. After 26–28 hr, neurons were fixed, stained and measured as in *A*.

vented the enhancement of neurite extension observed in neurons grown on wild-type $\beta APP_{770}$ transfectants. Because this result is consistent with the specific blocking effect of the B5 antibodies, our data strongly suggest that the activity of B5 is specific and that the sequence mediating the functional activity demonstrated in this study is located between amino acids 380 and 666 of $\beta APP_{770}$, most likely between residues 519 and 666 (the B5 region), which is distinct from the growth promoting region defined in $APP_s$ (Ninomiya et al., 1993).

## Discussion

Following the initial cloning of βAPP, the observations that neurons express particularly high levels of the precursor (Goldgaber et al., 1987; Tanzi et al., 1987; Neve et al., 1988; Hung et al., 1992) and that some molecules appear to be localized to the cell surface *in vivo* (Shivers et al., 1988) suggested that the protein might mediate cell-cell contact in the CNS. Subsequent studies showed that βAPP is conveyed to nerve terminals by fast axonal transport (Koo et al., 1990), that developing neurons undergo increased expression of βAPP which is preferentially localized to the growth cones of their processes (Ferreira et al., 1993), and that immature neurons metabolize very little of their abundant βAPP into the cleaved, secreted derivative ($APP_s$) (Hung et al., 1992). Taken together, these findings point to a possible role for full-length βAPP in neurite outgrowth via a specific cell-cell adhesion function, but no direct studies of the function of cell surface-inserted βAPP have been reported. Here, we demonstrate that fetal hippocampal neurons respond specifically to βAPP expressed at the surface of cells with which they come into contact by undergoing enhanced attachment and neurite outgrowth, and that this response is distinct from those previously described for secreted $APP_s$.

We used a system in which developing neurons are grown on transfected non-neural cells expressing various isoforms of βAPP in order to distinguish the functional properties of cell surface βAPP from those of soluble $APP_s$ and the Aβ and p3 peptides, all of which are released continuously by neuronal and glial cells in the CNS. This paradigm enables one to quantitate the amount of holoprotein inserted at the plasma membrane and to use recombinant techniques to delete or enhance putative functional domains of the precursor in order to establish their biological activities. We found that CHO cells stably overexpressing each of the three major isoforms of full-length βAPP produced a significant increase in initial neuronal attachment. This enhanced adhesion could be blocked by the βAPP ectodomain antibody, B5, when the neurons were grown on either βAPP-transfected CHO (Fig. 2*A*) or on primary astrocytes (data not shown). Therefore, the enhanced number of neurons seen at 26–28 hr (Fig. 2) resulted from increased cell adhesion. Furthermore, the degree of enhanced cell-cell interaction paralleled the amount of surface-expressed βAPP in both $\beta APP_{695}$ and $\beta APP_{770}$ clones (compare Figs. 1*D*, 2*B,C*). The adhesive characteristics of $APP_s$ were demonstrated previously in studies examining PC12 cells attached to $APP_s$-coated dishes (Schubert et al., 1989). Therefore, βAPP may function in both specific cell-cell and cell-matrix interactions *in vivo*. Like other examples of cell-surface expressed adhesion molecules which promote neuronal differentiation in the nervous system, for example, NCAM and F3/F11 (Doherty et al., 1990; Gennarini et al., 1991), cell-surface βAPP was also found to enhance neurite outgrowth in the rat fetal hippocampal neurons by increasing the mean neurite length, but it did not affect the mean number of primary neurites per cell (Figs. 3, 4).

Trophic activities of either $APP_s$ or Aβ on fibroblasts (Saitoh et al., 1989) and neurons (Schubert et al., 1989; Whitson et al., 1989; Yankner et al., 1990; Araki et al., 1991; Chen and Yankner, 1991; LeBlanc et al., 1992; Milward et al., 1992) have been demonstrated in previous studies. In this study, several experiments showed that no *enhanced* neurite promotion could be detected in the conditioned media of the βAPP-transfected cells, probably because the secreted derivatives (particularly $APP_s$) produced endogenously by the CHO cells saturated the neurons. Instead, the consistent and statistically significant increase in neurite outgrowth observed in our cocultures can be attributed to the increased surface expression of βAPP in the transfected cells on the basis of several findings. First, no difference was seen in the neurite outgrowth of hippocampal neurons grown on poly-D-lysine in transfectant conditioned medium markedly enriched in the secreted βAPP derivatives compared to that in plain CHO medium (Figs. 5, 6*A*). Second, neurite outgrowth on untransfected CHO cells supplemented with transfectant-conditioned medium rich in $APP_s$ also produced no neurite enhancement after 24 hr (Fig. 4*B*), excluding the possibility that $APP_s$ itself or an interaction between $APP_s$ and extracellular matrix molecules played a role in our response. Considering the basal

level of endogenous $APP_s$ produced by untransfected CHO cells that can saturate the neurons, the lack of response to the additional transfectant-derived $APP_s$ was seen whether the neurons were grown on a stronger growth-promoting substrate, poly-D-lysine or a weaker substrate, CHO cells. Third, the trophic effect of $APP_s$ has been localized to a five residue sequence, RERMS (Ninomiya et al., 1993). However, when our hippocampal neurons were treated with CHO medium supplemented with two different active peptides (T. Saitoh, personal communication) containing this sequence, no increase in neurite length/cell was obtained (Fig. 6*C*). Fourth, a partially overlapping βAPP peptide shown to antagonize the trophic effect of $APP_s$ (Ninomiya et al., 1993) did not block the neurite promoting activity of the βAPP-transfectants we used as a substrate for growth of hippocampal neurons (Fig. 6*D*). Therefore, all of our data strongly suggest that the increased neurite outgrowth appearing in the cocultures is caused by surface-expressed βAPP rather than $APP_s$, Aβ or other secreted derivatives. Although we believe it is unlikely, we can not completely exclude the possibility that βAPP overexpression could influence the CHO to secondarily produce a different neurite-promoting molecule. Because very small amounts of $APP_s$ are secreted by immature astrocytes, microglia and neurons *in vitro* (Haass et al., 1991; Hung et al., 1992), our results lead us to hypothesize that surface-inserted βAPP may play a more important role than $APP_s$ in the initial development and differentiation of CNS neurons.

Protease nexin I is a glial-derived serine protease inhibitor that has been shown to stimulate neurite outgrowth in cultured neuroblastoma cells and primary neurons (Gloor et al., 1986; Zurn et al., 1988). Although protease nexin II (the secreted form of the KPI-containing $\beta APP_{751/770}$ isoforms) has been proposed to have similar functions as protease nexin I, no enhancement of neurite outgrowth was observed when secreted $APP_s$ derived from $\beta APP_{751}$ transfectants was compared to that from $\beta APP_{695}$ transfectants (Araki et al., 1991; Milward et al., 1992). However, the surface-expressed forms of these molecules examined here produced a clear difference in growth promotion of axons and minor processes during 24 hr culturing of hippocampal neurons (Figs. 3, 4; Table 1). For example, although one of our $\beta APP_{695}$ CHO clones ($\beta APP_{695}$) expressed a substantially higher level of βAPP on the cell surface than a $\beta APP_{770}$ clone did, their ability to promote neurite outgrowth was approximately the same (Table 1). Likewise, clone $\beta APP_{695}$B4 had approximately equivalent surface expression of the precursor as clones $\beta APP_{770}$ and $\beta APP_{751}$D4, yet it displayed less neurite-promoting activity. Furthermore, two different antibodies to the KPI domain partially blocked the neurite promoting activity of $\beta APP_{770}$, bringing it down to the level mediated by clone $\beta APP_{695}$B4, which has a closely similar number of cell-surface βAPP molecules (Table 1). However, the fact that clone $APP_{770}$del, which contains the KPI motif but not the mid-region of the ectodomain, showed no neurite promoting activity (Fig. 7*B*) suggests that the protease inhibitory domain by itself is not sufficient to account for the effect of $\beta APP_{751/770}$ on neurite outgrowth in our system. Thus, we hypothesize that there are two neurite-promoting regions which work cooperatively in surface-expressed βAPP, one shared among all isoforms and the other localized to the Kunitz protease inhibitory motif.

The approximate region of surface βAPP which is apparently involved in the cell-cell adhesion and neurite outgrowth we observed was identified by antibody blocking experiments and analysis of deletion constructs (Fig. 7). Although the region defined to date, residues 380–666 of $\beta APP_{770}$, contains the RERMS sequence, the cell-cell interactions occurring in our system are not mediated through the latter motif (Fig. 6). However, the region does contain the 18 residue of exon 15 which is known to be absent in one alternatively spliced form of βAPP, L-APP (König et al., 1992). The expression of the L-APP form is high in nonadherent and low in adherent leukocytes suggesting that this region could be important for the function of adhesion in neurons, as well.

Our data further suggest that there are distinct epitopes underlying the growth promoting effects of surface-expressed βAPP and $APP_s$, although their extracellular domains have largely the same primary structure. This concept is supported by the recent finding of Small et al. that the region comprising residues 96–110 of $APP_s$ bound heparan sulfate proteoglycan (HSPG) and that this interaction, not the presence of $APP_s$ alone, promoted neurite outgrowth in hippocampal neurons (Small et al., 1994). Their data also suggested that the type of HSPG was critical for the neurite outgrowth-promoting effect of $APP_s$. Therefore, the lack of neuronal response to the increased amount of $APP_s$ in our culture system could relate in part to the absence of the correct type of HSPG. It is also of interest that the B5-reactive region of secreted $APP_s$ has been shown to have a calcium-lowering activity that mediates neuroprotection and neurite outgrowth in cultured hippocampal neurons (Mattson et al., 1993; Mattson, 1994). It is therefore possible that the same signal transduction mechanism (i.e., lowering of intraneuronal calcium levels) could mediate the neurite promotion conferred by surface-expressed βAPP in our experiments. It will now be interesting to determine whether the 18 amino acids of exon 15 or another motif in the B5-reactive region is the determinant for the neurite promotion mediated by cell surface βAPP. Moreover, the intriguing question of whether there are two different receptors for the distinct functional activities of βAPP and $APP_s$ or one common receptor which binds surface βAPP and $APP_s$ differently now needs to be addressed.

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