Selective ectodomain phosphorylation and regulated cleavage of β -amyloid precursor protein

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The β -amyloid precursor protein (β APP) is a highly conserved integral membrane protein expressed in most mammalian tissues and found at highest levels in the nervous system. Cerebral deposition of the amyloid β -peptide ($A\beta$), derived by proteolysis of β APP, is an early and invariant feature of Alzheimer's disease. Protein phosphorylation by protein kinase C (PKC) has been found to regulate the metabolism of β APP into non-amyloidogenic and amyloidogenic derivatives, but both the mechanism of these effects and the nature of β APP phosphorylation are unknown. When labeled *in vivo* with [32 P]orthophosphate, β APP was phosphorylated only on serine residues in the N-terminal half of the extracellular domain, resulting in the secretion of phosphorylated soluble β APP. PKC-mediated stimulation of β APP secretion and concurrent inhibition of $A\beta$ release did not involve enhanced phosphorylation of β APP and proceeded in the absence of cytoplasmic or extracellular phosphorylation of the precursor. The region of β APP required for this indirect regulation by PKC was largely restricted to a 64 amino acid stretch around the secretory cleavage site. Moreover, in a truncated molecule designed to release soluble β APP without the need for proteolytic cleavage, secretion was no longer regulated by PKC. Our data indicate that PKC-mediated pathways play a pivotal role in the control of β APP metabolism and amyloid formation. However, in contrast to current postulates, this regulation is independent of β APP phosphorylation and instead involves phosphorylation of other substrates that alter β APP processing, such as β APP-cleaving proteases.

Key words: Alzheimer's disease/amyloid/phosphorylation/protein kinase C/proteolysis

Introduction

The most characteristic pathological feature of Alzheimer's disease is the deposition of fibrillar aggregates of the amyloid β -peptide ($A\beta$) in the brain and cerebral blood vessels. This 39–43 amino acid peptide is generated by two proteolytic cleavages of the β -amyloid precursor protein (β APP), a 100–140 kDa integral membrane protein encoded by a gene on human chromosome 21 (Goldgaber *et al.*, 1987; Kang *et al.*, 1987; Robakis *et al.*, 1987; Tanzi *et al.*, 1987) that is expressed at highest levels in neurons (Goedert, 1987; Neve *et al.*, 1988). $A\beta$ comprises 28 residues just outside the putative single membrane-spanning domain of β APP plus

the first 11–15 amino acids of the transmembrane region. Although the precise role of $A\beta$ in the pathogenesis of Alzheimer's disease remains unclear, the identification of several different missense mutations, within and immediately flanking the $A\beta$ region of the β APP gene in certain families with autosomal dominant, early-onset Alzheimer's disease (reviewed by Hardy, 1992), strongly suggests that β APP metabolism is central to the disease mechanism.

In many cell types, proteolytic cleavage of β APP results in the secretion of an ~90–100 kDa soluble N-terminal fragment (APP_s) (Schubert *et al.*, 1989; Weidemann *et al.*, 1989) and membrane retention of an ~10 kDa C-terminal fragment (Selkoe *et al.*, 1988). This scission, mediated by an unidentified enzymatic activity termed 'APP secretase', occurs between a lysine residue and a leucine residue at positions 16 and 17 within the $A\beta$ region (Esch *et al.*, 1990; Sisodia *et al.*, 1990; Wang *et al.*, 1991). Thus, secretase cleavage of β APP at this bond precludes the formation and deposition of amyloid. The utilization of this secretory pathway appears to be regulated in a cell type-dependent manner, as both glial and neuronal cells produce very low amounts of the secreted derivative and the 10 kDa fragment compared with certain non-neural cells (Haass *et al.*, 1991; Hung *et al.*, 1992). Unexpectedly, secretion of the 4 kDa $A\beta$ peptide has recently been shown to occur as part of normal β APP metabolism in cultured cells, including neurons (Haass *et al.*, 1992b; Seubert *et al.*, 1992; Shoji *et al.*, 1992; Busciglio *et al.*, 1993). A 3 kDa peptide ($p3$) corresponding to a truncated form of $A\beta$ is also detected in conditioned media (Haass *et al.*, 1992b; Busciglio *et al.*, 1993) and appears to be derived from the 10 kDa fragment following secretase cleavage (Haass *et al.*, 1993). Importantly, a missense mutation immediately N-terminal to the $A\beta$ region of β APP in at least one family with autosomally-dominant Alzheimer's disease results in markedly increased production and release of $A\beta$ in transfected cells (Citron *et al.*, 1992; Cai *et al.*, 1993). This finding suggests further that factors governing the metabolic processing of β APP into $A\beta$ play a direct pathogenetic role in Alzheimer's disease.

Despite some cell-type differences in β APP processing, certain regulatory features controlling β APP metabolism appear to be shared by neuronal and non-neuronal cells. In particular, protein kinase C (PKC) activation by phorbol esters has been shown to stimulate secretion of APP_s and to increase the amounts of membrane-retained C-terminal fragments in PC12 pheochromocytoma cells (Buxbaum *et al.*, 1990; Caporaso *et al.*, 1992), as well as in human embryonic kidney 293 cells (Gillespie *et al.*, 1992). This mode of regulation could account for the increase of APP_s release from neuronal cells in response to a number of extracellular signals or to depolarization. In this regard, cells expressing muscarinic acetylcholine receptor subtypes m1 or m3 show increased secretion of APP_s upon addition of the cholinergic agonist carbachol, a process which is blocked

by the protein kinase inhibitor staurosporine (Buxbaum *et al.*, 1992; Nitsch *et al.*, 1992). Electrical stimulation of hippocampal slices also causes enhanced release of the soluble APP_s form (Nitsch *et al.*, 1993), indicating that neuronal activity and neurotransmitters may regulate β APP metabolism. Moreover, we and others have recently demonstrated that activation of PKC, either directly by phorbol esters or indirectly by cholinergic agonists, results in a marked inhibition of A β release (Buxbaum *et al.*, 1993; Hung *et al.*, 1993).

These data indicate that the PKC/phospholipase C second messenger system is likely to play a critical role in regulating the processing of β APP into amyloidogenic and non-amyloidogenic derivatives. However, the cellular mechanisms by which PKC mediates these effects are unknown. The metabolic fate of β APP could be influenced by direct phosphorylation of the precursor. Indeed, because PKC can phosphorylate a synthetic peptide corresponding to the cytoplasmic amino acids 645–661 of β APP695 specifically at a serine residue at position 655 (Gandy *et al.*, 1988) and because exogenous PKC appears to phosphorylate this residue in permeabilized PC12 cells *in vitro* (Suzuki *et al.*, 1992), it has been hypothesized that direct phosphorylation of the holoprotein is involved in upregulating APP_s secretion *in vivo* (Gandy and Greengard, 1992). Alternatively, PKC could act by activating β APP-cleaving proteases or by altering cellular trafficking pathways. In this study, we show that β APP in intact cells is phosphorylated on serine residues that are restricted to its ectodomain. Moreover, PKC-mediated activation of APP_s secretion and inhibition of A β production occur independently of precursor phosphorylation. In addition, we define a minimal region of β APP required for this PKC regulation. Taken together, our data indicate that PKC plays a pivotal role in the control of β APP metabolism and amyloid formation, but in contrast to current postulates, it does so indirectly, perhaps by activating β APP-cleaving proteases.

Results

β APP is phosphorylated exclusively on serine residues in 293 cells

To examine the phosphorylation state of β APP, we labeled cultured human embryonic kidney 293 cells with [³²P]orthophosphate. These cells were chosen because their expression and processing of β APP have been extensively characterized (Oltsdorf *et al.*, 1990) and because they have been previously demonstrated to undergo increased secretion of APP_s in response to activation of protein kinases (Gillespie *et al.*, 1992; Nitsch *et al.*, 1992). Furthermore, they synthesize primarily the 751 and 770 amino acid isoforms of β APP (β APP751/770) containing an insert homologous to the Kunitz family of serine protease inhibitors (Kitaguchi *et al.*, 1988; Ponte *et al.*, 1988; Tanzi *et al.*, 1988). We therefore used DNA constructs encoding the 695 amino acid isoform of β APP (β APP695) for transfection experiments to distinguish easily the introduced protein from endogenous forms. Upon immunoprecipitation of cell extracts from untransfected 293 cells with an antibody (C7) to the C-terminal 20 amino acids of β APP, we detected a single full-length phosphorylated β APP species (Figure 1A, lane 1). This is similar to that previously observed in HS683 human glioma cells (Oltsdorf *et al.*, 1990). The

Fig. 1. Serine phosphorylation of β APP *in vivo*. (A) Untransfected 293 cells (293; lane 1) and 293 cells stably transfected with β APP695 (293/695; lane 2) were labeled with [³²P]orthophosphate. Full-length β APP was immunoprecipitated from cell extracts with antibody C7 and analyzed by 8.5% SDS-PAGE. Note the presence of endogenously expressed phosphorylated β APP751/770 in both untransfected and transfected cells, with β APP695 detected only in the transfectants. (B) Phosphoamino acid analysis. Phosphorylated β APP immunoprecipitated from stably transfected 293/695 cells was subjected to SDS-PAGE and transferred to Immobilon-P. The β APP695 band was excised and subjected to 2-D phosphoamino acid analysis, separated along the horizontal axis by electrophoresis at pH 1.9, and along the vertical axis at pH 3.5. The positions of ninhydrin-stained phosphoamino acid standards are noted. The origin is denoted by an O.

phosphorylated protein migrates with an electrophoretic mobility identical to that of fully mature, N+O-glycosylated β APP751/770 (data not shown). We then examined 293 cells stably transfected with a cDNA expression plasmid encoding β APP695 (Selkoe *et al.*, 1988). In addition to the low levels of endogenous β APP751/770 forms, we detected substantial levels of phosphorylated β APP695 (Figure 1A, lane 2). Only the fully glycosylated forms of both endogenous and transfected APP were phosphorylated, although 293 cells express roughly equal amounts of the immature N-glycosylated and mature N+O-glycosylated species (Oltsdorf *et al.*, 1990; Haass *et al.*, 1991).

To determine which amino acids were phosphorylated in β APP, the phosphate-labeled β APP695 species was isolated following immunoprecipitation and electrophoresis and subjected to two-dimensional phosphoamino acid analysis. Figure 1B demonstrates that phosphate is added only to serine residues on β APP under basal, unstimulated conditions, with no detectable phosphorylation of either threonine or tyrosine residues.

β APP is phosphorylated on extracytoplasmic serine residues

The cytoplasmic domain of β APP contains two serine residues, at amino acid positions 655 and 675 (β APP695 numbering). Because PKC has been postulated to phosphorylate cytoplasmic serine residues of β APP (Gandy *et al.*, 1988; Suzuki *et al.*, 1992), we used oligonucleotide-directed mutagenesis to substitute either one or both serine residues with alanine. These mutations were introduced into the expression vector encoding the β APP695 cDNA and are denoted S655A, S675A and S655A/S675A. *In vivo* phosphate labeling of 293 cells stably transfected with the

Fig. 2. Phosphorylation of β APP serine mutants. (A) 293 cells stably transfected with β APP695 (lane 1) or serine mutants S655A (lane 2), S675A (lane 3) or S655A/S675A (lane 4) were labeled with [32 P]orthophosphate as in Figure 1. Full-length β APP molecules were immunoprecipitated with antibody C7 and analyzed by 8.5% SDS-PAGE. Extracts from 32 P-labeled 293 cells expressing C-terminal truncated 695 Δ C were immunoprecipitated with antibody B5 (lane 5); use of this antibody may account for the slightly weaker signal. The difference in electrophoretic mobility between wild-type β APP695 and the truncated 695 Δ C species is not readily seen; however, the bands can be resolved by 7% SDS-PAGE. (B) Phosphoamino acid analysis. Phosphorylated β APP695 containing various serine mutations was immunoprecipitated from 32 P-labeled cell extracts, separated by SDS-PAGE and transferred to Immobilon P. Isolated bands were analyzed by 2-D phosphoamino acid analysis. The positions of ninhydrin-stained phosphoamino acid standards are noted.

mutant constructs demonstrated that β APP is phosphorylated, even in the absence of one (Figure 2A, lanes 2 and 3) or both (Figure 2A, lane 4) serine residues. When these mutated proteins were isolated and subjected to phosphoamino acid analysis, they again revealed the sole presence of phosphoserine (Figure 2B). These data indicate that β APP must undergo serine phosphorylation in the ectodomain. To confirm this finding, we used a mutant β APP construct which introduced a stop codon after amino acid 653 (of β APP695), thus deleting the last 42 residues of the full-length precursor, including both cytoplasmic serine residues (Haass *et al.*, 1993). Immunoprecipitation of this truncated species (denoted 695 Δ C) with antibody B5 (generated against a bacterial fusion protein containing amino acids 444–592 of β APP695) confirmed that β APP undergoes ectodomain phosphorylation *in vivo* (Figure 2A, lane 5).

To determine whether the intracellular serine residues undergo phosphorylation in addition to those in the ectodomain, we labeled cells expressing either wild-type β APP695, S655A, S675A or S655A/S675A with [32 P]orthophosphate in the presence of 50 μ g/ml leupeptin, and immunoprecipitated cell lysates with antibody C7 to determine the phosphorylation state of the 10 kDa C-terminal fragment. Leupeptin has been found to increase significantly the levels of this fragment, presumably by inhibiting its proteolytic degradation (Golde *et al.*, 1992; Haass *et al.*, 1992a). Even under these conditions, we did not detect any phosphorylated 10 kDa fragment in cells stably transfected with either wild-type β APP695 or any of the serine-mutated constructs (data not shown), indicating that β APP is phosphorylated solely on serine residues in its ectodomain.

To establish that β APP phosphorylated in the ectodomain

Fig. 3. Soluble β APP is released as a phosphorylated molecule. 293 cells transfected with β APP695 were metabolically labeled for 12 h with [35 S]methionine or for 4 h with [32 P]orthophosphate. The conditioned media from 35 S- (lanes 1 and 2) and 32 P-labeled cells (lanes 3 and 4) were immunoprecipitated with either antibody B5 (lanes 1 and 3) or antibody R1736 (lanes 2 and 4). Note the comigration of 32 P-labeled APP_s with the 35 S-labeled species. The positions of APP_s (endogenous β APP751/770 and transfected β APP695) are indicated. Phosphorylated APP_s derived from β APP751/770 is more readily observed after longer autoradiographic exposure.

gives rise to a secreted species, we immunoprecipitated soluble APP_s from the media of 32 P-labeled β APP695-transfected 293 cells. Using antibody B5, we could specifically detect the secreted forms of both the endogenously expressed β APP751/770 and the transfected β APP695 (Figure 3). These phosphorylated molecules comigrated precisely with soluble APP_s secreted by [35 S]methionine-labeled cells (Figure 3, compare lanes 1 and 3). Furthermore, the labeled molecules were specifically immunoprecipitated from media by a second antibody which recognizes APP_s (Figure 3, lanes 2 and 4). This polyclonal antiserum (R1736) to the last 17 residues of APP_s (APP_{595–611}), which recognizes full-length C-terminal-containing β APP molecules very weakly, similarly demonstrates that APP_s is secreted after selective ectodomain phosphorylation. Phosphoamino acid analysis of the immunoprecipitated APP_s confirmed the sole presence of phosphoserine residues (data not shown). Our data leave open the possibility that both phosphorylated and non-phosphorylated forms of APP_s are secreted from 293 cells.

Localization of phosphorylation sites in soluble β APP

To define further the region of the ectodomain that is phosphorylated, we generated two mutants of β APP which delete large portions of either the N- or C-terminal parts of APP_s (Figure 4A). The construct 695/AX removes a 227 amino acid region in the N-terminal part of the extracellular domain of β APP695, whereas 695/XB deletes the non-overlapping 285 residues of the C-terminal portion of the ectodomain. Both deletion mutants retain the last 106 residues of β APP. Thus, their secreted derivatives can be efficiently immunoprecipitated with antibody R1736, and membrane-associated forms with antibody C7. Upon stable

Fig. 4. Localization of ectodomain phosphorylation sites. (A) Schematic of β APP ectodomain deletion constructs 695/AX and 695/XB. The 17 amino acid signal peptide is represented by a white bar and α and β by a striped bar. Vertical lines indicate transmembrane domains. The numbers above denote amino acid residues, with the restriction sites used to generate the constructs indicated (see Materials and Methods). (B) 293 cells stably transfected with wild-type β APP695, 695/AX or 695/XB were labeled with [35 S]methionine. Cell lysates were immunoprecipitated with antibody C7 (lanes 1–3), and conditioned media with R1736 (lanes 4–6), and analyzed by 8.5% SDS–PAGE. Note that both deletion mutants give rise to immature and mature species in cell lysates, but give rise to a single secreted soluble molecule. The secreted species derived from 695/XB has an electrophoretic mobility similar to that previously described (Golde *et al.*, 1992). (C) Conditioned medium from 32 P-labeled 293 cells expressing β APP695 (lane 1), 695/AX (lane 2) or 695/XB (lane 3) was immunoprecipitated with R1736 and analyzed by 8.5% SDS–PAGE. Like wild-type APP_s, soluble 695/XB is secreted as a phosphorylated species. However, phosphorylated 695/AX cannot be detected, although phosphorylated APP_s derived from endogenously expressed β APP751/770 is visible in the same lane ($\sim$ 116 kDa band).

transfection into 293 cells, both deletion constructs gave rise to immature and mature species in cell lysates (Figure 4B, lanes 2 and 3), and a single secreted protein in conditioned media (Figure 4B, lanes 5 and 6). To investigate the phosphorylation state of these proteins, soluble β APP molecules from the media of 32 P-labeled cells expressing 695/AX or 695/XB were immunoprecipitated with R1736 (Figure 4C). Significantly, while soluble 695/XB derivatives were secreted as phosphorylated molecules (Figure 4C, lane 3), no phosphate could be detected on the released 695/AX molecule (Figure 4C, lane 2), indicating that the sites of β APP ectodomain phosphorylation reside exclusively in the N-terminal half of APP_s. In this region, there are 10 serine residues; we are currently attempting to identify the specific sites and the stoichiometry of phosphorylation.

PKC activation does not increase β APP phosphorylation in vivo

To examine directly the effects of PKC on β APP phosphorylation, we stimulated 32 P-labeled β APP695-transfected cells with 1 μ M phorbol-12,13-dibutyrate (PDBu). Immunoprecipitates of full-length β APP from cells treated

Fig. 5. Phorbol esters do not increase the phosphorylation state of APP in vivo. (A) Stably transfected 293/695 cells were biosynthetically labeled for 4 h in total with [32 P]orthophosphate. 1 μ M PDBu was added in the presence of label for the indicated time at the end of the labeling period. Cell extracts were immunoprecipitated with antibody C7, analyzed by SDS–PAGE and transferred to Immobilon P. The slight decrease in phosphorylated β APP holoprotein after 15 and 30 min of treatment with PDBu is probably due to increased secretory cleavage upon PDBu addition (see text). (B) Phosphoamino acid analysis after PDBu treatment. β APP695 immunoprecipitated from 32 P-labeled 293/695 cells treated with 1 μ M PDBu for 7.5 min (lane 2 in A) was excised and subjected to 2-D phosphoamino acid analysis. The positions of the ninhydrin-stained standards are noted and the origin marked with an O.

with PDBu for 7.5 min failed to show any significant increase in the phosphorylation state of the molecule, compared with untreated cells (Figure 5A, compare lanes 1 and 2). Phosphoamino acid analysis of β APP695 isolated from PDBu-treated cells (7.5 min) again showed that the molecule contains only phosphoserine residues, indicating that no threonine or tyrosine residues are utilized as alternative phosphate acceptors, even after PDBu addition (Figure 5B).

One possible explanation for the reported effects of phorbol esters on β APP secretion (Caporaso *et al.*, 1992; Gillespie *et al.*, 1992) is that PKC activation leads to the phosphorylation and activation of additional kinases which subsequently utilize β APP as a substrate. This might result in a hyperphosphorylated state of β APP only after longer incubation times with phorbol ester. However, even after 15 or 30 min incubations with 1 μ M PDBu, no increase in total β APP phosphorylation was observed (Figure 5A, lanes 3 and 4). Instead, an apparent decrease in the level of phosphorylated full-length β APP molecules occurred after longer periods of incubation with phorbol ester. This result is most likely due to the increased turnover of the mature holoprotein into soluble forms upon activation of protein kinases (see below; Buxbaum *et al.*, 1990; Gillespie *et al.*, 1992). Thus, our data indicate that PKC activation does not increase the phosphorylation state of β APP in 293 cells *in vivo*.

Regulation of APP_s and A β secretion from cells expressing cytoplasmic serine mutants

Our finding that β APP phosphorylation is not stimulated by PKC activation suggests that cytoplasmic phosphorylation of the precursor molecule is not necessary for the observed enhancement of APP_s secretion. To confirm this directly, 293 cells stably transfected with wild-type β APP695 or site-directed mutants S655A, S675A and S655A/S675A were pulse-labeled with [35 S]methionine for 20 min and then

Fig. 6. Increased APP_s secretion and decreased Aβ release upon phorbol ester addition does not require cytoplasmic serine phosphorylation. (A) Untransfected 293 cells (lanes 1 and 2) or pooled stable transfectants expressing either wild-type βAPP695 (lanes 3 and 4) or serine mutants S655A (lanes 5 and 6), S675A (lanes 7 and 8) or S655A/S675A (lanes 9 and 10) were pulse-labeled for 20 min with [³⁵S]methionine. Conditioned medium was collected during a 60 min chase period in the absence (indicated by -) or presence (indicated by +) of 1 μM PDBu and immunoprecipitated with antibody B5. Immunoprecipitates were analyzed by 10% SDS-PAGE. The positions of endogenously expressed soluble βAPP751/770 and transfected soluble βAPP695 are indicated. Note that untransfected 293 cells express only low levels of βAPP695, observed in lane 2 after PDBu treatment. (B) Pooled stable transfectants expressing wild-type βAPP695 (lanes 1 and 2) or mutants S655A (lanes 3 and 4), S675A (lanes 5 and 6) or S655A/S675A (lanes 7 and 8) were labeled for 2 h with [³⁵S]methionine and chased for 5–6 h in the absence (-) or presence (+) of 1 μM PDBu. Conditioned media were immunoprecipitated with antibody R1280 and analyzed on 10–20% Tris-Tricine gels. The positions of Aβ and p3 are indicated. No bands were detected below p3; the increased smear is due to the elevated level of p3 secretion after PDBu addition. Differences in Aβ levels between the various constructs is most likely due to variations in expression levels of the transfected cDNAs.

chased for 60 min in the absence or presence of 1 μM PDBu. Both untransfected and βAPP695-transfected 293 cells showed increased levels of secreted APP_s (Figure 6A, lanes 1–4). The introduction of the non-phosphate acceptor alanine substitutions at either position 655 or 675, or both, had no effect on the ability of phorbol esters to upregulate the secretion of APP_s (Figure 6A). The response of these βAPP molecules to PDBu is indistinguishable from that of wild-type βAPP: in conjunction with an increase in APP_s, the levels of full-length, N+O-glycosylated precursor molecules in cell extracts is reduced after treatment (data not shown). This result is consistent with published reports of the effects of phorbol esters on βAPP metabolism (Buxbaum *et al.*, 1990; Gillespie *et al.*, 1992). In addition, mutation of the threonine residue at position 654, which has been reported to be phosphorylated *in vitro* by Ca²⁺/calmodulin-dependent protein kinase II in synthetic βAPP

Fig. 7. Lack of involvement of the ectodomain in PKC-activated secretion. 293 cells stably transfected with wild-type βAPP695 (lanes 1 and 2), 695/AX (lanes 3 and 4) or 695/XB (lanes 5 and 6) were pulse-labeled for 20 min with [³⁵S]methionine and chased for 60 min in the absence (-) or presence (+) of 1 μM PDBu. The chase medium was immunoprecipitated with antibody R1736 and analyzed by 8.5% SDS-PAGE. Soluble 695/AX in the absence of PDBu (lane 3) can be detected after longer autoradiographic exposure.

peptides (Gandy *et al.*, 1988), similarly failed to abolish the modulation of APP_s secretion by phorbol esters in intact cells (data not shown).

The increase in APP_s is accompanied by a corresponding decrease in Aβ release (Buxbaum *et al.*, 1993; Hung *et al.*, 1993). To confirm that phosphorylation of the cytoplasmic serine residues of βAPP also plays no role in this PKC-mediated inhibition of Aβ production, we pulse-labeled 293 cells expressing the serine-mutated βAPP molecules with [³⁵S]methionine for 2 h, and then chased in the absence or presence of PDBu. As shown previously, addition of phorbol esters resulted in decreased release of Aβ from cells expressing wild-type βAPP695 (Figure 6B, lanes 1 and 2). Similarly, release of Aβ was still downregulated from mutants S655A, S675A and S655A/S675A after PDBu treatment, indicating that, as for APP_s, Aβ regulation occurs independently of cytoplasmic serine phosphorylation (Figure 6B).

Requirements for PKC modulation of βAPP processing

To define the region within βAPP that is required for PKC-mediated stimulation of APP_s secretion, we first examined the ability of phorbol esters to activate release of soluble βAPP from 293 cells expressing the 695/AX or 695/XB deletion mutants. Addition of 1 μM PDBu after pulse-labeling with [³⁵S]methionine increased release of the secreted molecules from both truncated proteins (Figure 7). Since the 695/AX mutant is not phosphorylated (see above), these data further show that ectodomain phosphorylation is not a necessary event for PKC-activated secretion. The two large deletions indicate that epitopes between amino acids 78 and 589, comprising almost the entire extracellular domain of βAPP, are not required for the modulation of βAPP processing by PKC.

These data define a minimal region of βAPP required for activation of secretion to the first 77 amino acids of the precursor molecule or to the last 106 amino acid residues, including the Aβ region, the secretase cleavage site and the cytoplasmic domain. To examine the possibility that the cytoplasmic region is necessary for PDBu-activated

Fig. 8. Effect of C-terminal truncation on PKC-mediated APP_s release. (A) Schematic diagram of β APP C-terminal truncation constructs 695 Δ C and 695/S. The 17 amino acid signal peptide is represented by a white box and A β by a striped bar. 695 Δ C and 695/S were generated by introducing stop codons after Tyr653 or Gln611 (of β APP695), respectively. Vertical lines indicate transmembrane domains. (B) Stable transfectants expressing wild-type β APP (lanes 1 and 2), 695 Δ C (lanes 3 and 4) or soluble 695/S (lanes 5 and 6) were analyzed by pulse-chase experiments as in Figure 7. Immunoprecipitates were analyzed by 10% SDS-PAGE. Note that APP_s release from 695/S-expressing cells is not increased upon PDBu addition.

secretion, we performed pulse-chase experiments on 293 cells stably transfected with 695 Δ C (Figure 8A), which lacks almost the entire cytoplasmic domain of the precursor. Immunoblotting of cell extracts from 293 cells stably transfected with β APP695 and 695 Δ C expression plasmids confirmed similar levels of expression (data not shown). As previously demonstrated (Haass *et al.*, 1993), 695 Δ C showed elevated levels of APP_s secretion in the absence of PDBu (Figure 8B, compare lanes 1 and 3), consistent with other reports assessing the effect of similar deletions on β APP processing (Sisodia, 1992; DeStrooper *et al.*, 1993). However, addition of PDBu resulted in a further increase in secretion (Figure 8B), indicating that cytoplasmic epitopes are also not required for the effects of PKC activation on β APP processing. These data are consistent with the notion that the β APP secretase cleavage event may be directly regulated by PKC, which can activate APP_s release from molecules lacking major portions of the extracellular or intracellular domains. If this is true, secretion of soluble β APP which does not require cleavage by secretase should not be regulated by PKC. To investigate this possibility, we introduced a stop codon into β APP695 after the glutamine residue at position 611 (Figure 8A), which has been identified as the last amino acid of APP_s (Esch *et al.*, 1990). When expressed in 293 cells, this construct (denoted

695/S), which is not synthesized as a transmembrane molecule, gave rise to a secreted species which precisely comigrated with APP_s released from cells expressing wild-type β APP695 (Figure 8B). However, in contrast to β APP695 and 695 Δ C, addition of PDBu did not further stimulate secretion of soluble β APP from 695/S-transfected cells (Figure 8B, lanes 5 and 6), indicating that once APP_s is created, its release is no longer subject to regulation by PKC.

Discussion

The identification of β APP mutations genetically linked to familial Alzheimer's disease (Hardy, 1992) and the findings to date that at least one of these mutations substantially alters β APP processing (Citron *et al.*, 1992; Cai *et al.*, 1993) strongly support previous indirect evidence that regulation of β APP metabolism may be critically involved early in the disease process (for review see Selkoe, 1993). PKC has been directly implicated in activating the release of a large, soluble N-terminal derivative of β APP (Caporaso *et al.*, 1992; Gillespie *et al.*, 1992) and in inhibiting A β production (Buxbaum *et al.*, 1993; Hung *et al.*, 1993). Therefore, an understanding of the cellular mechanisms involved in this regulation may be significant both for defining the physiologic function of β APP and for elucidating its potential role in Alzheimer's disease. Among other findings, this study demonstrates that the PKC-mediated regulation of β APP processing does not require phosphorylation of the holoprotein. Furthermore, our data define a minimal region of the precursor that is required for this modulation of β APP processing and indirectly implicate APP secretase cleavage as the regulated event in this process.

Ectodomain phosphorylation of β APP

Analysis of β APP phosphorylation by exogenously added PKC in detergent-permeabilized PC12 cells led to the proposal that β APP is specifically phosphorylated by PKC on a cytoplasmic serine at position 655 (Suzuki *et al.*, 1992). In this study, we establish by phosphoamino acid analysis that β APP expressed in 293 cells is phosphorylated only on serine residues under both unstimulated and phorbol ester-stimulated conditions. However, analysis of β APP mutants containing substitutions of the two cytoplasmic serine residues indicates that, at least in 293 cells, neither of these amino acids is utilized as a phosphate acceptor. Furthermore, PKC activation by PDBu does not appear to increase the phosphorylation state of β APP in these cells.

Four independent results indicate that β APP is phosphorylated solely on its ectodomain. First, a mutant lacking both cytoplasmic serine residues still incorporates phosphate on serine. Second, a truncated form of β APP lacking almost the entire cytoplasmic domain is still phosphorylated *in vivo*. Third, soluble APP_s immunoprecipitated from the conditioned media of ³²P-labeled cells is released as a phosphorylated molecule which contains only phosphoserine residues. Fourth, the 10 kDa C-terminal fragment that is retained following secretase cleavage shows no phosphorylation, indicating that phosphate is added solely to the extracellular/intraluminal region. Furthermore, we have localized the site(s) of phosphorylation to a 227 amino acid portion in the N-terminal part of the β APP ectodomain.

We have examined ectodomain phosphorylation of β APP in cell types other than 293 cells. Our preliminary results

demonstrate that ^{32}P -labeled Chinese hamster ovary cells and HS683 human glioma cells both contain phosphorylated full-length βAPP , which is cleaved to yield labeled APP_s . Moreover, antibody C7 is able to precipitate a phosphorylated protein from primary rat hippocampal neurons that is likely to be βAPP (A.Y.Hung and D.J.Selkoe, unpublished data). Phosphorylated 10 kDa fragments could not be detected in any of the cells studied. Thus, the data we have reported here in 293 cells appear to pertain to a variety of cell types examined to date, including some of neuronal or glial origin. Nevertheless, the possibility remains that for some cell types the extent of phosphorylation or the specific residues utilized will differ from the pattern described here.

Our results indicate that βAPP may be a substrate for an ecto-protein kinase. Ecto-protein kinase activities have been identified on the cell surface of a variety of different cell types, including neurons (Ehrlich *et al.*, 1986, 1991). Although few physiological substrates for extracellular/intraluminal phosphorylation have been identified, it appears that basic fibroblast growth factor (Feige and Baird, 1989; Skubitz and Goueli, 1991) and possibly certain neuronal cell adhesion molecules (N-CAMs) (Ehrlich *et al.*, 1986) may serve as phosphate acceptors for these enzymes. Alternatively, βAPP may be phosphorylated by an intracellular kinase, as has been postulated for the insulin-like growth factor binding protein (Jones *et al.*, 1991). Ectodomain phosphorylation of βAPP and the release of soluble APP_s as a phosphorylated species could potentially affect its function as a growth- or adhesion-promoting molecule (Saitoh *et al.*, 1989; Schubert *et al.*, 1989; Milward *et al.*, 1992). Furthermore, our findings raise the intriguing possibility that APP_s might be secreted both as phosphorylated and non-phosphorylated molecules, and that these forms could potentially have distinct physiological properties. In this regard, the non-collagenous sialic acid-rich protein osteopontin has been demonstrated to be released from normal rat kidney cells in two forms (Nemir *et al.*, 1989). In its phosphorylated state, osteopontin interacts with the cell surface; however, the non-phosphorylated species forms complexes with plasma fibronectin.

Regulated cleavage and release of βAPP

Regulated secretory cleavage of membrane-bound precursor molecules by PKC activation is not unique to βAPP (reviewed by Ehlers and Riordan, 1991). Indeed, phorbol ester stimulates the cleavage and secretion of a number of other transmembrane proteins, including pro-TGF α (Pandiella and Massagué, 1991), the CSF-1 receptor (Downing *et al.*, 1989), the MEL-14 adhesion protein (Kishimoto *et al.*, 1989) and the TNF receptor (Lantz *et al.*, 1990). In particular, we demonstrate here that, like pro-TGF α and the CSF-1 and TNF receptors, the increased secretion of the soluble form of βAPP does not involve an increase in phosphorylation of the precursor molecule itself.

Although intracellular phosphorylation is not required for PKC-mediated stimulation of APP secretion, this finding alone does not rule out that distinct regions of the cytoplasmic tail could potentially mediate this effect in a phosphorylation-independent manner. Indeed, C-terminal truncated forms of βAPP have been previously reported to undergo an increased level of APP_s secretion in the absence of phorbol ester stimulation (Sisodia, 1992; DeStrooper *et al.*, 1993; Haass *et al.*, 1993). However, in this study we demonstrate further

that the 695 ΔC truncation mutant lacking almost the entire cytoplasmic domain retains the ability to be modulated by PKC. Thus, our results show that while cytoplasmic determinants in βAPP clearly affect its processing, they do not account for the entire effect of phorbol esters, indicating that PKC can activate APP_s release without any requirement for putative intracellular regulatory proteins that may bind to the C-terminus of βAPP . We hypothesize that the activation of PKC by phorbol esters and the removal of specific cytoplasmic epitopes of βAPP each result in increased secretion of APP_s by distinct mechanisms.

Our data indicate that the necessary epitopes for PKC regulation of βAPP processing lie either in the first 77 amino acids of the precursor, far from the site of secretory cleavage, or much more likely in a minimal 64 amino acid region of the precursor (amino acid residues 590–653 of βAPP_{695}) around the secretase site. The latter possibility is consistent with the hypothesis that PKC may directly activate APP secretase, rather than affecting βAPP localization or targeting dependent on interactions with distinct extra- or intracytoplasmic domains. Such a mechanism has been proposed for regulated cleavage of the TNF receptor, whose cytoplasmic domain is also dispensable for PKC-mediated cleavage (Brakebusch *et al.*, 1992), and the CSF-1 receptor (Downing *et al.*, 1989). The finding that release of APP_s , when synthesized *de novo* as a soluble molecule (695/S), is no longer PKC-regulated also supports the notion that PKC activates the cleavage of βAPP rather than the release of intracellularly cleaved material. That intracellular signaling events could influence a protease which cleaves the ectodomain of a substrate requires that messages be transduced across the membrane into the extracellular or intraluminal compartment. In this regard, APP secretase has been proposed to be a membrane-bound enzyme (Sisodia, 1992). Thus, PKC could stimulate secretase activity either by direct phosphorylation of the cytoplasmic portion of the enzyme or by phosphorylating a putative regulatory molecule. However, we cannot entirely rule out the possibility that PKC activation has multiple effects on βAPP metabolism, perhaps influencing both trafficking of the molecule and its enzymatic cleavage.

The significance of modulation of βAPP processing by PKC-mediated signaling pathways for its physiologic function remains to be determined. Interestingly, in the case of TGF α , both its membrane-bound and soluble forms are biologically active. Regulated cleavage converts one form to another (Massagué, 1990). Similarly, it appears that βAPP can also have distinct effects on various cell types when presented as a transmembrane or a soluble molecule. Indeed, APP_s has been shown to have growth-promoting properties on fibroblasts, PC12 and neuroblastoma cells (Saitoh *et al.*, 1989; Schubert *et al.*, 1989; LeBlanc *et al.*, 1992; Milward *et al.*, 1992). In contrast, cell surface-inserted βAPP appears to enhance neurite outgrowth from dissociated hippocampal neurons (W.Q.Qiu and D.J.Selkoe, submitted). Activation of βAPP secretory cleavage, for example through neurotransmitter (Nitsch *et al.*, 1992) or other stimulatory inputs (Nitsch *et al.*, 1993), could thus regulate the relative levels of distinct intercellular signals.

Implications for amyloid β -peptide regulation

The coordinate upregulation of APP_s secretion and inhibition of A β formation by PKC *in vitro* suggests that

agents capable of preferentially stimulating the secretase pathway could be potentially useful in decreasing $A\beta$ production *in vivo*. Our data demonstrate that this regulation is independent of β APP phosphorylation. The necessary involvement of other proteins in the complex regulation of β APP processing thus implies that a variety of genetic or metabolic alterations could have significant impacts on the generation of $A\beta$ *in vitro* and *in vivo*. Identifying the substrates of PKC in this process, whether proteases or otherwise, could serve to elucidate additional therapeutic approaches towards the retardation of $A\beta$ deposition early in the clinical course of Alzheimer's disease.

Materials and methods

In vitro mutagenesis of β APP cDNA and construction of plasmids

Oligonucleotide-directed mutagenesis of the β APP cDNA was performed according to standard procedures (Kunkel, 1985). The ~0.9 kb *EcoRI*–*HindIII* fragment of β APP was cloned into M13mp18 and used as template for mutagenesis of serine residues 655 and 675 to alanine (S655A and S675A). A mutant containing both substitutions (S655A/S675A) was generated by sequential mutagenesis. The mutated 563 bp *EcoRI*–*SpeI* fragments were isolated from the RF form of the M13 DNA and directly subcloned into the expression plasmid pCMV695 (Selkoe *et al.*, 1988), which encodes the 695 amino acid isoform of β APP. All point mutations were verified by DNA sequencing. To generate the ectodomain deletion constructs, the 2.7 kb *KpnI*–*HindIII* fragment of β APP695 was first subcloned into pGEM-3 (Promega), creating plasmid pGEM-K/H. pGEM-K/H was digested either with *AccI* and *XhoI* (695/AX), or *XhoI* and *BglII* (695/XB). The ends were filled with the Klenow fragment of DNA polymerase I and ligated to generate in-frame deletions in the β APP coding region. The *KpnI*–*EcoRI* fragments from plasmids bearing the deletions were isolated and substituted for the 1.7 kb *KpnI*–*EcoRI* fragment of pCMV695, thus creating 695/AX and 695/XB expression plasmids. A construct containing a C-terminal truncated form of β APP695 (695 Δ C) was generated by the PCR by introducing a stop codon after amino acid 653 (of β APP695), as previously described (Haass *et al.*, 1993). A construct encoding APP_s (695/S) was designed by introducing the annealed oligonucleotides AATTCGCACATG-CTCAGGATATGAAGTTTCATCATCAATAGT and CTAGACTAT-TGATGATGAACCTTCATATCCTGAGTCATGTCGG into pCMV695 linearized with *EcoRI* and *SpeI*, resulting in a stop codon after glutamine-611 of β APP695.

Transfection of 293 cells

Human embryonic kidney 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum. Cells were transfected with plasmids encoding wild-type and mutated β APP using Lipofectin (Gibco BRL), as described by the manufacturer. For generation of pooled stable transfectants, cells were cotransfected with β APP plasmids (10 μ g) and the neomycin resistance plasmid pRc/CMV (Invitrogen) (0.5 μ g), and selected in medium containing 400 μ g/ml G418 for 2–3 weeks.

Antibodies

To recognize full-length β APP and its C-terminal derivatives, we used antibody C7 raised against a synthetic peptide of the last 20 amino acid residues of β APP (Podlisny *et al.*, 1991). C-terminal truncated and soluble β APP forms were precipitated with an antiserum to synthetic β APP_{595–611} (R1736; Haass *et al.*, 1992b) or with an affinity-purified antibody raised against a fusion protein containing amino acids 444–592 (affinity-purified B5; Oltersdorf *et al.*, 1990). Antibody R1736 against the last 17 residues of APP_s preferentially recognizes this soluble form of β APP in immunoblots and immunoprecipitations and reacts very weakly with full-length β APP. For immunoprecipitation of $A\beta$ and 3 kDa forms, antibody R1280 (Tamaoka *et al.*, 1992) against $A\beta_{1–40}$ was used as described (Haass *et al.*, 1992b). Cell extracts were prepared by NP-40 lysis and analyzed by immunoprecipitation as previously described (Haass *et al.*, 1991).

Metabolic labeling and immunoprecipitation

Cells were metabolically labeled with 60–100 μ Ci/ml [³⁵S]methionine under pulse–chase conditions. For analysis of soluble β APP forms, cells were labeled for 20 min in methionine-free DMEM, washed once with

complete medium and chased with complete medium for 60 min. The effect of mutations on $A\beta$ release was examined as previously described (Hung *et al.*, 1993). Cells were labeled for 2 h, washed and then chased for 5–6 h to ensure processing and release of all labeled β APP species. PDBu was added at a final concentration of 1 μ M only during the chase period. Immunoprecipitations of [³⁵S]methionine-labeled cell extracts and culture supernatants were performed essentially as described (Haass *et al.*, 1991).

For *in vivo* labeling with [³²P]orthophosphate, 293 cells were incubated in phosphate-free DMEM for 60 min and then labeled with 1–2 mCi/ml H₃[³²P]O₄ for 4 h. For phorbol ester stimulation, 1 μ M PDBu was added in the presence of label for the indicated times. To examine the presence of the phosphorylated C-terminal 10 kDa fragment, cells were labeled in the presence of 50 μ M sodium orthovanadate (to inhibit phosphatases) and 50 μ g/ml leupeptin (to inhibit proteolytic degradation of the 10 kDa fragment) for 8 h. Cells were lysed with 1% NP-40 lysis buffer (Haass *et al.*, 1991) containing 50 mM β -glycerophosphate, 0.5 mM sodium orthovanadate and 2 mM DTT for 20 min on ice. Identical results were obtained when lysates were prepared in the presence of the additional phosphatase inhibitors sodium fluoride (50 mM) and sodium phosphate (10 mM). After centrifugation at 10 000 g for 10 min, lysates were immunoprecipitated as described (Haass *et al.*, 1991), with minor modifications. Briefly, following incubation with primary antibody and Protein A–Sepharose, immunocomplexes were layered onto a 200 μ l cushion of sucrose (1 M) (Sisodia *et al.*, 1990) and centrifuged for 2 min. The pellet was then washed as above in buffers containing phosphatase inhibitors. The conditioned medium from ³²P-labeled cells was supplemented with 10% fetal bovine serum (to decrease background), 50 mM β -glycerophosphate and 0.5 mM sodium orthovanadate prior to immunoprecipitation.

Immunoprecipitated proteins were separated by SDS–polyacrylamide gel electrophoresis or on 10–20% Tris–Tricine gels (Novex). Gels were either treated for autoradiography as described (Haass *et al.*, 1991) or transferred directly to nitrocellulose or Immobilon-P PVDF membranes (Millipore), according to Kamps and Sefton (1989).

2-D phosphoamino acid analysis

For phosphoamino acid analysis, separated proteins transferred to Immobilon membranes were excised after visualization by autoradiography. Isolated proteins were hydrolyzed for 60 min at 110°C in 5.7 N HCl (Kamps and Sefton, 1989), then lyophilized to dryness. The acid hydrolysates were subjected to 2-D phosphoamino acid analysis as described (Boyle *et al.*, 1991).

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