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Cell cycle-dependent regulation of the phosphorylation and metabolism of the Alzheimer amyloid precursor protein

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Accumulation of the amyloid Aβ peptide, which is derived from a larger precursor protein (APP), and the formation of plaques, are major events believed to be involved in the etiology of Alzheimer's disease. Abnormal regulation of the metabolism of APP may contribute to the deposition of plaques. APP is an integral membrane protein containing several putative phosphorylation sites within its cytoplasmic domain. We report here that APP is phosphorylated at Thr668 by p34^{cdc2} protein kinase (cdc2 kinase) *in vitro*, and in a cell cycle-dependent manner *in vivo*. At the G_2/M phase of the cell cycle, when APP phosphorylation is maximal, the levels of mature APP (mAPP) and immature APP (imAPP) do not change significantly. However, imAPP is altered qualitatively. Furthermore, the level of the secreted extracellular N-terminal domain (APP_S) is decreased and that of the truncated intracellular C-terminal fragment (APP_{COOH}) is increased. These findings suggest the possibility that phosphorylation-dependent events occurring during the cell cycle affect the metabolism of APP. Alterations in these events might play a role in the pathogenesis of Alzheimer's disease.

Key words: Alzheimer's disease/amyloid precursor protein/cell cycle/p34^{cdc2} protein kinase/protein phosphorylation

Introduction

Alzheimer Aβ amyloid precursor protein (APP) is an integral membrane glycoprotein, existing as several distinct isoforms arising by alternative splicing of a single gene. Each isoform also exists as immature (*N*-glycosylated) and mature (sulfated, *N*- and *O*-glycosylated) species. The amyloid domain (Aβ) consists of a 39-43 amino acid peptide, derived from a region at the junction of the ecto- and transmembrane domains of APP (Figure 1A) (Goldgaber *et al.*, 1987; Kang *et al.*, 1987; Robakis *et al.*, 1987; Tanzi *et al.*, 1987). Normal proteolytic processing of APP generates two fragments, a large extracellular N-terminal domain (APP_S) which is cleaved within the Aβ domain and secreted (Figure 1A) (Esch *et al.*, 1990; Sisodia *et al.*, 1990), and a truncated C-terminal domain (APP_{COOH}) which remains cell-associated and is degraded by lysosomes (reviewed in Gandy *et al.*, 1992a). Alternative proteolytic processing of APP gives rise to the production and secretion of Aβ and this process may be elevated in certain types of Alzheimer's disease (AD) (Haass *et al.*, 1992b; Seubert *et al.*, 1992).

APP is a phosphoprotein (Gandy *et al.*, 1988; Oltersdorf *et al.*, 1990; Suzuki *et al.*, 1992) and contains several putative phosphorylation sites in its cytoplasmic domain (Figure 1A). We have previously reported that Ser655 (numbering for APP_{695} isoform) is phosphorylated *in vitro* by both protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CaM kinase II), and Thr654 is also phosphorylated *in vitro* by CaM kinase II (Gandy *et al.*, 1988; Suzuki *et al.*, 1992). However, the phosphorylation of APP by PKC, CaM kinase II or any other protein kinase has not been characterized *in vivo*. In the present study, we now demonstrate that APP is phosphorylated at Thr668 by p34^{cdc2} protein kinase (cdc2 kinase) both *in vitro* and *in vivo*, and that the phosphorylation of APP at this site is regulated in a cell cycle-dependent manner in intact cells. Furthermore, in intact cells the phosphorylation of APP at Thr668 correlates with altered metabolism of APP.

Results

Phosphorylation of APP cytoplasmic peptides in vitro by cdc2 kinase

The amino acid sequences of the cytoplasmic C-terminal domains of the three major APP isoforms are identical and contain two seryl, three threonyl and two tyrosyl residues (Figure 1A). Preliminary studies using intact PC12 cells suggested that APP was phosphorylated at a threonyl site (data not shown). Examination of the amino acid sequences surrounding the three threonyl residues revealed that Thr668 was a potential site for phosphorylation by cdc2 kinase, a proline-directed protein kinase (Kemp and Pearson, 1990). Phosphorylation of a synthetic peptide ($APP^{645-694}$, corresponding to residues 645-694 of the cytoplasmic domain of APP_{695}) by purified cdc2 kinase was investigated. A purified cdc2 kinase-cyclin B complex phosphorylated $APP^{645-694}$ at threonyl residue(s) (Figure 1B). The catalytic efficiency for phosphorylation of $APP^{645-694}$ by cdc2 kinase approached that for a peptide substrate based on the sequence of the phosphorylation site for cdc2 kinase in the SV40 T antigen (Figure 1C and Table I), previously identified as an excellent substrate for this kinase (Marshak *et al.*, 1991). To confirm that Thr668 was a site of phosphorylation, a shorter peptide, $APP^{663-676}$, which contains only Thr668, was tested as a substrate for cdc2 kinase. As in the case of $APP^{645-694}$, $APP^{663-676}$ was phosphorylated, although weakly, at its threonyl residue (Figure 1B). These studies demonstrated that cdc2 kinase is able to phosphorylate APP at Thr668 *in vitro*.

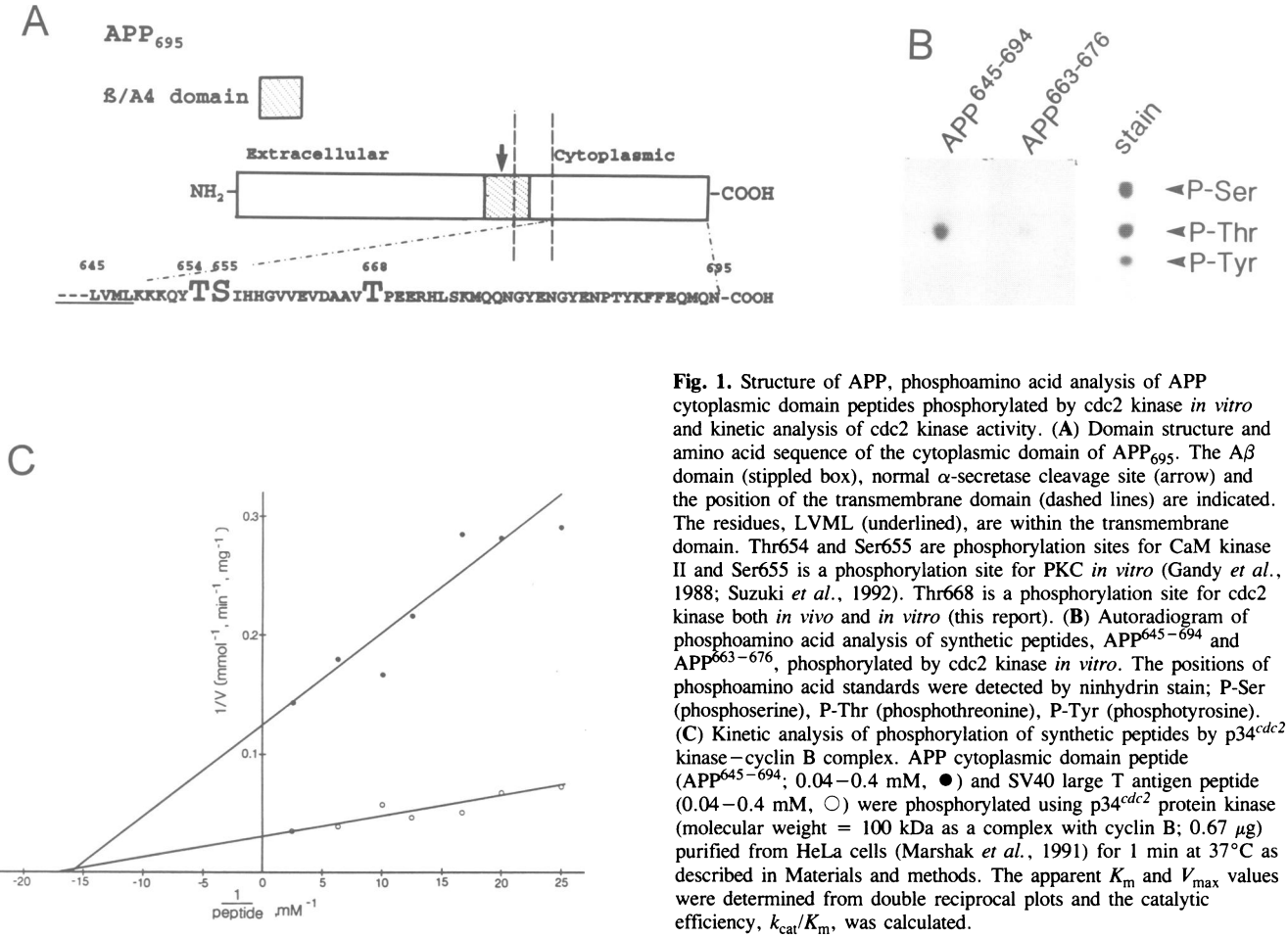

Fig. 1. Structure of APP, phosphoamino acid analysis of APP cytoplasmic domain peptides phosphorylated by cdc2 kinase *in vitro* and kinetic analysis of cdc2 kinase activity. (**A**) Domain structure and amino acid sequence of the cytoplasmic domain of APP_{695}. The Aβ domain (stippled box), normal α-secretase cleavage site (arrow) and the position of the transmembrane domain (dashed lines) are indicated. The residues, LVML (underlined), are within the transmembrane domain. Thr654 and Ser655 are phosphorylation sites for CaM kinase II and Ser655 is a phosphorylation site for PKC *in vitro* (Gandy *et al.*, 1988; Suzuki *et al.*, 1992). Thr668 is a phosphorylation site for cdc2 kinase both *in vivo* and *in vitro* (this report). (**B**) Autoradiogram of phosphoamino acid analysis of synthetic peptides, $APP^{645-694}$ and $APP^{663-676}$, phosphorylated by cdc2 kinase *in vitro*. The positions of phosphoamino acid standards were detected by ninhydrin stain; P-Ser (phosphoserine), P-Thr (phosphothreonine), P-Tyr (phosphotyrosine). (**C**) Kinetic analysis of phosphorylation of synthetic peptides by $p34^{cdc2}$ kinase–cyclin B complex. APP cytoplasmic domain peptide ($APP^{645-694}$; 0.04–0.4 mM, ●) and SV40 large T antigen peptide (0.04–0.4 mM, ○) were phosphorylated using $p34^{cdc2}$ protein kinase (molecular weight = 100 kDa as a complex with cyclin B; 0.67 μg) purified from HeLa cells (Marshak *et al.*, 1991) for 1 min at 37°C as described in Materials and methods. The apparent K_m and V_{max} values were determined from double reciprocal plots and the catalytic efficiency, k_{cat}/K_m, was calculated.

Table I. Phosphorylation of synthetic peptides by $p34^{cdc2}$ protein kinase

Substrate	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})	Catalytic efficiency (μM^{-1} s^{-1})
$APP^{645-694}$	62.5	8.0	0.016
SV40 T antigen peptide	58.8	33.3	0.070

Cell cycle-dependent phosphorylation of APP

If APP is a physiological substrate for cdc2 kinase *in vivo*, then APP should be phosphorylated in a cell cycle-dependent manner at the G_2/M phase, when cdc2 kinase is most active. Initial studies were performed using both PC12 cells, which express three APP isoforms, APP_{770}, APP_{751} and APP_{695}, and HeLa cells, which express APP_{770} and APP_{751}. PC12 cells were serum-starved at the G_1 phase and subsequently arrested at the G_2/M phase using nocodazole, a microtubule-destabilizing drug. The cells were incubated with [^{32}P]orthophosphate and APP was immunoprecipitated from cell lysates. Phosphorylation of the mature (mAPP) and the immature (imAPP) forms of APP was elevated at the G_2/M phase (Figure 2A). A truncated COOH-fragment (APP_{COOH}; 15 kDa) was also found to be phosphorylated at the G_2/M phase (Figure 2B).

The exact percentage of PC12 cells arrested in G_2/M phase by treatment with nocodazole could not be determined because these cells are adhesive and heterogenous in size, thus preventing the identification of individual cell phases. A more rigorous analysis of cell cycle-dependent phosphorylation of APP was performed using HeLa cells, in which the stage of the cell cycle was determined using fluorescence-activated cell sorting (FACS) analysis. Enhanced phosphorylation of imAPP and mAPP was found in HeLa cells at the G_2/M phase (>95% of cells at G_2/M) compared with cells at the G_1 phase (72% at G_1, 10% at S and 18% at G_2/M) (Figure 2C and D). The level of phosphorylation of APP_{COOH} in HeLa cells was relatively low at the G_2/M phase compared with that observed in PC12 cells (data not shown). The phosphorylation of $imAPP_{770/751}$ in HeLa cells at the G_2/M phase was 285 ± 80% (mean ± SD, n = 7) and phosphorylation of $mAPP_{770/751}$ at the G_2/M phase was 164 ± 16.5% (n = 7) relative to that at the G_1 phase. The absolute level of $mAPP_{770/751}$ (100 ± 7.4%, n = 5) and $imAPP_{770/751}$ (118 ± 14.3%, n = 5) did not change significantly at the G_2/M phase compared with that at the G_1 phase (see Figure 5A and D). Therefore, the net increases in the phosphorylation state of imAPP and mAPP were ~2.5- and 1.6-fold, respectively.

Phosphorylation of APP at Thr668 *in vivo* was confirmed by two-dimensional phosphopeptide mapping following digestion of ^{32}P-labeled samples by thermolysin (Figure 3).

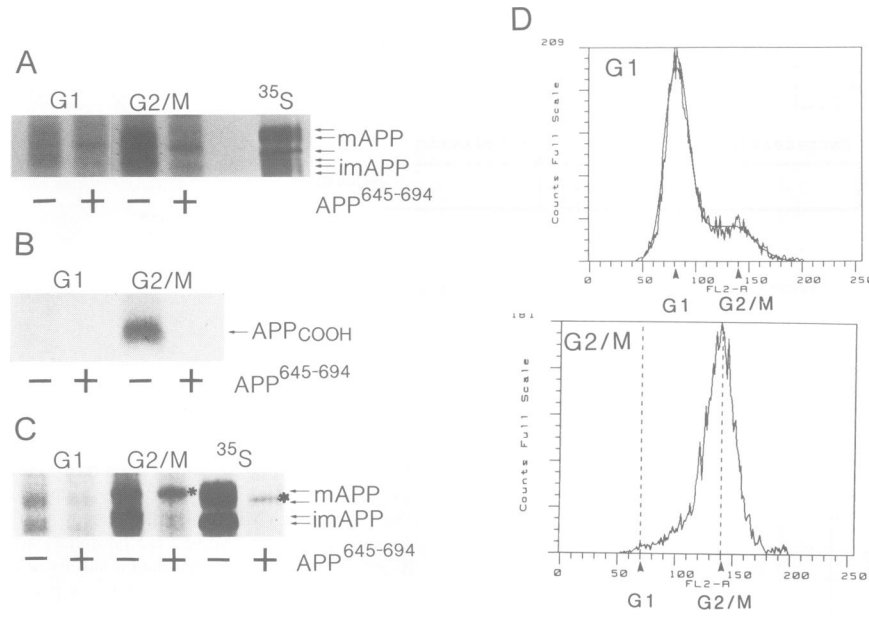

Fig. 2. Cell cycle-dependent phosphorylation of APP and APP_{COOH}. (**A**) Phosphorylation of holo-APP (7.5% polyacrylamide gel) in PC12 cells, (**B**) phosphorylation of the truncated APP_{COOH} (15% polyacrylamide gel) in PC12 cells and (**C**) phosphorylation of holo-APP (6% polyacrylamide gel) in HeLa cells. PC12 and HeLa cells were arrested at G_1 or G_2/M phase and labeled with [^{32}P]orthophosphate. The same cells arrested at the G_1 phase were also labeled with [^{35}S]methionine (^{35}S) as described in Materials and methods. APP was immunoprecipitated and analyzed by autoradiography following SDS−PAGE. Immunoprecipitations were carried out in the absence (−) or presence (+) of 10 μM $APP^{645-694}$ peptide. Radioactivity in the various APP isoforms was measured using a Molecular Dynamics Phosphorimager. The asterisk (*) in panel C indicates the position of a non-specifically immunoprecipitated protein that co-migrated with $mAPP_{770/751}$ and which also appeared to be phosphorylated in the G_2/M phase. The radioactivity in this band was subtracted to obtain the radioactivity in mAPP. (**D**) FACS analyses of HeLa cells arrested at the G_1 and G_2/M phase. Histograms (abscissa, relative fluorescence; ordinate, cell number) show the relative DNA content of HeLa cells.

$mAPP_{770/751}$ (panel 1), $imAPP_{770/751}$ (panels 2 and 4) as well as APP_{COOH} (panel 3) labeled with [^{32}P]orthophosphate in either intact PC12 or HeLa cells when cdc2 kinase was activated, produced phosphopeptide patterns similar to those derived from peptides $APP^{645-694}$ (panel 5) or $APP^{663-676}$ (panel 6), phosphorylated by cdc2 kinase *in vitro*. These results indicate that Thr668 was phosphorylated in both intact HeLa and PC12 cells.

cdc2 kinase phosphorylates APP at the G_2/M phase of the cell cycle

The following studies were designed to determine whether the activation of cdc2 kinase at the G_2/M phase (Norbury and Nurse, 1992) correlated with the phosphorylation of APP. An antibody prepared against the final six C-terminal amino acid residues of human $p34^{cdc2}$ kinase, which does not cross-react with other members of the cdc2 kinase family (Marshak *et al.*, 1991), was used to immunoprecipitate the kinase from lysates of HeLa cells synchronized either at the G_2/M or G_1 phases. The activity of cdc2 kinase was then measured using either histone H1 (Figure 4A) or $APP^{645-694}$ (Figure 4B) as substrate. Using either substrate, a much higher level of cdc2 kinase activity was detected in immunoprecipitated samples from G_2/M phase cells than in samples immunoprecipitated from G_1 phase cells. Identical results were also obtained using an anti-cdc2 kinase antibody (Z056) raised against a 15 amino acid peptide located near the C-terminal of human $p34^{cdc2}$ kinase (data not shown). The two-dimensional phosphopeptide maps of $APP^{645-694}$, phosphorylated by endogenous cdc2 kinase immunoprecipitated from G_2/M HeLa cells with the Z056 antibody (Figure 3, panel 9), showed an identical pattern to that of $APP^{645-694}$ phosphorylated by purified HeLa cdc2 kinase (Figure 3, panel 5).

To confirm further that the immunoprecipitated cdc2 kinase was responsible for the phosphorylation of $APP^{645-694}$, phosphorylation assays were performed in the presence and absence of the SV40 T antigen peptide (data not shown). The phosphorylation of $APP^{645-694}$ by immunoprecipitated cdc2 kinase was significantly inhibited by a 2-fold molar excess of the SV40 T antigen peptide (20% of control). In APP, the residues 668−671 represent a consensus sequence for phosphorylation by casein kinase II (Kemp and Pearson, 1990). However, an effective peptide substrate for casein kinase II, $DARPP^{95-107}$, (Kurihara *et al.*, 1988; Girault *et al.*, 1989) had no effect on the phosphorylation of $APP^{645-695}$ when present in a 10-fold molar excess (data not shown). Furthermore, purified casein kinase II does not phosphorylate $APP^{645-694}$ (T.Suzuki, unpublished observations) and the activity of casein kinase II is highest in the G_1 phase of the cell cycle (Carroll and Marshak, 1989).

These results strongly suggest that cdc2 kinase, activated at the G_2/M phase of the cell cycle, phosphorylated APP at Thr668. However, another class of protein kinases, the extracellular signal-regulated kinases (ERKs or MAP kinases) also phosphorylate consensus sequences containing serine-proline and threonine-proline motifs (Boulton *et al.*, 1991). MAP kinase was immunoprecipitated from lysates of HeLa cells synchronized either at the G_2/M or G_1 phases. The activity of MAP kinase was then measured using either myelin basic protein (Figure 4C) or $APP^{645-694}$ (Figure 4D) as substrate. Using myelin basic protein as substrate, approximately equal levels of MAP kinase activity

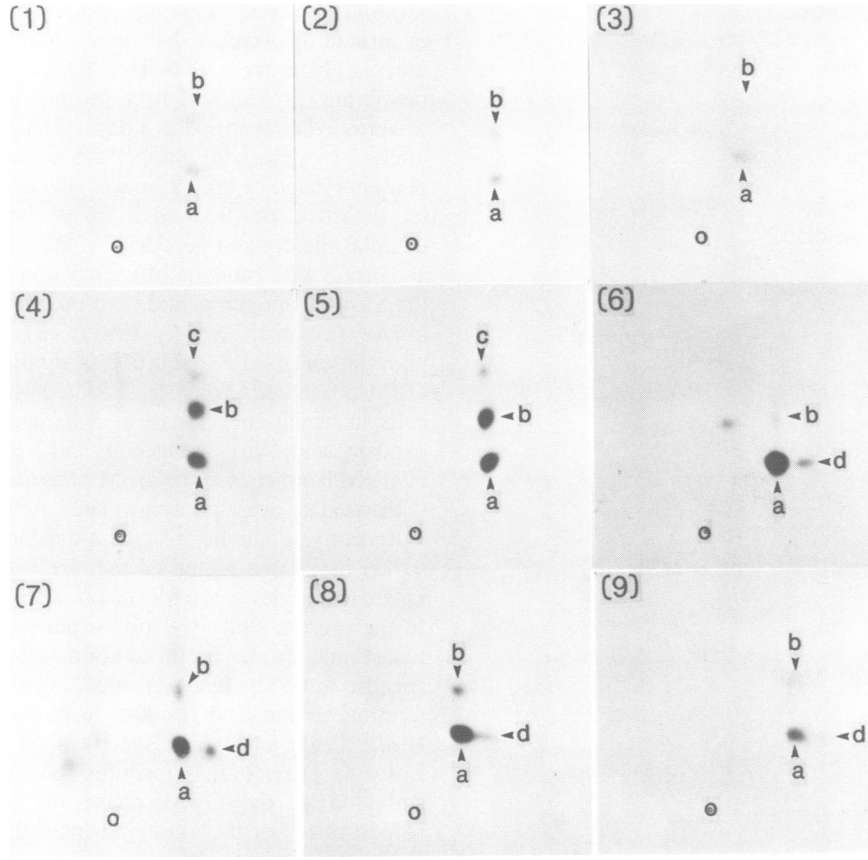

Fig. 3. Two-dimensional phosphopeptide map analysis of synthetic APP peptide and cell-associated forms of APP. ^{32}P-labeled APP and APP_{COOH} were isolated by immunoprecipitation from cells arrested at the G_2/M phase of the cell cycle or from cells treated with okadaic acid. Synthetic peptides were phosphorylated by purified cdc2 kinase. ^{32}P-labeled samples were digested using thermolysin and separated as described in Materials and methods. Phosphopeptides, a–d, contain phospho-Thr668. (**1**) $mAPP_{770/751}$ (PC12, G_2/M), (**2**) $imAPP_{770/751}$ (PC12, G_2/M), (**3**) APP_{COOH} (PC12, G_2/M), (**4**) $imAPP_{770/751}$ (HeLa, G_2/M), (**5**) $APP^{645-694}$ (phosphorylated with purified cdc2 kinase *in vitro*), (**6**) $APP^{663-676}$ (phosphorylated with purified cdc2 kinase *in vitro*), (**7**) $imAPP_{770/751}$ (PC12, okadaic acid), (**8**) APP_{COOH} (PC12, okadaic acid) and (**9**) $APP^{645-694}$ (phosphorylated by immunoprecipitated cdc2 kinase from G_2/M phase HeLa cells).

were found in samples immunoprecipitated from either G_1 or G_2/M phase cells. In contrast, no phosphorylation of $APP^{654-694}$ was detected in either of the immunoprecipitated samples. These results indicated that MAP kinase was not activated in a cell cycle-dependent manner and that this kinase did not phosphorylate APP. In support of this conclusion, we have found that activation of MAP kinase by incubation of HeLa or PC12 cells (G_1 phase) with EGF, and PC12 cells with NGF, did not result in increased phosphorylation of APP at Thr668 (data not shown).

Cell cycle-dependent processing of APP

Previous studies indicated that agents regulating protein kinase and/or protein phosphatase activities increased the normal metabolic processing of APP (Buxbaum *et al.*, 1990; Caporaso *et al.*, 1992b). Therefore, we analyzed the potential effect of phosphorylation of APP by cdc2 kinase on maturation and processing of the protein (Figure 5). In G_2/M phase cells, the content of mAPP (100 ± 7.4%, n = 5) and imAPP (118 ± 14.3%, n = 5) measured by immunoblot analysis did not change significantly compared with the levels found in G_1 phase cells. However, a qualitative change was observed for imAPP at the G_2/M phase. In G_2/M phase cells, imAPP appeared as a doublet on SDS–PAGE, with both bands showing reduced mobility. The level of secreted APP_S decreased (66.7 ± 4.45%, n = 3) and the level of the truncated APP_{COOH} increased (230 ± 49.2%, n = 5) relative to the level of these two fragments at the G_1 phase. The results indicate that cleavage of APP and secretion of APP_S are altered in a cell cycle-dependent manner, and that the qualitative change of imAPP and the accumulation of APP_{COOH} correlate with the enhanced phosphorylation of APP at the G_2/M phase of the cell cycle.

Activation of cdc2 kinase by okadaic acid and phosphorylation of APP

Recently, it was demonstrated that treatment of cells with okadaic acid, a potent inhibitor of protein phosphatases 1 and 2A, results in the activation of cdc2 kinase (Fèlix *et al.*, 1990) through the indirect stimulation of protein phosphatase cdc25 (Kumagai and Dunphy, 1992). This method for activation of cdc2 kinase was used to analyze further the phosphorylation of APP by cdc2 kinase. First, the activation of cdc2 kinase was tested in PC12 cells treated with okadaic acid. Endogenous cdc2 kinase was immunoprecipitated from lysates from control cells or cells treated with okadaic acid and activity was measured using either the SV40 T antigen peptide (data not shown) or $APP^{645-694}$ (Figure 6A) as substrate. Using either substrate, high levels of cdc2 kinase activity ($APD^{645-694}$; 640 ± 61%, n = 2) were found in lysates from PC12 cells incubated with okadaic acid (Figure

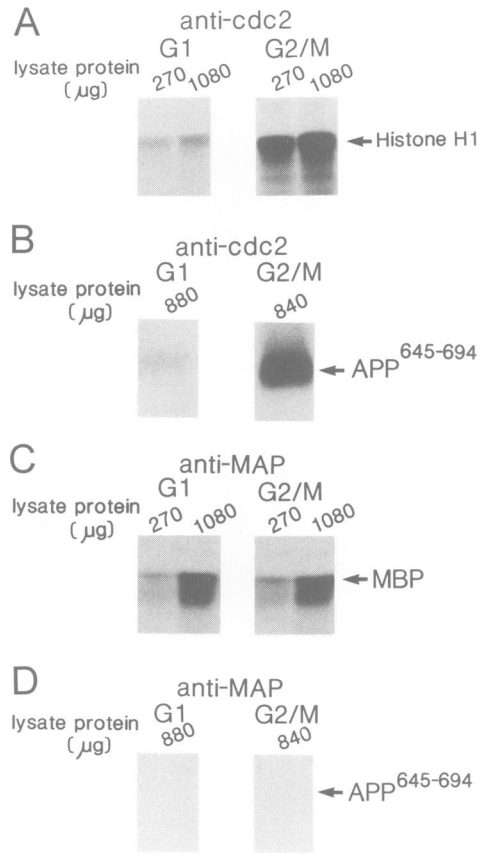

Fig. 4. Cell cycle-dependent activity of cdc2 and MAP kinases. cdc2 kinase (A and B) or MAP kinase (C and D) activities were immunoprecipitated from lysates from HeLa cells treated with (>95% at G_2/M phase) or without (70% at G_1 phase) nocodazole. Histone H1 (5 μg) (**A**) or $APP^{645-694}$ (50 μM) (**B**) was used to measure cdc2 kinase activity. Myelin basic protein (MBP, 5 μg) (**C**) or $APP^{645-694}$ (50 μM) (**D**) was used to measure MAP kinase activity. The amounts of lysate protein from which the kinases were immunoprecipitated are indicated. Phosphorylated proteins and $APP^{645-694}$ recovered by immunoprecipitation were analyzed by autoradiography following SDS–PAGE (15% polyacrylamide).

6A). Treatment of PC12 cells with okadaic acid also resulted in an increase in the phosphorylation of mAPP, imAPP (Figure 6B) and APP_{COOH} (Figure 6C). The imAPP (360 ± 49%, $n = 2$, relative to that in G_1 cells) was found to be phosphorylated to a greater extent than mAPP (198 ± 51%, $n = 2$), consistent with the results observed at G_2/M phase in both HeLa and PC12 cells (Figure 2). Two-dimensional phosphopeptide mapping analyses of samples obtained from okadaic acid-treated PC12 cells (Figure 3) confirmed that Thr668 was the sole phosphorylation site in $imAPP_{770/751}$ (Figure 3, panel 7) and APP_{COOH} (Figure 3, panel 8). The phosphorylation of APP observed under two different conditions, synchrony of cells at G_2/M phase or activation of cdc2 kinase by okadaic acid, supports the contention that APP phosphorylation by cdc2 kinase occurs in intact cells.

Discussion

In the present study we found that APP is phosphorylated at Thr668 by cdc2 kinase *in vitro* and in intact cells. Furthermore, cell cycle-dependent phosphorylation of APP at Thr668 is associated with altered APP metabolism. Other substrates for cdc2 kinase have been identified as cytoskeletal or structural proteins that undergo reorganization during mitosis (Norbury and Nurse, 1992). APP is an integral membrane protein and may be the first of this class of proteins to be identified as a physiological substrate for cdc2 kinase. Evidence to support the conclusion that APP is phosphorylated by cdc2 kinase *in vivo* includes the following: (i) *in vitro*, purified cdc2 kinase phosphorylates APP cytoplasmic domain peptides, $APP^{645-694}$ and $APP^{663-676}$, at Thr668 with catalytic efficiency comparable with that of the SV40 T antigen peptide, an excellent substrate for this kinase (Marshak *et al.*, 1991); (ii) the state of phosphorylation of APP at Thr668 is significantly increased in G_2/M phase cells when cdc2 kinase is most active and in cells in which cdc2 kinase is activated by treatment with okadaic acid; (iii) endogenous cdc2 kinase, immunoprecipitated from cells at the G_2/M phase, or from cells treated with okadaic acid, phosphorylated $APP^{645-694}$ at Thr668.

Recently, a number of cyclin-dependent protein kinases (cdks) have been identified that are highly related to cdc2 kinase (for reviews, see Meyerson *et al.*, 1992; Sherr, 1993). In the present study we used a purified complex of cdc2 kinase and cyclin B. In addition, we also used a highly specific antibody that was made against the final six C-terminal amino acid residues in human $p34^{cdc2}$ (Marshak *et al.*, 1991) and which specifically recognized only this isoform. Therefore, our results indicate that cdc2 kinase activated at the G_2/M phase of the cell cycle was responsible for the observed phosphorylation of APP. However, these results do not rule out the possibility that other cdc2-related kinases in complexes with different cyclins could, under certain circumstances, also phosphorylate APP. Another class of protein kinases, the ERKs or MAP kinases, also phosphorylate consensus sequences containing serine-proline and threonine-proline motifs (Boulton *et al.*, 1991). However, our results indicate that MAP kinases are not regulated in a cell cycle-dependent manner and that they do not appear to phosphorylate APP in intact cells.

An important question raised by the present results concerns the potential function of phosphorylation of APP by cdc2 kinase in the adult nervous system. We have recently found, using phosphorylation state-specific antibodies raised against a synthetic peptide containing phospho-Thr668, that APP isolated from adult rat brain is phosphorylated at Thr668 (M.Oishi, unpublished results). This result does not distinguish whether APP phosphorylation occurs in post-mitotic neurons and/or in glial cells which are capable of cell division. One possibility is that APP is phosphorylated in glial cells, a major source of secretion of Aβ peptide (Busciglio *et al.*, 1993). However, APP may also be phosphorylated in neuronal cells. As discussed above, other cdc2-related protein kinases have been identified and some of these isoforms are highly expressed in the nervous system. For example, the PSSALRE isoform (cdk5), which has 57% identity to cdc2 kinase based on its predicted amino acid sequence, was expressed at highest levels in human adult brain (Meyerson *et al.*, 1992). In addition, cdk5 was found to be expressed at high levels in terminally differentiated neurons which are no longer in the cell cycle (Hellmich *et al.*, 1992). These observations, taken together with the present results, suggest the possibility that members of a family of protein kinases, initially identified as being associated with regulation of the cell cycle, have important functions in adult brain. Recent studies have also shown that

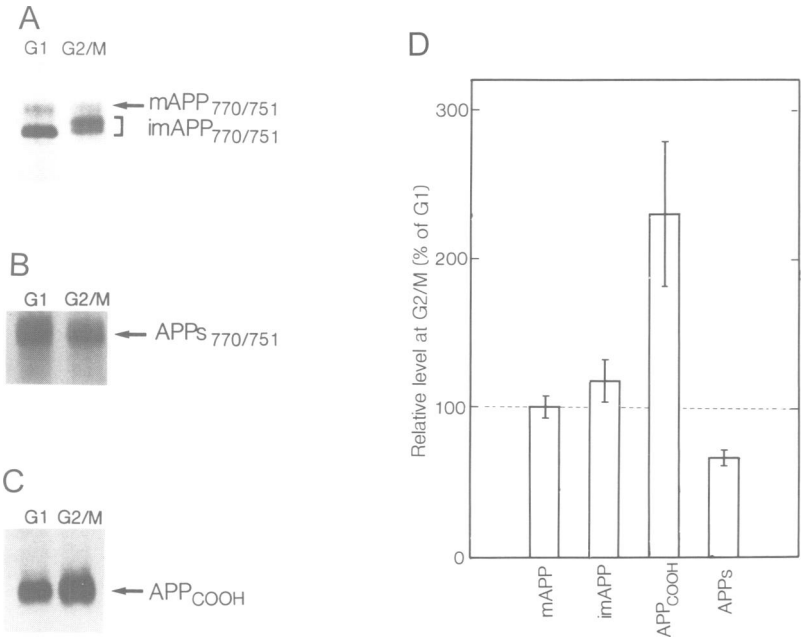

Fig. 5. Cell cycle-dependent metabolism of APP. Immunoblot analysis of the levels of (**A**) mAPP and imAPP, (**B**) APP_S and (**C**) APP_{COOH}. Parallel experiments were performed at the same time as those shown in Figure 2, using the same number (5×10^6 cells) of an identical batch of HeLa cells enriched for G_1 (72% at G_1 phase) and G_2/M (>95% at G_2/M phase). Cells were then cultured in fresh serum-free DMEM instead of phosphate-free DMEM for 2.5 h with (G2/M) or without (G1) nocodazole (1 μg/ml) as described in the legend to Figure 2. Immunoprecipitated protein was resolved by SDS–PAGE using 6% (A and B) or 15% (C) polyacrylamide gels, transferred to PVDF membrane and probed with antibodies G-369 (A and C) or 22C11 (B). (**D**) The levels of mAPP (n = 7), imAPP (n = 7), APP_{COOH} (n = 5) and APP_S (n = 3) were quantitated using a Molecular Dynamics Phosphorimager. The relative ratios of the levels of APP-related proteins in cells at the G_2/M and G_1 phase are indicated.

tau protein (Mawal-Dewan *et al.*, 1992) and neurofilament protein (Hisanaga *et al.*, 1991; Lew *et al.*, 1992) are phosphorylated by cdc2 kinase *in vitro*. Aberrantly phosphorylated tau is a major constituent of the paired helical filaments (PHF) observed in the brains of AD patients (Grundke-Iqbal *et al.*, 1986). Furthermore, neurofilament proteins are specifically expressed in neuronal cells. However, it is not yet known whether cdc2 kinase is involved in the phosphorylation of PHF tau in intact cells, since it appears likely that MAP kinase may be responsible for much of the aberrant phosphorylation of tau (Drewes *et al.*, 1992).

The results presented above suggest that activation of cdc2 kinase and phosphorylation of APP may be involved in the regulation of APP metabolism. In the last several years, a large number of studies have begun to clarify various steps involved in the processing and metabolism of APP, and have suggested that defects in these processes may be associated with the pathogenesis of certain types of AD (for review, see Gandy *et al.*, 1992a). APP is a protein with a receptor-like structure which possesses one transmembrane domain (Figure 1A). During maturation, imAPP (*N*-glycosylated in the endoplasmic reticulum) moves to the Golgi complex, where complete maturation occurs with the protein becoming more highly glycosylated (*N*-, *O*-glycosylation and sulfation) (Figure 7, G1). Normal secretory cleavage of the protein, catalyzed by the so-called 'α-secretase', occurs at a site present within the Aβ domain. Subsequently, the large extracellular N-terminal domain, APP_S, is released into the extracellular milieu ('secretion') and the C-terminal fragment, APP_{COOH}, is internalized and degraded by the endosomal/lysosomal pathway. APP can also be cleaved at an alternative site in the extracellular domain by an alternative secretase, 'β-secretase', giving rise to a longer C-terminal fragment which contains the entire Aβ sequence (reviewed in Gandy *et al.*, 1992a,b). This latter process appears to occur at a low level in all cells and may be elevated in AD (Haass *et al.*, 1992b; Seubert *et al.*, 1992), although the increased production of Aβ has not been found in all types of AD.

Studies from our laboratory have shown that several steps in the processing of APP are subject to regulation by protein phosphorylation (Gandy *et al.*, 1992a). Treatment of cells with phorbol ester, which activates PKC, and/or okadaic acid, which inhibits protein phosphatases 1 and 2A, increases the production of APP_{COOH} and APP_S (Buxbaum *et al.*, 1990; Caporaso *et al.*, 1992b) by a mechanism which may involve either direct phosphorylation of APP (Suzuki *et al.*, 1992) and/or activation of the α-secretase pathway (reviewed in Gandy *et al.*, 1992a). Under these conditions, the increased secretion of APP_S is associated with a concomitant decrease in the production of Aβ (Buxbaum *et al.*, 1993), suggesting that stimulation of the α-secretase pathway by agents that regulate these protein phosphorylation pathways might represent a viable strategy in treatments designed to reduce the production of Aβ. Stimulation of PKC and inhibition of protein phosphatases 1 and/or 2A in intact cells also results in the production of C-terminal fragments of APP which migrate with higher apparent molecular weight on SDS–PAGE (Buxbaum *et al.*, 1990). It is possible that these fragments represent cleavage products that might include the intact Aβ domain. Alternatively, the altered migration of these peptides on SDS–PAGE may be caused by phosphorylation (Suzuki *et al.*, 1992).

The present studies indicate that protein phosphorylation catalyzed by cdc2 kinase may also regulate the processing of APP. At least two distinct effects on APP metabolism are observed at the G_2/M phase of the cell cycle (Figure 7, G2/M). The decrease in the level of APP_S suggests the

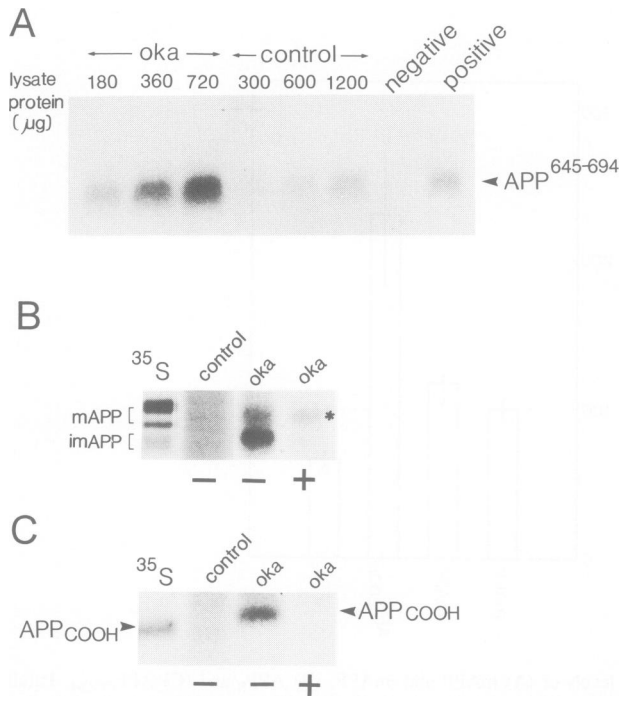

Fig. 6. Activation of cdc2 kinase and phosphorylation of APP and APP_{COOH} in PC12 cells treated with okadaic acid. (**A**) Activity of cdc2 kinase immunoprecipitated from PC12 cells treated with (oka) or without (control) okadaic acid (1 μM). cdc2 kinase activity was measured using $APP^{645-694}$ as substrate as described in the legend to Figure 4. The amount of lysate protein used for cdc2 kinase immunoprecipitation is indicated. Buffer alone (negative) and purified human cdc2 kinase (0.5 μg, positive) were used as controls. Autoradiograms showing (**B**) holo-APP and (**C**) APP_{COOH}. PC12 cells were prelabeled with [^{32}P]orthophosphate in the presence (oka) or absence (control) of 1 μM okadaic acid or prelabeled with [^{35}S]methionine (^{35}S) in the absence of okadaic acid. Immunoprecipitations were carried out in the absence (−) or presence (+) of 10 μM $APP^{645-694}$. Immunoprecipitated samples were analyzed by autoradiography following SDS−PAGE using 7.5% (B) and 15% (C) polyacrylamide gels. Radioactivity in the various APP isoforms was measured using a Molecular Dynamics Phosphorimager. The asterisk (*) in panel B indicates the position of a protein that is non-specifically immunoprecipitated that co-migrated with $mAPP_{770/751}$ and which also appeared to be phosphorylated. The radioactivity in this band was subtracted to obtain the radioactivity in mAPP.

possibility that the activity of the APP α-secretase toward APP could be affected by phosphorylation of the protein in G_2/M phase cells. The decreased level of APP_S would be expected to correlate with a reduction in the level of APP_{COOH}, since both fragments result from cleavage of APP by α-secretase. However, an increase in APP_{COOH} was observed at the G_2/M phase, suggesting that phosphorylation of APP_{COOH} by cdc2 kinase may inhibit the movement of the C-terminal fragment through the endosomal/lysosomal pathway or inhibit its degradation within this pathway. Alternatively, the effects on APP processing observed at the G_2/M phase of the cell cycle may be produced entirely, or in part, by cdc2 kinase-dependent phosphorylation of other interacting components. It has previously been suggested that cdc2 kinase activity is involved in the inhibition of vesicle fusion during membrane trafficking (Tuomikoski *et al.*, 1989; Woodman *et al.*, 1992, 1993). Also, the subcellular localization of rab4, a protein believed to be important in vesicle trafficking, is regulated upon phosphorylation by cdc2 kinase (Sluijs *et al.*, 1992).

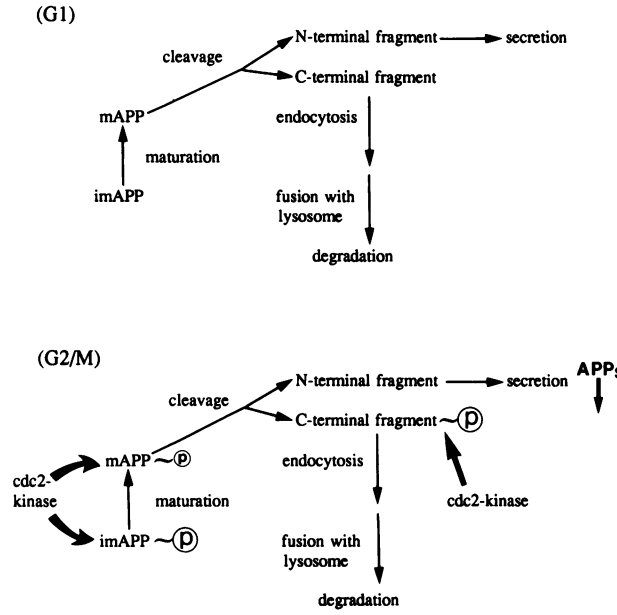

Fig. 7. Cell cycle-dependent regulation of APP metabolism by cdc2 kinase. Direct phosphorylation of APP by cdc2 kinase during G_2/M phase may alter the pathways involved in the maturation of APP and the degradation of APP_{COOH}. See text for further discussion.

The molecular mechanism(s) of Aβ production remains unclear, and alternative pathways for the processing of APP to Aβ have been proposed (Caporaso *et al.*, 1992a; Estus *et al.*, 1992; Golde *et al.*, 1992; Haass *et al.*, 1992a; Shoji *et al.*, 1992). In the present study, an increased accumulation of APP_{COOH} was observed during the mitotic phase of normally dividing cells. This result raises the possibility that activation of cdc2 kinase may be associated with altered production of potentially amyloidogenic fragments containing the entire Aβ domain. Such a regulatory mechanism may be important in the brain since it has recently been demonstrated that glial cells are a major source of Aβ (Busciglio *et al.*, 1993). In addition, as discussed above, cdc2-related kinase(s) may also be active in post-mitotic neurons.

APP isoforms are ubiquitously expressed in cells (Marotta *et al.*, 1992) and cell cycle-regulated phosphorylation of these proteins may be important in modulating their normal physiological function. A recent study has suggested that a region of the cytoplasmic domain of APP may interact with the GTP binding protein G_0 (Nishimoto *et al.*, 1993), although these results require confirmation. In normal cells, binding of an unknown ligand to the extracellular domain of APP may achieve its physiological effects by modifying intracellular second messenger pathways via an interaction with G_0 (Hanley and Selkoe, 1993). The site of phosphorylation of APP by cdc2 kinase (Thr668) is within the proposed domain of interaction of APP with G_0, suggesting the possibility that phosphorylation could modulate this process.

In conclusion, the present results have demonstrated the cell cycle-dependent phosphorylation of APP by cdc2 kinase. This mechanism may be involved in the normal functioning of mAPP or in various aspects of its processing or metabolism in normal dividing cells in the brain as well as in non-nervous tissue. Phosphorylation of APP by cdc2-related kinase(s) may also play a role in regulation of the function or metabolism of APP in neurons. Alterations

in the activity of one or more members of the cdc2 kinase family in any of these cell types may have important ramifications in terms of the altered processing of APP that is believed to be important in the pathogenesis of AD.

Materials and methods

Synthetic peptides

The peptide, ADAQHATPPKKKRKVEDPKDF, synthesized as a different batch, has an identical amino acid sequence to CSH103 (Marshak *et al.*, 1991) derived from the sequence of the large tumor antigen of SV40 and was used as a specific substrate for cdc2 kinase. $APP^{645-694}$ and $APP^{663-676}$ (Figure 1A) were synthesized at the W.M.Keck Foundation Biotechnology Resource Laboratory (Yale University) and the Protein Sequencing/Biopolymer Facility (The Rockefeller University) respectively. Peptide identity was confirmed by amino acid analysis and mass spectroscopy. Histone H1, myelin basic protein and nocodozole were obtained from Sigma.

Antibodies

Polyclonal antibody G-369 was prepared against the cytoplasmic domain peptide $APP^{645-694}$ (Buxbaum *et al.*, 1990). Anti-cdc2 kinase antibody was raised against a synthetic peptide corresponding to the final six C-terminal amino acid residues of human $p34^{cdc2}$ (Marshak *et al.*, 1991). Anti-cdc2 kinase monoclonal antibody, Z056, raised against a 15 amino acid peptide located near the C-terminus of human $p34^{cdc2}$ kinase and anti-MAP kinase monoclonal antibody, Z033, were obtained from Zymed Lab Inc. (South San Francisco, CA). Monoclonal antibody 22C11 was raised against the N-terminus of APP (Weidemann *et al.*, 1989). Anti-mouse IgG antibody (M-7023) was purchased from Sigma.

Phosphorylation assays

cdc2 kinase (as part of a cyclin B complex) was purified from HeLa cells (Brizuela *et al.*, 1989; Marshak *et al.*, 1991). Synthetic peptides and enzymes were incubated in a reaction volume of 50 μl containing (final concentration): 50 mM Tris−acetate (pH 8.0), 10 mM magnesium acetate, 1 mM EDTA and 1 mM 2-mercaptoethanol. Reactions were initiated by addition of 0.1 mM [γ-^{32}P]ATP (4 Ci/mmol). The reactions were terminated by the addition of 450 μl of 30% acetic acid containing bovine serum albumin (2 mg/ml). The phosphorylated peptides were then separated from free ATP by sequential chromatography using two AG1-X8 resin (Bio-Rad) columns (2 ml bed volume) equilibrated in 30% (v/v) acetic acid (Kemp *et al.*, 1976). Recovered samples were dried, dissolved in 60 μl of SDS sample buffer [50 mM Tris−HCl (pH 6.8), 12.5 mM EDTA, 3.75% (w/v) SDS, 10% (v/v) glycerol, 4 M urea, 2.5% (v/v) 2-mercaptoethanol and 0.015% (w/v) bromophenol blue], boiled for 5 min, then subjected to SDS−PAGE (final acrylamide concentration of 15% for $APP^{645-694}$; 17.5% for the SV40 T antigen peptide). Phosphorylated samples were recovered as dried gel pieces and incorporation of phosphate was measured by scintillation counting. The apparent K_m and V_{max} values were determined from double reciprocal plots and the catalytic efficiency, k_{cat}/K_m, was calculated.

Prelabeling of cells and immunoprecipitation of APP

PC12 cells ($2-3 \times 10^6$ cells) were grown in complete Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated fetal calf serum (FCS) and 5% (v/v) heat-inactivated horse serum. HeLa cells (5×10^6 cells) were grown in complete DMEM containing 10% (v/v) heat-inactivated FCS. Cells were subjected to serum starvation in DMEM containing 0.5% (v/v) FCS (limited DMEM) for 28 h. G_1 phase cells were further cultured in fresh limited DMEM for 12 h and G_2/M phase cells were further cultured in complete DMEM containing nocodazole (1 μg/ml) for 12 h. Cells were labeled for 2.5 h at 37°C with [^{32}P]orthophosphate (1 mCi/ml) or [^{35}S]methionine (0.5 mCi/ml) in 3 ml of phosphate- or methionine-free DMEM, respectively, in the presence (G_2/M) or absence (G_1) of nocodazole (1 μg/ml). In separate experiments, PC12 cells ($2-3 \times 10^6$ cells) were prelabeled with [^{32}P]orthophosphate or [^{35}S]methionine in the presence or absence of okadaic acid (1 μM) for 2.5 h at 37°C. Prelabeled cells were recovered by pipetting from the plate, washed with Dulbecco's phosphate-buffered saline (DPBS) and subjected to centrifugation at 800 *g* for 5 min. The cells were then lysed in buffer containing (final concentration): 120 μl of 50 mM Tris−acetate (pH 8.0), 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 1% (v/v) Triton X-100, 0.1% (v/v) 2-mercaptoethanol, 2 μM okadaic acid, 200 μg/ml (w/v) pepstatin A, 200 μg/ml (w/v) chymostatin and 200 μg/ml (w/v) leupeptin for 30 min on ice with occasional vortexing. Samples were centrifuged (10 000 *g* for 5 min), and then 100 μl of a solution containing 0.1 M Tris−HCl (pH 7.4), 2.2% (w/v) SDS, 5.44 M urea were added to the supernatant (120 μl) and the mixture was boiled. Samples were centrifuged (10 000 *g* for 5 min) and APP in the supernatant (200 μl) was immunoprecipitated using antibody G-369 as described (Suzuki *et al.*, 1992). Control immunoprecipitations were carried out in the presence of excess peptide, $APP^{645-694}$ (10 μM). Separation of immunoprecipitated samples using 7.5% or 6% polyacrylamide gels was performed as described (Laemmli, 1970) and separation of samples using 15% polyacrylamide gels was performed using a modified version of the Laemmli procedure (Suzuki *et al.*, 1992).

cdc2 and MAP kinase activities in cell lysates

HeLa cells (2×10^7 cells) incubated with nocodazole (1 μg/ml) ('G_2/M'; >95% at G_2/M phase) or control cells ('G_1'; 70% at G_1 phase) were prepared as described above. In separate experiments, PC12 cells ($4-5 \times 10^6$ cells) were incubated with or without okadaic acid (1 μM) for 2.5 h at 37°C. The cells were lysed in 350 μl of extraction buffer [50 mM Tris−HCl (pH 7.4), 0.25 M NaCl, 0.1% (v/v) Nonidet P-40, 5 mM EDTA, 50 mM NaF, 50 μg/ml (w/v) pepstatin A, 50 μg/ml (w/v) chymostatin and 50 μg/ml (w/v) leupeptin] for 30 min on ice and centrifuged (10 000 *g* for 10 min at 4°C). The supernatants were incubated with either cdc2 kinase (5 μl of rabbit serum or 5 μg of Z056) or MAP kinase antibodies (4 μg) for 1 h on ice and endogenous cdc2 kinase−antibody complex was recovered by addition of protein A−Sepharose (Pharmacia) or agarose-linked anti-mouse IgG (HyClone, EGK-1060). The beads were washed twice with extraction buffer and twice with cdc2 kinase reaction buffer as described above or with MAP kinase reaction buffer [40 mM HEPES (pH 7.4), 3 mM $MgCl_2$, 5 mM EGTA and 2 mM 2-mercaptoethanol]. The endogenous kinase−antibody complexes coupled to resin were incubated with 5 μg of histone H1 or myelin basic protein, or with 50 μM $APP^{645-694}$ peptide for 30 min at 37 °C as described above. After centrifugation (10 000 *g* for 5 min), the supernatant was boiled in 0.1 M Tris−HCl (pH 7.4), 2.2% (w/v) SDS and 5.44 M urea. Histone H1 and myelin basic protein were separated directly by SDS−PAGE using 15% polyacrylamide. $APP^{645-694}$ was immunoprecipitated using G-369 antibody and samples were separated by SDS−PAGE using 15% polyacrylamide gels. The incorporation of radioactivity was determined by autoradiography.

FACS analysis

HeLa cells (1×10^6 cells) were fixed with 50% methanol and DNA was stained by stain solution [50 μg/ml (w/v) propidium iodide, 100 U/ml RNase A and 0.1% (w/v) glucose/DPBS]. The cells were then analyzed on a Becton Dickinson FACScan Flow Cytometer.

Two-dimensional phosphopeptide analysis

Gel pieces containing ^{32}P-labeled $APP_{770/751}$, APP_{COOH}, $APP^{645-694}$ and $APP^{663-676}$ were reswollen and digested with thermolysin (0.3 mg/ml) as previously described (Suzuki *et al.*, 1992). Phosphopeptides were applied to thin layer chromatography (TLC) plates (O, origin) and separated by electrophoresis at pH 3.5 in the first dimension (positive, left), followed by chromatography in the second dimension, then analyzed by autoradiography.

Phosphoamino acid analysis

Synthetic peptides (100 μM) were phosphorylated as described above. Gel pieces containing ^{32}P-labeled $APP^{645-694}$ and $APP^{663-676}$ were reswollen, digested with thermolysin (0.3 mg/ml) and then hydrolyzed in 6 M HCl as described previously (Suzuki *et al.*, 1992). The resulting samples were separated on TLC plates by electrophoresis. The TLC plates were stained with ninhydrin and analyzed by autoradiography.

Immunoblot analysis

Proteins separated by SDS−PAGE were electroblotted onto PVDF membrane (Immobilon-P, Millipore) in a buffer containing 10 mM CAPS (pH 11)/10% methanol. Membranes were probed with antibody (G-369) and [^{125}I]protein A (Amersham), or with antibodies (Z056 or 22C11), anti-mouse IgG antibody and [^{125}I]protein A.

Measurement of APP, APP_{COOH} and APP_S

HeLa cells were lysed and mAPP, imAPP and APP_{COOH} were immunoprecipitated using G-369 antibody as described above. Protein in the cell culture medium was precipitated by addition of trichloroacetic acid [final conconcentration 10% (w/v)], the pellet washed twice with cold acetone, dried and resuspended in 100 μl of 0.1 M Tris−HCl (pH 7.4), 2.2% (w/v) SDS and 5.44 M urea. Samples were diluted by addition of 120 μl of lysis buffer, boiled (5 min), centrifuged (10 000 *g* for 5 min), and the supernatant (200 μl) was recovered. APP_S was immunoprecipitated

from this supernatant using 22C11 antibody plus agarose resin-linked anti-mouse IgG antibody (HyClone). Immunoprecipitated samples were separated with SDS–PAGE, electroblotted onto PVDF membrane and analyzed by immunoblotting.

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