Phosphorylation of Alzheimer β-Amyloid Precursor-like Proteins†

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ABSTRACT: Amyloid precursor-like proteins (APLPs), APLP1 and APLP2, are members of a gene family which include the Alzheimer β-amyloid precursor protein (APP). APLP1, APLP2, and APP contain highly homologous amino acid sequences, especially in their cytoplasmic domains, although APLPs lack the β-amyloid domain derived by proteolytic processing from APP. APP is phosphorylated at three sites in the cytoplasmic domain in cultured cells and adult rat brain [Suzuki et al. (1994) *EMBO J. 13*, 1114−1122; Oishi, et al. (1997) *Mol. Med. 3*, 109−121] and at sites in the extracellular domain in cultured cells [Knops et al. (1993) *Biochem. Biophys. Res. Commun. 197*, 380−385; Hung & Selkoe (1994) *EMBO J. 13*, 534−542; Walter et al. (1997) *J. Biol. Chem. 272*, 1896−1903]. We report here that a cytoplasmic domain peptide from APLP1 is phosphorylated *in vitro* by protein kinase C and that a cytoplasmic domain peptide from APLP2 is phosphorylated *in vitro* by protein kinase C and cdc2 kinase. APLP2 is phosphorylated by cdc2 kinase at a site homologous to the cdc2 kinase site phosphorylated in APP. Furthermore, phosphorylation of this site occurs in a cell cycle-dependent manner in cultured cells. These findings indicate that in intact cells the phosphorylation of APLP2 appears to be regulated in a similar fashion to that of APP.

The principal protein component of extracellular cerebral amyloid deposits in patients with Alzheimer's disease (AD) is the β-amyloid peptide (Aβ) which is derived from a large precursor protein, the Alzheimer β-amyloid precursor protein (APP) (Goldgaber et al., 1987; Kang et al., 1987; Robakis et al., 1987; Tanzi et al., 1987). APP is an integral membrane glycoprotein with a receptor-like structure, existing as several distinct isoforms (Goldgaber et al., 1987; Tanzi et al., 1988; Ponte et al., 1988; Kitaguchi et al., 1988; De Sauvage & Octave, 1989). Recent evidence indicates that APP is a member of a gene family encoding membrane proteins that are remarkably conserved (Wasco et al., 1992, 1993; Sprecher et al., 1993; Slunt et al., 1994). APP-like genes have been isolated from various mammalian species (Wasco et al., 1992, 1993), from fly (Rosen et al., 1989), and from nematode (Daigle & Li, 1993). In APP, three phosphorylation sites (Thr654, Ser655, and Thr668) in the cytoplasmic domain have been identified *in vitro* for protein kinase C (PKC), calcium/calmodulin-dependent protein kinase II (CaMkII), and cdc2 kinase, respectively (Gandy et al., 1988; Suzuki et al., 1992, 1994). Thr668 of APP has been shown to be phosphorylated at the G2/M phase of the cell cycle by cdc2 kinase (Suzuki et al., 1994), and Thr654 and Ser655 have been found to be phosphorylated in adult brain tissue by unidentified protein kinases (Oishi et al., 1997). Amino acid sequence analysis suggests that the cytoplasmic domains of the APP-like proteins (APLPs) contain potential phosphorylation sites similar to those identified in APP. In the present study, we demonstrate directly that the cytoplasmic domains of APLP1 and APLP2 are both phosphorylated. A peptide encompassing the cytoplasmic domain of APLP1 was found to be efficiently phosphorylated by PKC but to be phosphorylated to a lesser extent by CaMkII or cdc2 kinase. A peptide encompassing the cytoplasmic domain of APLP2 was found to be efficiently phosphorylated by PKC and cdc2 kinase but to be phosphorylated to a lesser extent by CaMkII. Notably, APLP2 is phosphorylated at Thr736 (numbering for $APLP2_{763}$ isoform) at the G2/M phase of the cell cycle. This site corresponds to Thr668 (numbering for APP_{695} isoform) of APP, the site of APP phosphorylation in G2/M phase cells (Suzuki et al., 1994) and in brain tissues (Oishi et al., 1997; Satoh et al., manuscript in preparation). Characterization of the phosphorylation state of both APP and the APLPs will help to clarify the biological function(s) of the proteins which is (are) presently unknown. Further characterization of the phosphorylation of APP and the APLPs may also be relevant to understanding the pathogenesis of AD.

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MATERIALS AND METHODS

Materials. Fetal bovine serum (FBS) was a product of Iansa (Mexico). Horse serum (HS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (Gaithersburg, MD). Phorbol 12,13-dibutyrate (PDBu) was purchased from Calbiochem-Novabiochem Co. (San Diego, CA), and aphidicolin and nocodazole were purchased from Sigma (St. Louis, MO).

Synthetic Peptides. $APP^{645-694}$ (corresponding to residues 645−694 of the cytoplasmic domain of APP_{695}), $APLP1^{608-653}$ (corresponding to residues 608−653 of the cytoplasmic domain of $APLP1_{653}$), $APLP2^{717-763}$ (corresponding to residues 717−763 of the cytoplasmic domain of $APLP2_{763}$), and the shorter peptides $APLP1^{616-637}$ and $APLP2^{725-746}$ were synthesized at the W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University). $APLP2^{732-740}$[Cys] phosphorylated at Thr736 was synthesized at Quality Controlled Biochemicals Inc. (Hopkinton, MA). [Cys]$APLP2^{744-763}$ and [Cys]$APP^{676-695}$ were synthesized using a Shimadzu peptide synthesizer, PSSM-8 (Kyoto, Japan).

Phosphorylation Assays. PKC and CaMkII were purified from rat brain as previously described (Gandy et al., 1988; Suzuki et al., 1992). Recombinant cdc2 kinase/cyclin B complex was purchased from New England BioLabs (Beverly, MA). The substrate peptide (40 μM) was incubated with PKC (1 μg) in a reaction volume of 50 μL containing (final concentration): 50 mM HEPES, pH 7.4/1 mM EGTA/2 mM $MgCl_2$/1 mM $CaCl_2$/1 mM ATP and PDBu (1 μM) or incubated with CaMkII (1 μg) in the above reaction buffer containing bovine calmodulin (20 μg/mL) instead of PDBu. The peptide (40 μM or the amounts indicated in Figure 3) was incubated with cdc2 kinase/cyclin B (0.13 μg in Figure 2a and 0.67 μg in Figure 3) in a reaction volume of 50 μL containing (final concentration): 50 mM Tris-acetate, pH 8.0/10 mM magnesium acetate/1 mM EDTA/1 mM 2-mercaptoethanol. Reactions were initiated by addition of 0.1 mM [γ-^{32}P]ATP (4 Ci/mmol) and terminated by addition of 50 μL of 30% (v/v) 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 columns (Bio-Rad, Hercules, CA) (2 mL bed vol) equilibrated in 30% (v/v) acetic acid. Shorter peptides were dried, and incorporation of ^{32}P into peptide was measured by liquid scintillation spectrometry. In identical studies, the cytoplasmic domain peptides 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, and then subjected to SDS−PAGE [15% (w/v) acrylamide gel]. Phosphorylated samples were recovered as dried gel pieces, and incorporation of ^{32}P into peptide was measured by scintillation spectrometry. The apparent K_m and V_{max} values were determined from double-reciprocal plots, and the catalytic efficiency, k_{cat}/K_m, was calculated.

Phosphoamino Acid Analysis. Dried gel pieces containing ^{32}P-labeled peptides were reswollen, digested with thermolysin (0.1 mg/mL), and then hydrolyzed in 6 M HCl as described (Suzuki et al., 1992). The resulting digests were separated on thin layer chromatography (TLC) plates by electrophoresis. The TLC plates were stained with ninhydrin and analyzed by autoradiography.

Production and Specificity of Antibodies. Polyclonal antibodies, AbAPLP2 (UT-422 and UT-424), were prepared against the peptide [Cys]$APLP2^{744-763}$ and affinity purified using antigen peptide coupled to SulfoLink gel (Pierce, Rockford, IL). The affinity-purified antibodies were further purified using a column of [Cys]$APP^{676-695}$ coupled to SulfoLink gel. The flow-through fractions for either UT-422 or UT-424 were specific for the APLP2 cytoplasmic peptide $APLP2^{717-763}$ and for the holoAPLP2 molecule. The antibodies did not cross-react with the APP cytoplasmic peptide $APP^{645-694}$ or holoAPP molecules at a concentration of 1 μg/mL purified IgG (Satoh et al., manuscript in preparation). A polyclonal phosphorylation state-specific antibody, pAbThr736 (UT-425), was raised against a chemically phosphorylated synthetic peptide antigen corresponding to $APLP2^{732-740}$[Cys][PiThr736] and affinity purified using antigen peptide coupled to SulfoLink gel. UT-425 was specific for the APLP2 cytoplasmic peptide $APLP2^{717-763}$ phosphorylated at Thr736 with cdc2 kinase and did not react with dephosphorylated APLP2, APP cytoplasmic peptides, or an APP cytoplasmic peptide phosphorylated at Thr668 at a concentration of 1 μg/mL purified IgG. AbAPLP2 and pAbThr736 could both detect as little as 0.002 nmol of antigen as determined using dot blot analysis and standard amounts of antigens.

Analysis of APLP2 Phosphorylation in HeLa Cells and Rat Glioma C6 Cells. HeLa cells ($2-3 \times 10^6$ cells) were grown in DMEM containing 10% (v/v) heat-inactivated FBS (complete DMEM); C6 cells were grown in complete DMEM supplemented with 5% (v/v) heat-inactivated HS. Cells were subjected to serum starvation in DMEM containing 0.5% FBS (v/v) (limited DMEM) for 24 h. G1 phase cells were prepared by further culture in fresh limited DMEM for 12 h. For S phase synchronization, cells were cultured in complete DMEM (HeLa) or complete DMEM supplemented with 5% (v/v) heat-inactivated HS (C6) containing aphidicolin (5 μg/mL). For G2/M phase synchronization, nocodazole (1 μg/mL) replaced aphidicolin. When HeLa cells were so treated, over 90% of cells were synchronized, respectively, in S phase, over 90% of cells were synchronized, respectively, in G2/M phase, and approximately 70% of cells were synchronized in G1 phase, as previously described (Suzuki et al., 1994; Oishi et al., 1997). C6 cells were not analyzed for cell cycle stages because these cells are adhesive and form aggregates, thus preventing the identification of individual cell phases. Synchronization of C6 cells was confirmed by the fact that APP is phosphorylated at G2/M phase and APP phosphorylation is negligible at G1 and S phases (data not shown; Suzuki et al., 1994; Oishi et al., 1997).

APLP2 was immunoprecipitated from cell lysates using AbAPLP2 and protein A-Sepharose (Pharmacia, Uppsala, Sweden) as described (Suzuki et al., 1994; Oishi et al., 1997). Immunoprecipitates were analyzed by SDS−PAGE [6% (w/v) polyacrylamide gel] and electrophoretically transferred to nitrocellulose membranes. Membranes were probed with AbAPLP2 and pAbThr736 antibodies followed by [^{125}I]-proteinA (Amersham, Buckinghamshire, U.K.). Radioactivity on immunoblots was quantitated using a Fuji BAS 2000 imaging analyzer (Tokyo, Japan) or by autoradiography.

(a) Potential PKC phosphorylation site

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                 655
APP  :   -KKKQYTSIHHG-
                 614
APLP1 :  -KKKPYGTISHG-
                 723
APLP2 :  -RKRQYGTISHG-
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(b) Identified cdc2 kinase phosphorylation site

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                 668
APP  :   -DAAVTPEERHL-
                 736
APLP2 :  -DPMLTPEERHL-
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FIGURE 1: Phosphorylation sites in the cytoplasmic domain of APP and APLPs. (a) The site phosphorylated by PKC in APP has been previously identified as Ser655 (Gandy et al., 1988; Suzuki et al., 1992). The sites in APLP1 and APLP2 are predicted based on their conserved location and from the *in vitro* phosphorylation results obtained in the present study. (b) The site phosphorylated by cdc2 kinase in APP has been previously identified as Thr668 (Suzuki et al., 1994; Oishi et al., 1997). The site in APLP2 phosphorylated by cdc2 kinase is based on its conserved location and from the *in vitro* and intact cell results in the present study.

Treatment of APLP2 with Chondroitinase. The APLP2−AbAPLP2 complex was recovered from HeLa or C6 cell lysates following addition of protein A-Sepharose. The beads were washed twice with a buffer containing 100 mM Tris-HCl (pH 8.0)/30 mM sodium acetate and then incubated with 0.2 unit of protease free chondroitinase ABC (Seikagaku Co., Tokyo, Japan), 200 μg/mL (w/v) pepstatin A, 200 μg/mL (w/v) chymostatin, 200 μg/mL (w/v) leupeptin, and 1 μM microcystin-LR (Calbio Chem, La Jolla, CA) in the same buffer for 1 h at 37 °C under agitation. After incubation, the antigen-antibody complexes were washed as described (Suzuki et al., 1994), treated with SDS sample buffer, and separated with SDS−PAGE [6% (w/v) polyacrylamide gel]. Samples were electroblotted onto nitrocellulose membranes and analyzed by immunoblotting using AbAPLP2 and pAbThr736.

RESULTS

Phosphorylation of the Cytoplasmic Domains of APP, APLP1, and APLP2 in Vitro. The phosphorylation of APLP1 and APLP2 has not previously been determined. However, comparison of the amino acid sequences of APP, APLP1, and APLP2 indicated that the cytoplasmic domains of APLP1 and APLP2 contain potential phosphorylation sites for the protein kinases known to phosphorylate APP (Figure 1). Thr614 in APLP1 and Thr723 in APLP2 are located in an analogous position to Ser655 in APP and are potential sites for PKC (Figure 1). Furthermore, Thr736 in APLP2, which is located in a position analogous to Thr668 in APP, is a candidate site for phosphorylation by cdc2 kinase. The NPTY sequence of APLP1, APLP2, and APP does not represent a consensus amino acid sequence for any known threonine protein kinase (Kemp & Pearson, 1990).

Synthetic peptides corresponding to the cytoplasmic domain of APLPs were incubated with cdc2 kinase, CaMkII, or PKC and [γ-^{32}P]ATP under conditions in which incorporation of ^{32}P into peptide (40 μM) was linear for at least 1 min. The phosphorylation levels of APLP1 and APLP2

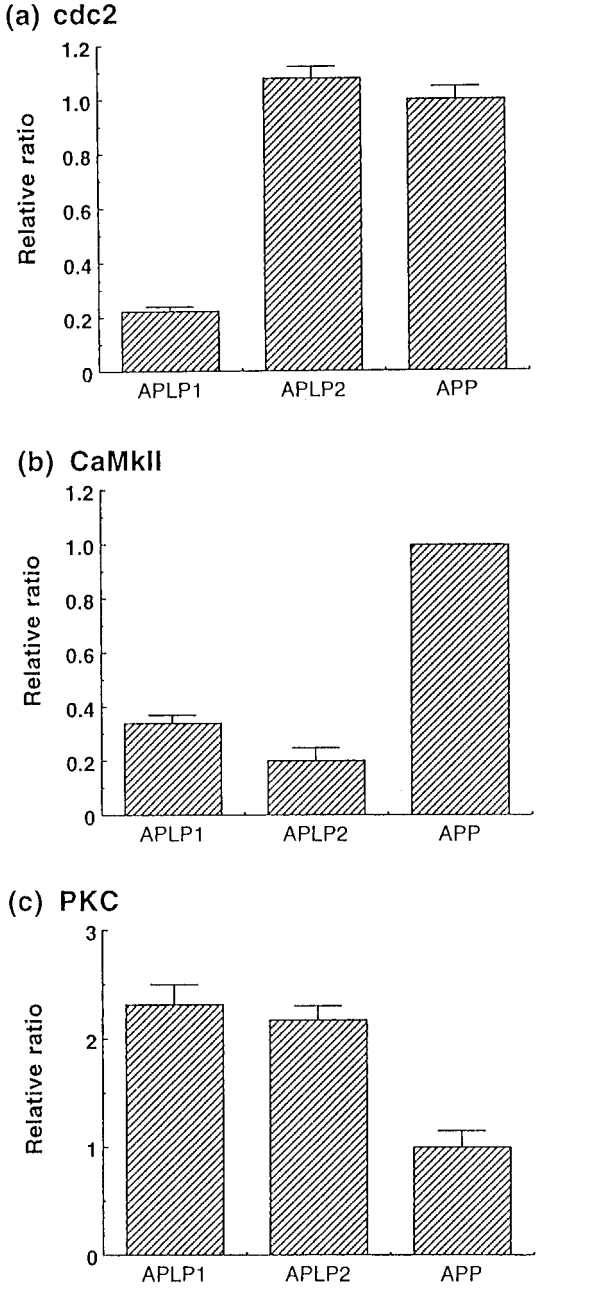

FIGURE 2: Phosphorylation of cytoplasmic domain peptides from APLP1, APLP2, and APP *in vitro*. Peptides were phosphorylated with (a) cdc2 kinase, (b) CaMkII, or (c) PKC. The relative ratios of the levels of phosphorylation in APLP1$^{608-653}$ and APLP2$^{717-763}$ peptides are indicated relative to APP$^{645-694}$, which was assigned a reference value of 1.0. Values are averages from duplicate studies, and error bars are indicated.

peptides were estimated relative to the level of phosphorylation of APP peptide (Figure 2). cdc2 kinase phosphorylated APLP2$^{717-763}$ at a rate similar to that for phosphorylation of APP$^{645-694}$ but phosphorylated APLP1$^{608-653}$ less efficiently (approximately 20% of the rates of APP and APLP2 peptides) (Figure 2a). CaMkII phosphorylated the peptides from either APLP1 or APLP2 at 20−30% of the rate observed for the APP peptide (Figure 2b). PKC phosphorylated the APLP1 and APLP2 peptides at rates more than 2-fold higher than that for the APP peptide (Figure 2c). The catalytic efficiencies for phosphorylation of APLP2$^{717-763}$ and APP$^{645-694}$ peptides by cdc2 kinase were also determined and shown to be similar (Figure 3 and Table 1). The peptide APLP2$^{725-746}$, but not APLP1$^{616-637}$, was phosphorylated by

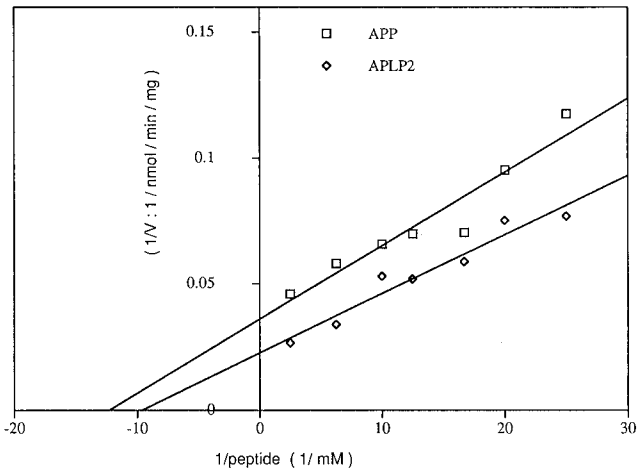

FIGURE 3: Kinetic analysis of phosphorylation of the cytoplasmic domain peptides from APLP2 and APP by p34^{cdc2} protein kinase. APP$^{645-694}$ and APLP2$^{717-763}$ were phosphorylated using p34^{cdc2} protein kinase (molecular weight = 100 kDa as a complex with cyclin B; 0.67 μg) for 1 min at 37 °C. The apparent K_m and V_{max} values, determined from double-reciprocal plots, and the catalytic efficiency, k_{cat}/K_m, were calculated and are presented in Table 1.

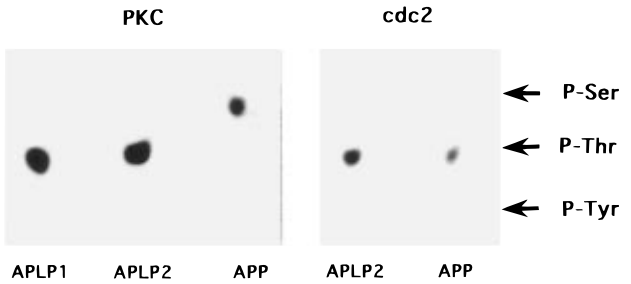

FIGURE 4: Phosphoamino acid analysis of the cytoplasmic domain peptide from APLP1, APLP2, and APP, phosphorylated by PKC and/or cdc2 kinase *in vitro*. The autoradiogram shows the position of phosphoamino acids. Standards (arrows) were detected by ninhydrin stain (data not shown). P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

Table 1: Phosphorylation of Cytoplasmic Domain Peptides of APP and APLP2 by p34^{cdc2} Protein Kinase

substrate	K_m (μM)	V_{max} (nmol/min/mg)	catalytic efficiency (μM-1/sec)
APP$^{645-694}$	80	27.7	0.22
APLP2$^{717-764}$	104	44.4	0.27

cdc2 kinase (data not shown). Shorter peptides, APLP1$^{616-637}$ and APLP2$^{725-746}$, which contain other potential phosphorylated threonyl residues were not phosphorylated by PKC (data not shown).

To identify the amino acid residues phosphorylated by cdc2 kinase or PKC, the phosphorylated cytoplasmic domain peptides from APP, APLP1, and APLP2 were subjected to phosphoamino acid analysis (Figure 4). PKC phosphorylated serine in APP (Ser655) as described previously (Gandy et al., 1988; Suzuki et al., 1992) and phosphorylated only threonine in the APLP1 and APLP2 peptides. Threonine was phosphorylated by cdc2 kinase in both the APP and APLP2 peptides. APLP2$^{725-746}$ contains only a single threonyl residue, Thr736. To confirm that Thr736 is the site in APLP2 phosphorylated by cdc2 kinase, and to assist in the studies described below, a phosphorylation state-specific antibody was raised to phospho-Thr736 (pAbThr736). pAbThr736 recognized APLP2$^{717-763}$ that had been phosphorylated by cdc2 kinase and did not recognize APP phosphorylated at Thr668 (data not shown), clearly indicating that Thr736 is the site of phosphorylation by cdc2 kinase. Together the *in vitro* data indicate that Thr614 of APLP1 and Thr723 of APLP2 are phosphorylated by PKC and that Thr736 of APLP2 is phosphorylated by cdc2 kinase. The results raise the possibility that APLP2 is a potential substrate for cdc2 kinase and that APLP1 and APLP2 are potential substrates for PKC. The lower efficiency of phosphorylation suggests that these substrates are less likely to be potential substrates for CaMkII.

Phosphorylation of APLP2 in Intact Cells. The *in vitro* studies utilizing purified protein kinases and cytoplasmic domain peptides from APP, APLP1, and APLP2 indicate that APLP2 is a potential substrate for cdc2 kinase and APLP1 and APLP2 are potential substrates for PKC. If APLP2 is a physiological substrate for cdc2 kinase *in vivo*, then APLP2 should be phosphorylated in a cell cycle-dependent manner at the G2/M phase where cdc2 kinase is most active. HeLa cells and rat C6 glioma cells at different stages of the cell cycle were prepared. APLP2 was immunoprecipitated from cell lysates obtained from the G1, S, or G2/M phase and probed with pAbThr736 and AbAPLP2, and the levels of APLP2 phosphorylated at Thr736 were measured (Figure 5). As described previously (Thinakaran & Sisodia, 1994) and shown in Figure 5, both nonsulfated (NS-APLP2) and chondroitin-sulfated (CSGAG-APLP2) glycosaminoglycan forms of APLP2 were detected in cultured cells. Phosphorylation level of the APLP2 was very low at the G1 or S phase in either cell type. However, a dramatic increase in phosphorylation of NS-APLP2 was observed at the G2/M phase in HeLa cells (approximately 10-fold) and C6 cells (approximately 6-fold). Notably, the level of NS-APLP2 increased slightly (~1.5-fold in both HeLa and C6 cells) at the G2/M phase relative to that of G1 phase cells, and this was associated with a decreased level of the CSGAG-APLP2 in G2/M phase cells (Figure 5).

The phosphorylation level of CSGAG-APLP2 at G2/M phase was extremely low and could only be detected as a weak band following longer exposure of the autoradiogram (Figure 5e). Notably the phosphorylation of CSGAG-APLP2 at G2/M phase was less than that of the phosphorylation level of NS-APLP2 observed at the G1 or S phase. The sensitivities of the pAbThr736 and AbAPLP2 were found to be similar using standard amounts of peptide antigen (data not shown).

The specificity of APLP2 immunoprecipitation with AbAPLP2 was confirmed by competition studies using the cytoplasmic domain peptides (3 μM) from APLP1, APLP2, or APP (Figure 6a). In the absence of added peptide, both NS- and CSGAG-APLP2 were recovered (Figure 6, lane 1). Addition of APLP2$^{717-763}$ completely abolished APLP2 immunoprecipitation (lane 2). APP$^{645-694}$ and APLP1$^{608-653}$ did not decrease immunoprecipitation of APLP2 (lanes 3 and 4) from HeLa cells. An identical result was obtained in C6 cells (data not shown). The identification of CSGAG-APLP2 from HeLa and C6 cells was also confirmed by treatment of the APLP2−antibody complex with chondroitinase (Figure 6b), the high molecular weight CSGAG species being decreased by 60−70% by this treatment. Total APLP2 recovered from a G2/M HeLa cell lysate by immunoprecipitation with AbAPLP2 was also incubated in the absence or presence of chondroitinase prior to being subjected to immunoblotting using pAbThr736, and the amount of total

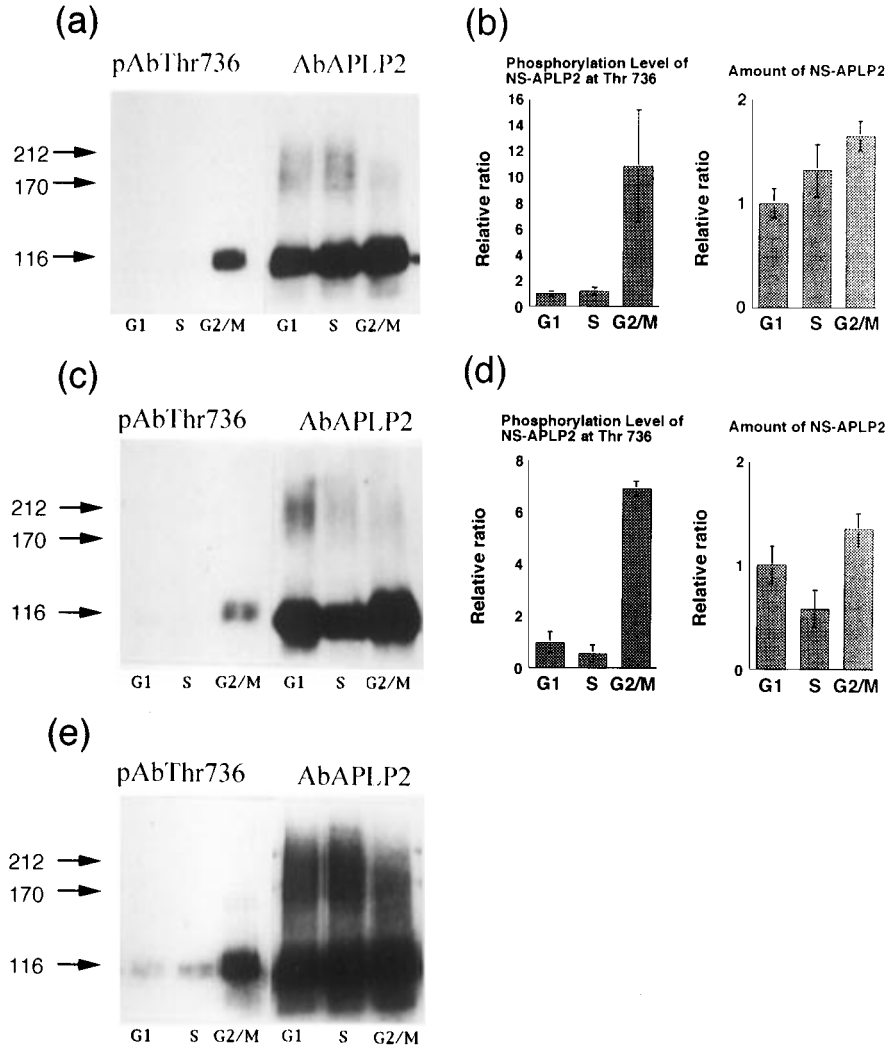

FIGURE 5: Cell cycle-dependent phosphorylation and metabolism of APLP2. HeLa cells (a, b, and e) and C6 cells (c and d) were synchronized at various cell cycle stages: G1 (synchronized by serum withdrawal), S (synchronized with aphidicolin), and G2/M (synchronized with nocodazole). APLP2 was immunoprecipitated from cell extracts using AbAPLP2, and samples were subjected to SDS−PAGE using 6% (w/v) polyacrylamide and transferred to nitrocellulose membranes. (a, c, and e) Immunoblots were probed with pAbThr736 (left panel) or AbAPLP2 (right panel) and [^{125}I]protein A, and then autoradiography was performed. Arrows indicate protein molecular weight markers (kDa). (b and d) Relative levels of phosphorylation and total amounts of the nonsulfated form (NS-APLP2) of APLP2 at various cell cycle stages. Immunoblots shown in panels a and c were quantitated using a Fuji BAS 2000 imaging analyzer. The levels of APLP2 phosphorylated at Thr736 and nonsulfated APLP2 were standardized to the amount of protein in the cell extracts, and the relative ratios of phosphorylated Thr736 at G1, S, and G2/M phases are indicated. Values are averages from duplicate studies, and error bars are indicated. (e) A longer exposure of the autoradiogram shown in panel a.

NS-APLP2 detected with pAbThr736 was measured. The amount of phosphorylated NS-APLP2 increased slightly after the treatment with chondroitinase (data not shown). This result was consistent with low phosphorylation of CSGAG-APLP2.

DISCUSSION

In the present study we have found that cytoplasmic domain peptides from APLP1 and APLP2 are phosphorylated by PKC and that the cytoplasmic domain peptide from APLP2 is phosphorylated by cdc2 kinase. Our previous data demonstrated that the cytoplasmic domain of APP is phosphorylated by PKC *in vitro* (Gandy et al., 1988) and in semi-intact cells (Suzuki et al., 1992). *In vivo*, the cytoplasmic domain of APP does not appear to be subject to phosphorylation at Ser655 by phorbol ester-sensitive PKCs (Oishi et al., 1997). However, Ser655 of APP is phosphorylated in cultured cell lines and rat brain tissues as demonstrated using a phosphorylation state-specific antibody (Oishi et al., 1997). Potentially, PKC may phosphorylate Ser655 in intact tissue. A novel Ser655 protein kinase activity has also been identified in rat brain (Isohara et al., manuscript in preparation), suggesting the possibility that a kinase distinct from PKC might be responsible for phosphorylation of this site. The phosphorylation sites for PKC, identified in the present study with respect to the domain organization of the proteins, as Thr614 in APLP1 and Thr723 in APLP2, correspond to Ser655 in APP. Thus, the sites in APLP1 and APLP2 may possibly also be phosphorylated *in vivo* by protein kinase(s) distinct from PKC.

Kinetic analysis indicated that *in vitro* cdc2 kinase phosphorylated APP and APLP2 with similar rates. Furthermore, Thr736 of APLP2, like Thr668 of APP (Suzuki et al., 1994; Oishi et al., 1997), was phosphorylated in a cell cycle-dependent fashion, with very high levels of phosphorylation being found at the G2/M phase and very low levels being detected at other phases of the cell cycle. In this study we did not determine directly whether activation

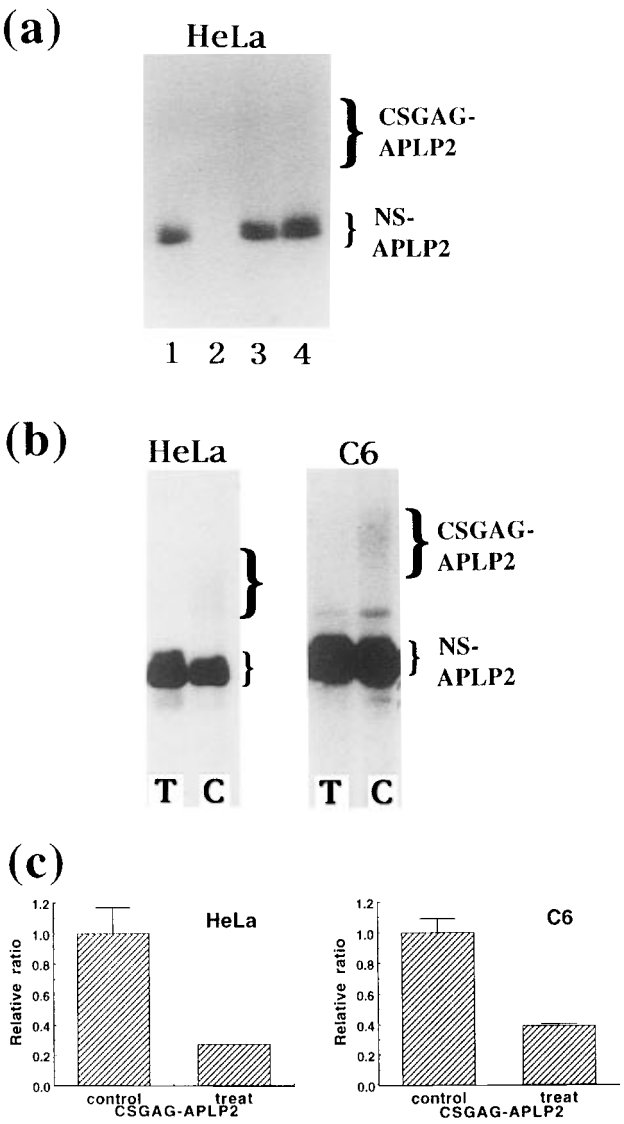

FIGURE 6: Specificity of AbAPLP2 and identification of chondroitin sulfate glycosaminoglycan APLP2. (a) APLP2 was immunoprecipitated from cell extracts of HeLa cells (5×10^6 cells) with AbAPLP2 in the absence (lane 1) or presence of cytoplasmic domain peptides (3 μM): $APLP2^{717-763}$ (lane 2), $APLP1^{608-653}$ (lane 3), and $APP^{645-694}$ (lane 4). (b) APLP2 was recovered from cell extracts of HeLa and C6 cells (5×10^6 cells) with AbAPLP2. The APLP2−antibody complex coupled to resin was incubated with (T) or without (C) chondroitinase as described in Materials and Methods. (a and b) Immunoprecipitated proteins were resolved by SDS−PAGE using 6% (w/v) polyacrylamide gels, transferred to nitrocellulose membrane, and probed with AbAPLP2 and [^{125}I]-protein A; then autoradiography was performed. (c) The levels of chondroitin sulfate glycosaminoglycan APLP2 from HeLa (left) and C6 (right) cells (5×10^6 cells) with (treat) or without (control) chondroitinase treatment were quantitated using a Fuji BAS 2000 imaging analyzer. The relative ratios with (treat) or without (control) treatment are indicated. Values are averages for duplicate studies, and error bars are indicated.

of cdc2 kinase at the G2/M phase correlated with the phosphorylation of APLP2. However, we have previously shown that active cdc2 kinase, but not MAP kinase, is responsible for phosphorylation of Thr668 of APP in intact cells (Suzuki et al., 1994). Therefore it is likely that endogenous cdc2 kinase phosphorylates Thr736 of APLP2 at the G2/M phase of the cell cycle. The development of a highly specific antibody that detects only the phosphorylated form at Thr736 should be useful for studies of the regulation of APLP2 phosphorylation in intact cells and tissues. Indeed, in preliminary studies in rat brain tissues, APLP2 phosphorylation at Thr736 can be detected with the antibody (Satoh et al., manuscript in preparation).

Phosphorylation of Thr668 of APP is detected preferentially in immature APP (N-glycosylated isoforms) rather than in fully mature isoforms (N- and O-glycosylated isoforms) when cultured cell lines are tested (Suzuki et al., 1994; Oishi et al., 1997). In the present study, NS-APLP2 was phosphorylated to a much higher level than CSGAG-APLP2 at the G2/M phase. Possibly the high level of phosphorylation results from intracellular colocalization of the immature form of the proteins with active cdc2 kinase. An additional factor that contributed to the difficulty in detecting phosphorylation of CSGAG-APLP2 at the G2/M phase might be the fact that its level was reduced at this phase of the cell cycle. Four alternatively spliced isoforms of APLP2 exist, and only two of these are subject to chondroitin sulfation (Thinakaran et al., 1995). It is likely that the processing of APLP2 by chondroitin sulfation is suppressed during mitosis. Less likely is the possibility that alternative splicing of APLP2 is modified in a cell cycle-dependent manner.

Recently, Fe65 was identified as a protein that binds the cytoplasmic domain of APLP2 and APP (Fiore et al., 1995). Thr668 in APP and Thr736 in APLP2 are within the region of the cytoplasmic domains of the two proteins that are believed to interact with Fe65. APP-BP1 (Chow et al., 1996) and a novel 130 kDa protein (Watanabe et al., manuscript in preparation) have also been identified as APP cytoplasmic domain-binding proteins, and it is possible that both proteins may also interact with the cytoplasmic domain of APLP2. Thus, phosphorylation may regulate the interaction between the cytoplasmic domain of APP/APLP2 and binding proteins such as Fe65, APP-BP1, and/or the130 kDa protein.

In conclusion, the present results demonstrate that APLPs are phosphoproteins and that APLP2 like APP is phosphorylated in a cell cycle-dependent manner. The results support the possibility that APP and APLP2 are subject to similar regulation and metabolism. Further elucidation of the biological function of APLPs and their phosphorylation will hopefully help in the understanding of the pathogenesis of AD.

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