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Two different protein kinases act on a different time schedule as glial filament kinases during mitosis

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Glial fibrillary acidic protein (GFAP) is a component of glial filaments specific to astroglia. We now report the spatial and temporal distributions of four phosphorylated sites in the GFAP molecule during mitosis of astroglial cells, determined by antibodies which can distinguish phosphorylated epitopes from non-phosphorylated-epitopes. Immunofluorescence microscopy showed that the Ser8 residues in the entire cytoplasmic glial filament system are initially phosphorylated when the cells enter mitosis. In cytokinesis, the phosphoSer8 residues become dephosphorylated, whereas Thr7, Ser13 and Ser34 in glial filaments at the cleavage furrow become the preferred sites of phosphorylation. The cdc2 kinase purified from mitotic cells can phosphorylate GFAP at Ser8 but not at Thr7, Ser13 or Ser34, *in vitro*. These results suggest that cdc2 kinase acts as a glial filament kinase only at the G_2–M phase transition while other glial filament kinases are probably activated at the cleavage furrow before final separation of the daughter cells.

Key words: cdc2 kinase/intermediate filament/mitosis/phosphorylation

Introduction

One of the profound changes in cellular morphology occurring during mitosis is the reorganization of the cytoskeletal structure, including microfilaments, microtubules and intermediate filaments (IFs). Several lines of evidence reveal that various types of IF proteins are hyperphosphorylated during mitosis; thus, phosphorylation appears to play an important role in regulating the organization of IFs (O'Connor *et al.*, 1981; Evans and Fink, 1982; Gard and Lazarides, 1982; Celis *et al.*, 1983; Evans, 1988a; Chou *et al.*, 1989). The possibility that phosphorylation might regulate the *in vivo* polymerization of IFs has been supported by *in vitro* studies with purified cAMP-dependent protein kinase, protein kinase C and Ca^{2+}–calmodulin-dependent protein kinase II (Inagaki *et al.*, 1987, 1988, 1990; Evans, 1988b; Geisler and Weber, 1988; Ando *et al.*, 1989; Geisler *et al.*, 1989; Kitamura *et al.*, 1989; Hisanaga *et al.*, 1990; Tokui *et al.*, 1990). It has also been shown that vimentin is a substrate for cdc2 kinase and that phosphorylation of *in vitro* polymerized vimentin IFs by the kinase causes their disassembly (Chou *et al.*, 1990).

IF proteins are arranged into the following three major domains: amino-terminal head, central helical rod and carboxyl-terminal tail domains (Steinert and Roop, 1988). It has been reported that cAMP-dependent protein kinase, protein kinase C, Ca^{2+}-calmodulin-dependent protein kinase II and cdc2 kinase phosphorylate the head domains of IF proteins, and that this phosphorylation induces depolymerization of the IFs *in vitro* (Inagaki *et al.*, 1987, 1988, 1990; Evans, 1988b; Geisler and Weber, 1988; Ando *et al.*, 1989; Geisler *et al.*, 1989; Kitamura *et al.*, 1989; Chou *et al.*, 1990; Gonda *et al.*, 1990). However, each protein kinase differs with regard to site recognition on this domain (Ando *et al.*, 1989, 1991; Kitamura *et al.*, 1989; Inagaki *et al.*, 1990; Chou *et al.*, 1991).

To examine the role of site-specific phosphorylation of IF proteins in the distribution and function of IFs during mitosis and to define mitotic IF kinase(s), we developed four antibodies recognizing functional phosphorylated sites in glial fibrillary acidic protein (GFAP). We have now obtained immunocytochemical evidence that phosphorylation of each site occurs at a different time-point during mitosis, thereby indicating that multiple glial filament kinases are activated with different time schedules in astroglial cells. In addition, as each phosphorylated site showed a spatially different distribution pattern in the mitotic cells, a causal relationship probably exists between the site-specific phosphorylation of GFAP and the specific organizational change of glial filaments.

Results

Characterization of epitope specificity of antibodies on phosphopeptides

To examine the role of site-specific phosphorylation of GFAP in the distribution and function of glial filaments, we prepared four anti-phosphoGFAP antibodies that react with distinct phosphorylated sites of phosphoGFAP. These sites on GFAP are important for assembly of glial filaments *in vitro* (Inagaki *et al.*, 1990).

Antibodies YC-10, pG1-T, pG1-II and pG2 were produced against four phosphopeptides, Arg-Arg-Arg-Val-Thr-phosphoSer-Ala-Ala-Arg-Arg-phosphoSer, Arg-Arg-Arg-Val-phosphoThr-Ser-Ala-Ala-Arg-Arg, Ser-Ala-Ala-Arg-Arg-phosphoSer-Tyr-Val-Ser-Ser-Leu and Pro-Gly-Pro-Arg-Leu-phosphoSer-Leu-Ala-Arg-Met-Pro, respectively, which correspond to the amino acid sequences of porcine GFAP (Geisler and Weber, 1983).

After ruling out any reactivity with unphosphorylated forms of the peptides, the specificity of the antibodies was tested. The four phosphopeptides described in Figure 1 were

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separated by reversed-phase HPLC and each peptide was immobilized onto microtitre wells. Antibodies YC-10, pG1-T, pG1-II and pG2 specifically reacted with phosphopeptides that contain the phosphorylated forms of Ser8, Thr7, Ser13 and Ser34, respectively (Figure 1). Polyclonal antibodies pG1-T, pG1-II and pG2 were affinity purified by Sepharose 4B coupled with specific phosphopeptides and used for further analysis.

Antibodies YC-10, pG1-T, pG1-II and pG2 can distinguish four phosphorylated sites on GFAP

Non-phosphorylated GFAP and the phosphorylated GFAP by cAMP-dependent protein kinase were examined by Western blotting. All of the antibodies reacted with the latter but not with the former (Figures 2A–D, lanes a and b). To examine the epitope specificity of these antibodies on the phosphorylated GFAP, peptide competition experiments were performed. As shown in Figure 2A, the reactivity of anti-phosphoSer8 antibody was blocked by preincubation with the phosphopeptide which included phosphoSer8 but not with the other phosphopeptides. The reactivities of antibodies pG1-T, pG1-II and pG2 (the antibodies to phosphoThr7, phosphoSer13 and phosphoSer34, respectively) were also abolished in a specific manner by preincubation with phosphopeptides containing respective epitopes (Figures 2B–D). Antibodies YC10, pG1-T, pG1-II and pG2 can recognize the phosphorylated forms of Ser8, Thr7, Ser13 and Ser34 on the phosphopeptides and on phosphoGFAP.

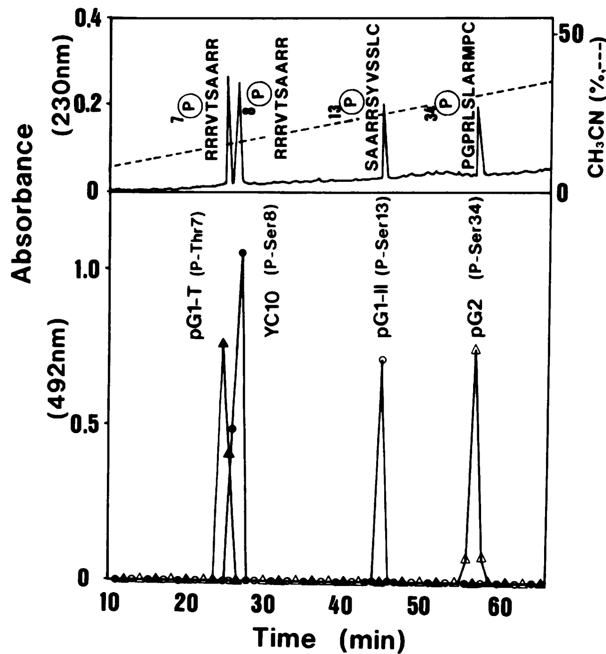
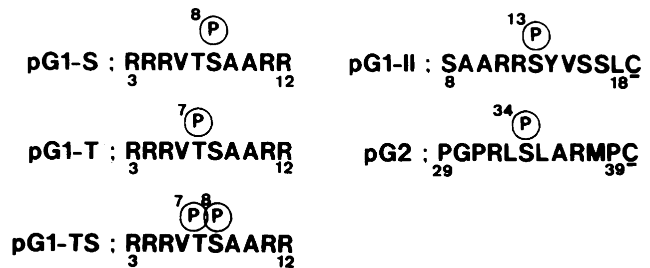


Fig. 1. Reactivity of four antibodies against different phosphopeptides. **Top panel.** HPLC separation over a YMC-PakODS-AP column of four phosphopeptides. Fractions were collected once per minute. The phosphorylation sites are indicated by a P within a circle. **Bottom panel.** Optical density, monitored at 492 nm, of ELISA of aliquots of phosphopeptides reacted with four antibodies. Wells were coated with 50 μl of each fraction in 150 μl of 50 mM Na_2CO_3, pH 9.6. Immunoreactivity was detected with 1:1000 peroxidase conjugated anti-mouse IgG antibody (Bio-Rad) or anti-rabbit IgG antibody (Bio-Rad) and visualized with *o*-phenylenediamine. Antibodies YC-10 (●), pG1-T (▲), pG1-II (○) and pG2 (△) show single immunoreactive peaks.

Similar results for antibodies YC-10 and pG2 were presented in the previous papers (Nishizawa *et al.*, 1991; Yano *et al.*, 1991). Interestingly, preincubation of antibody YC-10 with



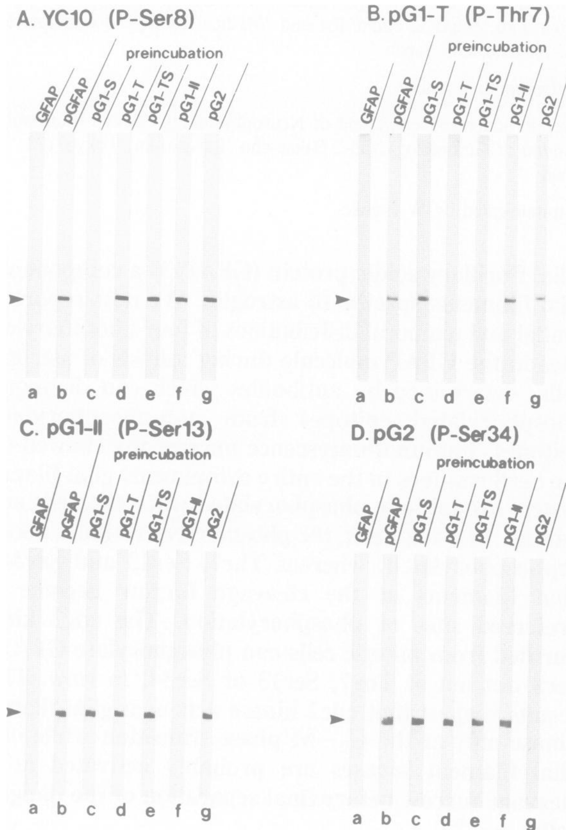


Fig. 2. Analysis of the epitopes detected by four antibodies YC-10, pG1-T, pG1-II and pG2. **Top panel.** List of the phosphopeptides used in peptide competition experiments. The amino acid sequences correspond to that of porcine GFAP. The peptides phosphorylated by cAMP-dependent protein kinase were purified with reversed phase HPLC. The phosphorylation sites are shown by a P within a circle. **Bottom panel.** Western blotting analysis. Thr7, Ser8, Ser13 and Ser34 of porcine GFAP correspond to Thr7, Ser8, Ser13 and Ser38 of human GFAP, respectively (Reeves *et al.*, 1989). We describe human GFAP as GFAP and Ser38 of human GFAP as Ser34, unless otherwise indicated. A–D, 75 ng of unphosphoGFAP (lane a) and the phosphorylated GFAP by cAMP-dependent protein kinase (lanes b–g) were transferred from 10% SDS–polyacrylamide gels to nitrocellulose membranes and reacted with four antibodies. Immunoreactivity was detected with 1:1000 peroxidase conjugated anti-mouse IgG antibody (Bio-Rad) or anti-rabbit IgG antibody (Bio-Rad) and visualized with diaminobenzidine. The position of GFAP is indicated (arrowhead). A, monoclonal antibody YC-10 (1 μg/ml) which reacts with the peptide containing phosphoSer8 was preincubated with 0.1 mg/ml of phosphopeptides pG1-S (lane c), pG1-T (lane d), pG1-TS (lane e), pG1-II (lane f) and pG2 (lane g). Lane b shows reactivity of the antibody in the absence of phosphopeptide. B–D, the same sets of experiments were carried out with antibodies pG1-T (1 μg/ml, B), pG1-II (10 μg/ml, C) and pG2 (67 μg/ml, D). Essentially, the same results were obtained using porcine GFAP (data not shown).

phosphopeptide Arg-Arg-Arg-Val-phosphoThr-phosphoSer-Ala-Ala-Arg-Arg (pG1-TS) had no effect on reactivity of the antibody (Figure 2A, lane e). This means that antibody YC-10 no longer recognizes GFAP when it is phosphorylated at both Thr7 and Ser8. Antibody YC-10 did not react with up to 100 μg/ml of pG1-TS coated on microtitre wells (data not shown). In contrast, antibody pG1-T was blocked by phosphopeptide pG1-TS as well as by phosphopeptide pG1-T, thus it can recognize GFAP phosphorylated at both Thr7 and Ser8 (Figure 2B, lanes d and e).

Four sites in GFAP are phosphorylated in different places and at different times during mitosis

Based on the foregoing biochemical analysis, the spatial and temporal distribution of four phosphorylated sites of GFAP within astroglial cells was analysed using these anti-phosphoGFAP antibodies. To elucidate the dynamics of phosphorylation on GFAP during the cell cycle, astrocytoma cells, U251, were partially synchronized and doubly stained with anti-GFAP antibodies and propidium iodide. Although monoclonal antibody G-A-5, which recognizes the tail domain of GFAP, did stain an intricate mesh of glial filaments of the interphase cells, none of the anti-phosphoGFAP antibodies stained filaments in the cells (Figures 3B–E, background).

In the mitotic cells, the fine fibrillar filaments stained by G-A-5 reorganized, yet the filament structure could still be discerned (Figure 3A). All of the anti-phosphoGFAP antibodies specifically reacted with the mitotic cells (Figures 3B–D). Antibody YC-10 intensely stained filamentous structures throughout the cytoplasm of the rounded-up cells. At the onset of furrowing, the filaments were less stained. Fluorescence from the filaments continued to be faint through anaphase and finally disappeared at early telophase (Figure 3B). Thus, in U251 cells, Ser8 of GFAP may be phosphorylated at the onset of mitosis in the entire glial filament network.

The spatial and temporal distributions of pG1-T, pG1-II and pG2 immunoreactivity (i.e. phosphoThr7, phosphoSer13 and phosphoSer34, respectively) in the cells were similar but very different from that seen with YC-10 immunoreactivity. Antibodies pG1-T, pG1-II and pG2 also stained a filamentous structure during anaphase and telophase, but stained no cellular component at prometaphase and metaphase. Furthermore, the immunoreactivities were concentrated in the centre of the dividing cells (Figures 3C–E). The data obtained using antibody pG2 provide further support for our previous results (Nishizawa *et al.*, 1991). Thr7, Ser13 and Ser34 on GFAP may be phosphorylated at the cleavage furrow during cytokinesis.

The reactivity of antibody YC-10 was abolished by preincubation with the phosphopeptide containing phosphoSer8, but not with the other phosphopeptides used (Figure 4A). Similar results were obtained for antibodies pG1-T, pG1-II and pG2 (Figure 4B; data not shown). These observations were comparable to the data obtained at the level of immunoblotting in Figure 2.

The mitosis specific appearance of immunoreactivities of anti-phosphoGFAP antibodies is not an artefact of the immunocytochemical method. Whole cell lysates of the interphase cells and the mitotic cells treated by SDS sample buffer were resolved by SDS–PAGE, transferred to nitrocellulose and the strips were incubated with the anti-phosphoGFAP antibodies. A prominent immunoreactive band at 50 kDa was visible in the lysate of the mitotic cell fraction. In contrast, no significant reactivity was detected at 50 kDa in the interphase cells lysate (Figure 5). Two other faint bands were observed in both the lysates for antibody pG1-T at ~88 kDa and 77 kDa (Figure 5B). The unphosphorylated GFAP and the phosphoGFAP migrated on SDS–PAGE at a position corresponding to a molecular weight of 50 kDa. Our results provide strong evidence that the appearance of immunoreactivities with anti-phosphoGFAP antibodies in cell stainings truly represents the presence of antigens in the mitotic but not in the interphase cells.

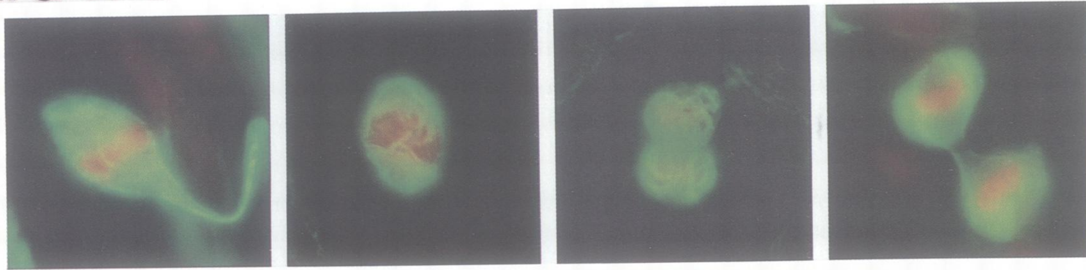
cdc2 kinase phosphorylates GFAP at Ser8 but not at Thr7, Ser13 or Ser34 in vitro

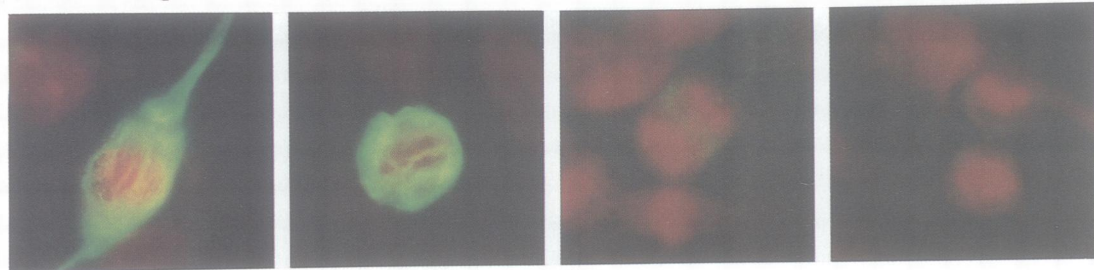
cdc2 kinase phosphorylates vimentin and the phosphorylation of vimentin contributes to M phase reorganization of the vimentin IF network (Chou *et al.*, 1990). To determine whether or not cdc2 kinase is the mitotic glial filament kinase, we purified this kinase from mitotic Hela cells (Kusubata *et al.*, submitted). The kinase incorporated 0.5 mol phosphate into 1 mol human GFAP. The blots of GFAP that had been phosphorylated by either cdc2 kinase or cAMP-dependent protein kinase were probed, using the anti-phosphoGFAP antibodies. The phosphoGFAP by cdc2 kinase reacted only with antibody YC-10, while the phosphoGFAP by cAMP-dependent protein kinase reacted with all the antibodies (Figure 6). Therefore, cdc2 kinase appears to phosphorylate GFAP predominantly at Ser8 but not at Thr7, Ser13 or Ser34.

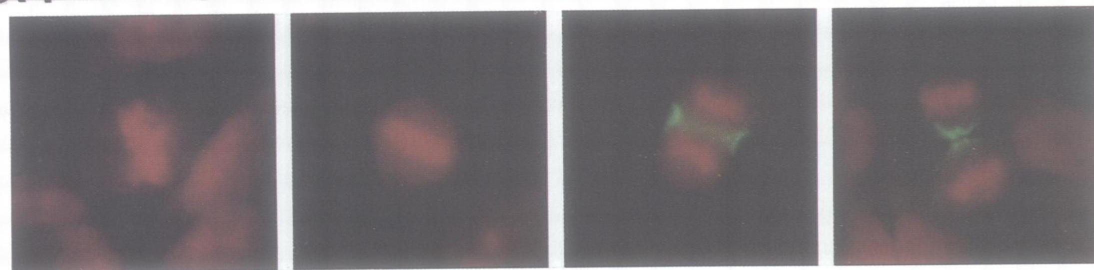
Discussion

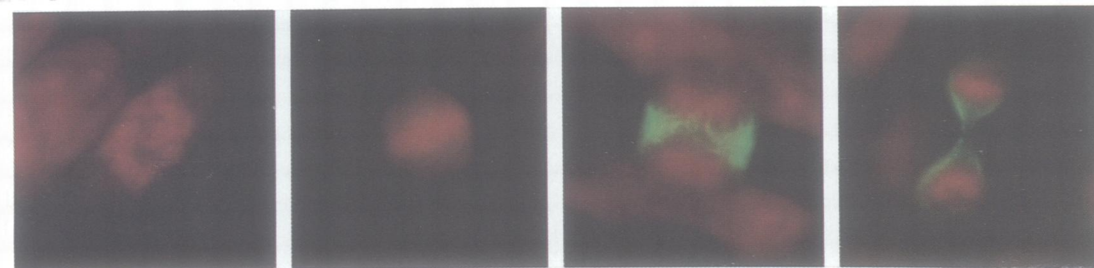
Using four antibodies which can distinguish four phosphorylated sites (phosphoThr7, phosphoSer8, phosphoSer13 and phosphoSer34) in GFAP, we examined phosphorylation of each site occurring on glial filaments in a cell cycle of human astrocytoma (U251) cells. All of the antibodies reacted with glial filaments in the mitotic cells but not with the filaments in the interphase cells. Immunoreactivity of the anti-phosphoSer8 antibody appeared in the entire cytoplasm at the onset of mitosis. In contrast, immunoreactivities of the other antibodies used appeared in the late mitotic stage and were restricted to the cleavage furrow.

Recent observations have demonstrated that IFs can undergo dynamic changes in supramolecular organization during mitosis (Blose and Chacko, 1976; Aubin *et al.*, 1980; Zieve *et al.*, 1980; Horwitz *et al.*, 1981; Blose and Bushnell, 1982; Franke *et al.*, 1982, 1983; Lane *et al.*, 1982; Celis *et al.*, 1983; Jones *et al.*, 1985; Tolle *et al.*, 1987). Analysis of desmin and vimentin from interphase and mitotic cells revealed that increased phosphorylation of the proteins observed during mitosis occurs within the amino-terminal head domains (Evans, 1988a; Chou *et al.*, 1990) and phosphorylation of the head domains of IF proteins by cAMP-dependent protein kinase, protein kinase C, Ca^{2+}-calmodulin-dependent protein kinase II and cdc2 kinase was shown to contribute to depolymerization of IFs *in vitro* (see Introduction). All of the antibodies used here recognize the phosphorylated sites in the amino-terminal head domain of GFAP. Therefore, mitosis-specific appearance of immunoreactivities with all of the antibodies is









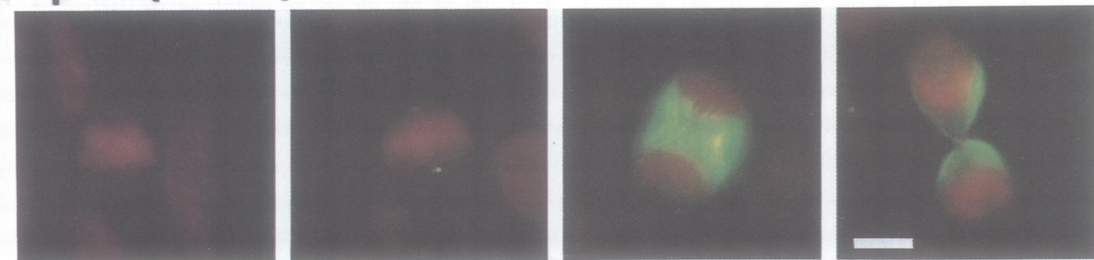
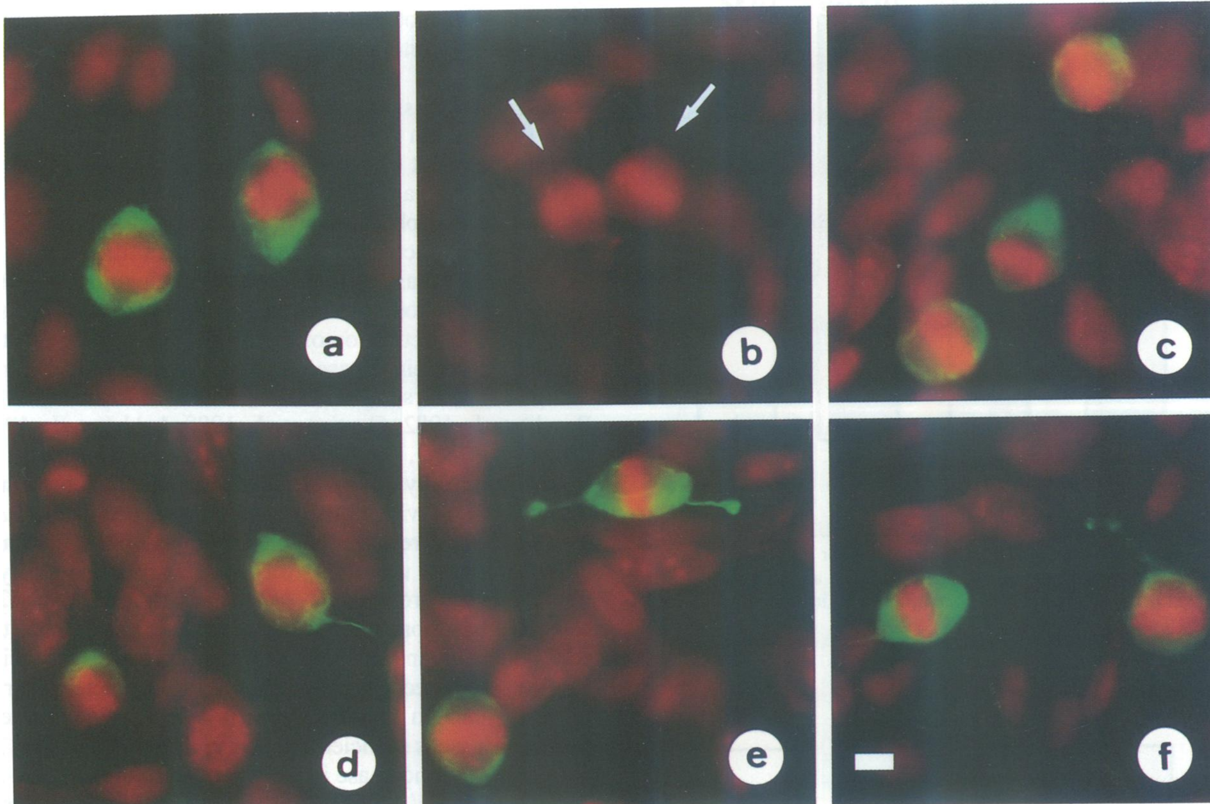


Fig. 3. Immunofluorescence microscopy of human astrocytoma cells, U251, with anti-GFAP antibodies. The cells on cover glasses were stained with either a monoclonal anti-GFAP (unphosphoGFAP and phosphoGFAP) antibody (**A**) or the anti-phosphoGFAP antibodies (**B**–**E**). Propidium iodide was mixed in the embedding solution to visualize chromosomes (orange). There are interphase cells in the background of B–E. Bar represents 10 μm.



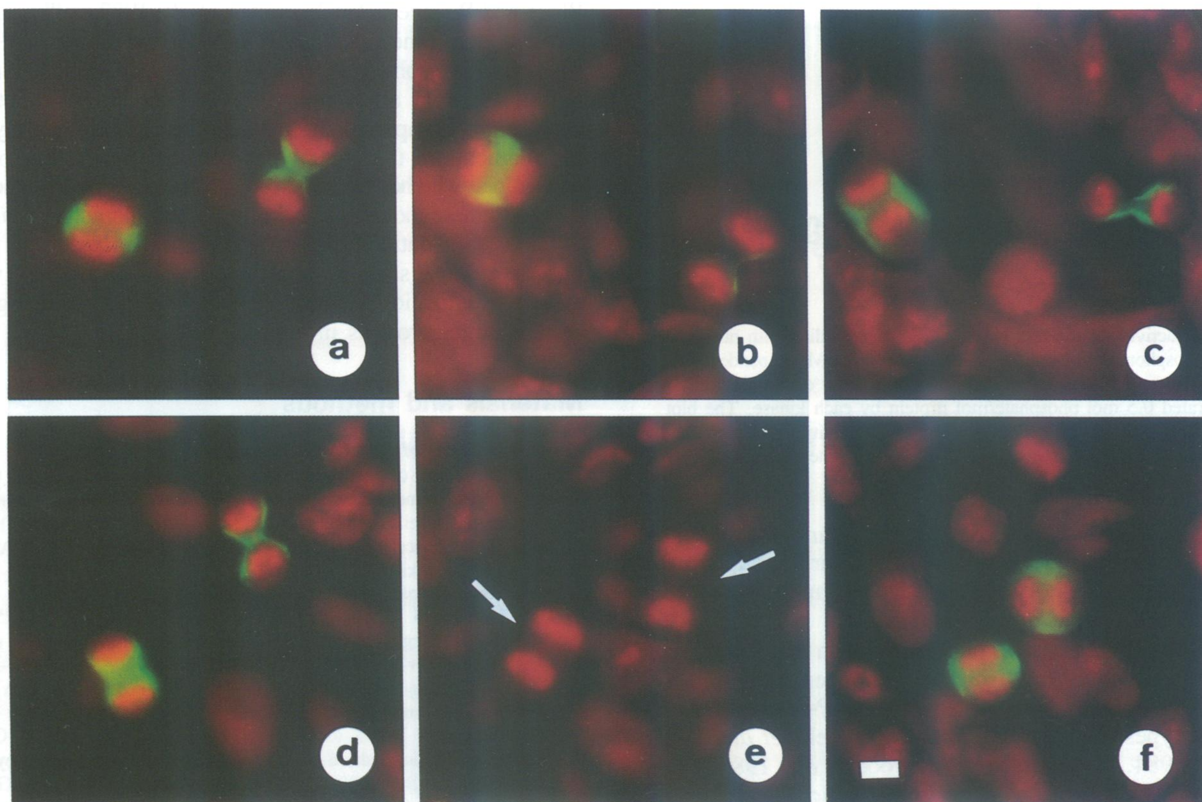


Fig. 4. Demonstration of epitope specificity of the anti-phosphoGFAP antibodies at the level of immunofluorescence.-Antibodies YC-10 (**A**) and pG1-II (**B**) were preincubated with 0.1 mg/ml of phosphopeptides pG1-S (b), pG1-T (c), pG1-TS (d), pG1-II (e) and pG2 (f). a, reactivities of the antibodies in the absence of phosphopeptide. Arrows indicate the mitotic cells. Bar represents 10 μm.

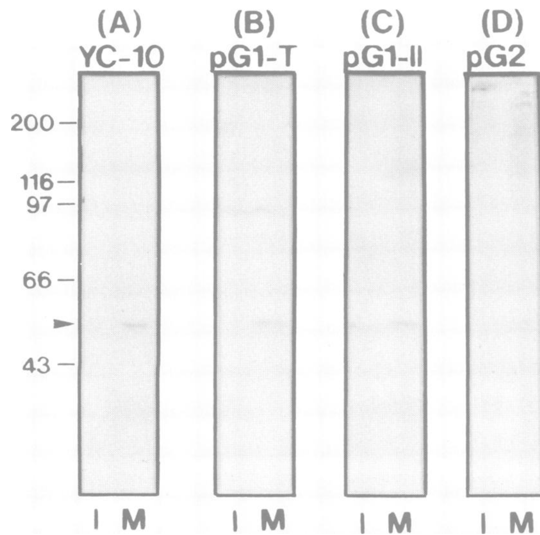


Fig. 5. Western blotting of interphase and mitotic U251 cells lysates. The same amounts of sample (5 μl) were loaded for each lane following adjustment of protein concentrations with a densitometer. The position of GFAP is indicated (arrowhead). I, interphase cells; M, mitotic cell fraction.

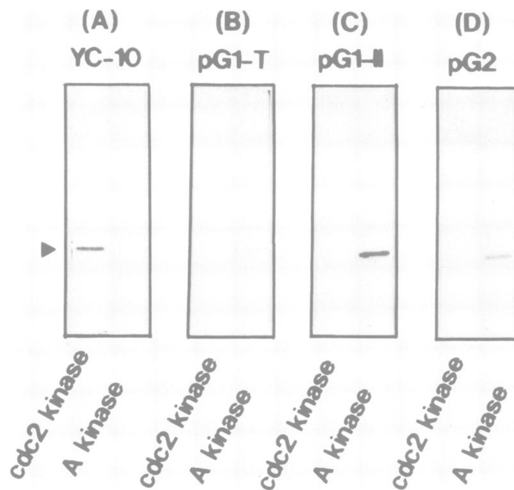


Fig. 6. Reactivity of the anti-phosphoGFAP antibodies with human GFAP phosphorylated by either cdc2 kinase or cAMP-dependent protein kinase. 75 ng of the protein was loaded for each lane and incorporated 0.5 mol phosphate/mol protein by each kinase. The blot was reacted with the antibodies and immunoreactivity was determined. The position of GFAP is indicated (arrowhead). The same results were obtained using porcine GFAP (data not shown).

consistent with previous observations (Evans, 1988a; Chou *et al.*, 1990). Our observations provide further support for the view that phosphorylation of IF plays an important role in IF reorganization during mitosis.

Although an increase in phosphorylation of several types of IF proteins during mitosis has been observed by SDS−PAGE analysis of proteins from ^{32}P-labelled cells of various types (Evans and Fink, 1982; Celis *et al.*, 1983; Evans, 1984, 1988a, 1989; Westwood *et al.*, 1985; Chou *et al.*, 1989), phosphorylation events which take place on IFs can be analysed only at G_2−metaphase transition when antimitotic drugs such as colcemid and nocodazole are used. Since there is no antimitotic drug available that can synchronize cells at anaphase or telophase, biochemical approaches cannot be used to study the phosphorylation of IFs in the late mitotic phase. Our data obtained using anti-phosphoGFAP antibodies clearly show that the phosphorylated sites (phosphoThr7, phosphoSer13 and phosphoSer34) in GFAP during cytokinesis differ from that (phosphoSer8) at G_2−metaphase transition in U251 cells. The efficient separation of glial filaments to daughter cells may follow phosphorylation of the former sites by glial filament kinase(s) activated at anaphase. Identification of the participating kinase(s) is the subject of ongoing studies.

The entire mitotic process in higher eukaryotes seems to be initiated by activation of the M phase promoting factor, the mitotic protein kinase complex consisting of cyclin B and p34^{cdc2} (Nurse *et al.*, 1976; Nurse and Bissett, 1981; Beach *et al.*, 1982; Gautier *et al.*, 1988; Labbé *et al.*, 1988; Draetta *et al.*, 1989). cdc2 kinase was seen to stoichiometrically phosphorylate vimentin and to induce disassembly of vimentin filaments *in vitro* (Chou *et al.*, 1990). In the present study, cdc2 kinase phosphorylated GFAP at Ser8 *in vitro*. This was found to be a phosphorylation site of the protein at G_2−metaphase transition in U251 cells. Our data provide further support for the notion that cdc2 kinase is an endogeneous protein kinase responsible for the increased phosphorylation of IF protein during mitosis. Although phosphorylation of cytoplasmic IF proteins by cdc2 kinase is sufficient to induce disassembly of the IFs at an early mitotic phase in baby hamster kidney (BHK-21) cells (Chou *et al.*, 1990), the IFs of many other types of cells such as U251 cells appear to be intact throughout mitosis (Figure 3A; Aubin *et al.*, 1980; Horwitz *et al.*, 1981; Blose and Bushnell, 1982; Franke *et al.*, 1982, 1983; Lane *et al.*, 1982; Jones *et al.*, 1985; Nishizawa *et al.*, 1991). The initial phosphorylation of GFAP by cdc2 kinase might loosen the tight association of glial filaments with other cytoskeletal components rather than induce disassembly of the filaments in the latter types of cells. It remains to be determined whether or not phosphorylation of glial filaments by cdc2 kinase induces disassembly of the filaments *in vitro*.

The use of antibodies which specifically react with the phosphorylated sites in phosphoproteins of interest will enable visualization of phosphorylation events occurring on certain target proteins in a single cell.

Materials and methods

Phosphorylation of GFAP and peptides

The catalytic subunit of cAMP-dependent protein kinase was prepared from bovine heart by the method of Beavo *et al.* (1974). cdc2 kinase was purified from mitotic Hela cells (Kusubata *et al.*, submitted). GFAP (4.0 μM) was phosphorylated by incubation with 5 μg/ml of the catalytic subunit of cAMP-dependent protein kinase, 0.1 mM [γ-^{32}P]ATP, 0.3 mM $MgCl_2$ and 25 mM Tris−HCl, pH 7.0 at 25°C or with 0.5 μg/ml cdc2 kinase, 0.1 mM [γ-^{32}P]ATP, 3 mM $MgCl_2$ and 25 mM Tris−HCl, pH 7.0 at 25°C. 0.5 mM of G1-I (Arg-Arg-Arg-Val-Thr-Ser-Ala-Ala-Arg-Arg), G1-II (Ser-Ala-Ala-Arg-Arg-Ser-Tyr-Val-Ser-Ser-Leu-Cys) and G2 (Pro-Gly-Pro-Arg-Leu-Ser-Leu-Ala-Arg-Met-Pro-Cys) were phosphorylated by incubation with 5 μg/ml catalytic subunit of cAMP-dependent protein kinase, 1 mM [γ-^{32}P]ATP, 2 mM $MgCl_2$ and 25 mM Tris−HCl, pH 7.5 at 25°C.

The amount of phosphate in protein and peptide was determined by the method of Stull and Buss (1977). The amount of phosphate bound to pG1-S, pG1-T, pG1-TS, pG1-II and pG2 was ~1, 1, 2, 1 and 1 mol of phosphate/mol of peptides, respectively. The amount of phosphate bound to GFAP phosphorylated by cAMP-dependent protein kinase and cdc2 kinase

was ~2.2 and 0.5 mol of phosphate/mol of GFAP, respectively. These preparations were used for the experiments.

Isolation of the phosphorylated peptides

The phosphorylated peptides pG1-S, pG1-T, pG1-TS, pG1-II and pG2 present in the reaction mixture were isolated by reversed-phase HPLC on a YMC-Pak ODS-AP column (4.6 × 250 mm). Elution of the peptides was monitored by UV at 230 nm and radioactivity. Peptides pG1-T, pG1-TS, pG1-S and G1-I were eluted at 23, 25, 26 and 24 min, respectively, using a linear gradient of 5–20% acetonitrile in 0.1% trifluoroacetic acid (TFA) over 30 min. Peptides pG1-II, G1-II, pG2 and G2 were eluted at 19, 23, 31 and 33 min, respectively, using a linear gradient of 15–40% acetonitrile in 0.1% TFA over 40 min. Chromatography was carried out at a flow rate of 0.8 ml/min. Purity of the isolated phosphopeptides was assessed by analytical reversed phase HPLC. The unphosphorylated peptides were not observed in the phosphopeptides fractions.

Assignment of phosphorylated amino acid residues in phosphopeptides

Phosphopeptides pG1-S, pG1-T, pG1-TS, pG1-II and pG2 were hydrolysed in a 6 N HCl for 1.5 h at 110°C and subjected to phosphoamino acid analysis by electrophoresis at pH 3.5 on a cellulose thin layer plate. Phosphoserine was the phosphoamino acid in pG1-S, pG1-II and pG2 and phosphothreonine was the one in pG1-T. pG1-TS contained both phosphoserine and phosphothreonine. Since pG1-II peptide contained four serine residues, the exact location of phosphoserine in the peptide was determined according to the method described by Ando *et al.* (1989). In brief, pG1-II peptide was treated with ethanethiol in alkaline condition to specifically convert the phosphoserine residue to *S*-ethylcysteine and was then subjected to gas phase sequence analysis. The release of *S*-ethylcysteine was high at the sixth cycle of Edman degradation, thereby indicating that the sixth serine residue in pG1-II peptide was phosphorylated. Sequence analysis of the peptide without ethanethiol treatment ruled out that the first serine residue was a phosphoserine because of the lack of conversion of the phosphoserine at the aminoterminus to *S*-ethylcysteine. The serine residues are found to be phenylthiohydantoin–serine (PTH–serine) and the dithiothreitol adduct of PTH–serine (DTT–serine) in gas phase sequencing. The phosphoserine residues provide exclusively DTT–serine (Meyer *et al.*, 1986). Both PTH–serine and DTT–serine were observed at the first cycle for the peptide, thereby confirming that the sixth serine residue was the phosphoserine in pG1-II peptide.

Production and purification of anti-phosphopeptide antibodies

Antibodies pG1-T and pG1-II were raised in rabbits against two phosphopeptides, Arg-Arg-Arg-Val-phosphoThr-Ser-Ala-Ala-Arg-Arg-Cys and Ser-Ala-Ala-Arg-Arg-phosphoSer-Tyr-Val-Ser-Ser-Leu-Cys, coupled to keyhole limpet hemocyanin, as described by Nishizawa *et al.* (1991). Production and characterization of antibodies YC-10 and pG2 were reported by Yano *et al.*, (1991) and Nishizawa *et al.* (1991).

Affinity columns were prepared by coupling pG1-T, pG1-II and pG2 peptides to CNBr-activated Sepharose 4B (Pharmacia), as suggested by the manufacturer. The monospecific antibodies pG1-T, pG1-II and pG2 were affinity-purified by Sepharose 4B coupled with specific phosphopeptides and were used for experiments.

Immunoblotting and immunofluorescence microscopy

All the procedures have been described in detail elsewhere (Nishizawa *et al.*, 1991; Yano *et al.*, 1991).

Cell fractionation

Human astroglioma cells, U251, were synchronized at S phase by incubation with 0.5 mM hydroxyurea (Nakarai Chemicals, Ltd., Kyoto) for 12 h and then treated with 10% trichloroacetic acid at 0 h (interphase cells) or 11 h (mitotic cells), after release. The mitotic cells fraction contained 10% metaphase cells and 20% anaphase and telophase cells. After washing with cold PBS to remove trichloroacetic acid, cell pellets (3 × 10^6 cells) were dissolved in SDS sample buffer containing 8 M urea, with brief sonication.

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