Mitotic Mechanisms in Alzheimer's Disease?

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Abstract. The mechanism(s) leading to widespread hyper-phosphorylation of proteins in Alzheimer's disease (AD) are unknown. We have characterized seven new monoclonal antibodies recognizing independent phospho-epitopes in the paired helical filament proteins (PHF) found in AD brain. These antibodies show pronounced immunoreactivity with cultured human neuroblastoma cells that are in the M phase of cell division, but have no discernible reactivity with interphase cells. Immunoreactivity with these antibodies does not localize to the microtubule spindles or chromosomes in M phase, but is confined to the surrounding cytoplasm. Similar staining in M phase is observed with cultured cells of various tissue types and species. Cells arrested in M phase with the microtubule depolymerizing agent, nocodazole, show marked increases in immunoreactivity with the antibodies by immunofluorescence staining, ELISA, and immunoblotting. In neuroblastoma cells, the appearance of the TG/MC phospho-epitopes coincides with activation of mitotic protein kinases, but not with the activity of the neuronal specific cyclin-dependent kinase, cdk5. These data suggest that the TG/MC epitopes are conserved mitotic phospho-epitopes produced as a result of increased mitotic kinase activity. To investigate this possibility in AD, we examined the staining of human brain tissue with MPM-2, a marker antibody for mitotic phospho-epitopes. It was found that MPM-2 reacts strongly with neurofibrillary tangles, neuritic processes, and neurons in AD but has no staining in normal human brain. Our data suggest that accumulation of phospho-epitopes in AD may result from activation of mitotic posttranslational mechanisms which do not normally operate in mature neurons of brain.

M UCH evidence (Grundke-Iqbal et al., 1986; Wood et al., 1986; Lee et al., 1991; Goedert et al., 1992a; Kanemaru et al., 1992) has indicated that the PHF found in neurofibrillary tangles (NFT)[1] and neurites in Alzheimer's disease (AD) brain are composed predominantly of hyperphosphorylated tau proteins. Whereas normal tau has 3 moles of phosphate per mole of protein, paired helical filament proteins (PHF) have 11 moles of phosphate per mole of protein (Ksiezak-Reding et al., 1994). Aberrant protein phosphorylation has therefore been hypothesized to be a critical step in the formation of PHF in AD. Thus, a considerable amount of research has focused on the identification of protein kinases responsible for excessive phosphorylation in the disease.

In vitro studies have shown that virtually every serine–threonine protein kinase phosphorylates tau, producing some characteristic of PHF (Hoshi et al., 1987; Baudier and Cole, 1987; Litersky and Johnson, 1992; Steiner et al., 1990; Mandelkow et al., 1992; Ishiguro et al., 1992). The mapping of several PHF phospho-epitopes to serine/threo-

onine–proline motifs in the normal tau sequence (reviewed by Kosik and Greenberg, 1994) has directed attention to the interaction of various members of the proline-directed protein kinase family and tau. The results indicate that nearly all members of this kinase family which include the ERK/MAP kinases (Drewes et al., 1992; Ledesma et al., 1992; Goedert et al., 1992b; Lu et al., 1993), the cdc2 kinase (Hall et al., 1990; Mawal-Dewan et al., 1992), cdk2 (Bauman et al., 1993), cdk5 (Paudel et al., 1993; Ishiguro et al., 1994), and the glycogen synthase kinase-3 (Mandelkow et al., 1992), phosphorylate recombinant tau producing PHF epitopes. Although in vitro phosphorylation of a protein by a given kinase is an important criteria for establishing an in vivo kinase–substrate relationship, the promiscuity of proline-directed kinases in vitro makes it difficult to correlate in vitro activities with physiological relevance (Hall and Vulliet, 1991; Nigg, 1993). What would substantially assist in the clarification of these in vitro data is a cellular model system in which PHF epitopes are expressed, so that mechanisms leading to their formation may be more clearly defined.

In the present study we have characterized monoclonal antibodies raised against and specific for PHF from AD brain in a model system of human neuroblastoma cells. We have found that the phospho-epitopes recognized by seven new anti-PHF monoclonal antibodies are expressed in a cell cycle–regulated fashion in cultured cells of a vari-

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1. *Abbreviations used in this paper:* AD, Alzheimer's disease; APP, amyloid precursor protein; NFT, neurofibrillary tangles; PHF, paired helical filament proteins.

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ety of species. The PHF-phospho-epitopes are markedly elevated in M phase, and are not detectable in any other phases of the cell cycle. We have therefore hypothesized that mitotic protein kinases are involved in the production of PHF phospho-epitopes.

Materials and Methods

Purification of PHF

PHF were purified from AD brain supernatant using a column of immobilized Alz-50 antibody as previously described (Vincent et al., 1992).

Antibodies

The TG and MC monoclonal antibodies were raised in mice immunized with immunoaffinity purified PHF preparations from AD brain. Alz-50 was produced using basal forebrain homogenates from AD brain as immunogen (Wolozin et al., 1986), and PHF-1 was made against detergent-extracted PHF (Greenberg et al., 1992). All the above hybridomas were grown to yield titers of ~10 μg antibody per ml culture supernatant. A polyclonal antibody recognizing residues 264–288 of the COOH terminus of mouse cdc2, an anti-PSTAIR monoclonal antibody, a polyclonal antibody recognizing the COOH-terminal residues 268–283 in human cdk5, and the MPM-2 monoclonal antibody were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). A mouse monoclonal antibody specific for cdk5 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibody against β-tubulin (IgG2a) was obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). All isotype-specific secondary antibodies were purchased from Southern Biotechnology Assoc. (Birmingham, AL). FITC- and rhodamine-conjugated subtype specific antibodies were also obtained from Southern Biotechnology Assoc. and Cy5-conjugated mouse IgG secondary antibody for triple labeling experiments was bought from Jackson ImmunoResearch Labs. (West Grove, PA).

Immunocytochemistry with Human Brain Tissue

All the cases used in this study were evaluated by neuropathological examination of postmortem brain specimens that were immersion fixed in 10% formalin. Those cases that were clinically diagnosed with senile dementia of the Alzheimer type were confirmed histopathologically according to the distribution and number of plaques and tangles observed with thioflavin S fluorescence staining and with the monoclonal antibody, Alz-50. Age matched normal cases had no clinical history of dementia, and no thioflavin S or Alz-50 positive lesions, except where indicated. For immunocytochemical studies, fixed tissue was sectioned with a vibratome at a thickness of 50 μm, and immunocytochemistry was performed on free-floating sections after blocking endogenous peroxidase with 3% hydrogen peroxide and nonspecific antibody binding with 5% nonfat milk. All primary antibodies were used at dilutions of 1:10 in the 5% milk blocking solution. The MPM-2 antibody was used at a 1:250 dilution. The specific binding of primary antibodies was detected using HRP-labeled goat anti-mouse isotype-specific secondary antibodies at dilutions of 1:500 in blocking solution, and diaminobenzidine (Sigma Chemical Co., St. Louis, MO) as the chromogen.

Cell Culture

The human neuroblastoma line (MSN) was cultured in RPMI medium containing 10% fetal bovine serum, as described in Vincent et al. (1994). All other cell lines were maintained in DME, supplemented with 10% fetal bovine serum. Hela cells were kindly provided by Ruchira Das Gupta and Howard Sirotkin (Albert Einstein College of Medicine, Bronx, NY). For arresting cells in metaphase, exponentially growing cells were treated overnight with 10 μM nocodazole (Sigma Chemical Co.) in 10% DMSO. The final concentration of DMSO in the cultures was less than 0.1%. The mitotic index of the cultures was determined by propidium iodide staining of formalin-fixed cells. In general, ~15% of the cells in asynchronous cultures were mitotic, whereas ~85% of the cells displayed condensed chromosomes after nocodazole treatment.

Immunofluorescence and Confocal Microscopy

Cells cultured on glass coverslips (Bellco Glass, Inc., Vineland, NJ) were fixed with 10% formalin for 40 min at 4°C. Cells were permeabilized using two 5 min washes in TBS containing 0.25% Triton X-100. Nonspecific antibody binding was blocked with 5% milk in TBS and incubations with primary antibody were carried out overnight at 4°C. Bound antibody was visualized by incubation with FITC-conjugated isotype-specific secondary antibodies for 1 h. Interphase nuclei and condensed chromosomes were distinguished by double staining with propidium iodide (Sigma Chemical Co.). In triple labeling studies, Cy5-conjugated IgG secondary antibody was used for visualization of anti-tubulin antibody binding. After sequential dehydration with alcohols, the coverslips were mounted in Gurr fluoromount (Bio/medical Specialties, Santa Monica, CA). Immunofluorescence data was analyzed using the Bio-Rad MRC 600 laser scanning confocal microscope fitted with Nikon Diaphot optics.

Alkaline Phosphatase Treatment of PHF Preparations

PHF preparations were prepared by immunoaffinity chromatography as described in Vincent and Davies, (1992). Aliquots were incubated with 15 U/ml of Escherichia coli alkaline phosphatase (type IIIN; Sigma Chemical Co.), for 1 h at 65°C. Samples were boiled with SDS sample buffer before electrophoreses in SDS–polyacrylamide gels and transfer to nitrocellulose.

Preparation of Cultured Cell Lysates for Immunoblot Analysis

MSN cells were harvested by mechanical shaking and centrifugation. Cell pellets were resuspended in Lysis buffer (TBS containing 2 mM PMSF, 2 mM EGTA, 25 μM leupeptin, 5 mM NaF, 0.5% Triton X-100, and 0.2% SDS), and frozen at −70°C. Lysates were obtained by thawing frozen cell suspensions and centrifuging for 2 min. The resulting supernatants were diluted 1:10 with water to reduce the Triton X-100 concentration to 0.05% and protein was assayed using the micro Bio-Rad procedure (Bio-Rad Laboratories, Richmond, CA). Aliquots of equivalent protein were boiled with SDS-PAGE sample buffer and used for electrophoresis and direct immunoblotting experiments. In general, 50 μg protein were loaded per lane of a 10% mini gel. Electrophoresis and immunoblotting were performed as previously described (Vincent et al., 1994), except that with some antibodies (where indicated) binding was detected using the highly sensitive enhanced chemiluminescence reagent, i.e., ECL (Amersham Corp., Arlington Heights, IL).

ELISA

Whole cell lysates were assayed by ELISA and were prepared as described above except without detergents. Lysates were titered into 96-well microtiter plates (Nunc, Roskilde, Denmark) starting with 30 μg protein in the first well. Dilutions were made in water. Antigen was bound to the plate by drying the samples under a current of air at 37°C for 1 h. Antibody binding was detected with isotype-specific secondary antibodies and ABTS substrate solution (Bio-Rad Laboratories). OD was determined using an SLT SPECTRA scanner (Tecan Technicals US Incorporated, Durham, NC), and the data were analyzed by totaling the OD from the entire titration as an estimate of the area under the curve.

Immunoprecipitation

MSN supernatants generated from cell lysates in the presence of detergents (see above) were used for immunoprecipitation. Supernatants containing 100 μg protein were incubated with 2–3 μg of the respective rabbit primary antibody for 2–3 h at room temperature. 50 μl of a 30% slurry of Sepharose beads coupled to protein A (Sigma Chemical Co.) were used to bring down the antibody–antigen immune complex. The immunoprecipitates were washed three times with lysis buffer, reconstituted to the original volume with 1x sample buffer and boiled for 3 min. The proteins released from the beads were resolved by SDS-PAGE and analyzed by immunoblotting.

For precipitation of mitotic kinase activity with p13suc1-beads aliquots of the supernatants were diluted 1:10 with water to decrease the detergent concentration below 0.1%, prior to assay of protein concentration by the micro Bio-Rad procedure (Bio-Rad Laboratories). 100 μg protein of MSN supernatant were used for the precipitation of p13-bound mitotic kinases. 5μl of p13suc1-bound to agarose beads (Upstate Biotechnology

Inc.) were incubated with the appropriate supernatants for 1 h at 4°C. The mixture was centrifuged for 2 min to isolate the beads, and the beads were washed with 15 vol of lysis buffer twice, and then twice with 25 vol TBS to reduce the concentration of detergent for the kinase assay.

Histone H1 Kinase Assays

Kinase assays were conducted according to the procedure recommended by Upstate Biotechnology Inc. The p13-bound proteins were reconstituted to 20 µl with TBS. 3–5 µl of the slurry were incubated with 5–8 µg of histone H1 (Boehringer Mannheim Biochemicals), and 0.25 mCi γ-^{32}P-ATP (Amersham Corp.) in a final volume of 30 µl kinase buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM MgCl$_2$, 2 mM EGTA, 1 mM DTT, 2 µM ATP, and 0.5 µg/ml protein kinase A inhibitor (Sigma Chemical Co.). The reaction was allowed to proceed for 30 min at room temperature, and stopped with sample buffer and boiling. Samples were loaded onto SDS gels for Coomassie blue staining and autoradiography. The incorporation of labeled phosphate into the histone bands in the gel was quantitated using a phosphoimager (Molecular Dynamics, Sunnyvale, CA).

Results

The TG and MC Monoclonal Antibodies React with Phosphorylated Epitopes in PHF

The TG and MC monoclonal antibodies were raised against PHF purified by affinity chromatography using a column of immobilized Alz-50 antibody. This purification procedure (Vincent and Davies, 1992) does not use any harsh detergents which are typically used in conventional PHF purification schemes. Our method thereby enriches for PHF in their native form and allows for studies of other proteins in physiological association with the filaments. That the TG/MC antibodies are bona fide markers for AD pathology is illustrated by their reactivity with the immunoaffinity purified PHF preparation on immunoblots (Fig. 1). All the antibodies react with the principal 57–68-kD triplet PHF proteins, and also with aggregation and degradation products of the triplet proteins. Thus, the pattern of immunoreactivity of the TG and MC antibodies with PHF resembles the pattern of immunoreactivity with the established PHF antibodies, PHF-1 (Greenberg et al., 1992), and Alz-50 (Wolozin et al., 1986; Ksiezak-Reding et al., 1988). Immunoreactivity with all of the antibodies and PHF is eliminated or greatly reduced following treatment of the PHF preparation with alkaline phosphatase (Fig. 1, +). This is direct evidence that these antibodies are directed against phosphorylated epitopes in PHF, an idea supported by the observation that none of the antibodies react with normal tau (data not shown). As is usually observed with Alz-50 immunoreactivity, an increase in electrophoretic mobility is observed after phosphatase treatment but the intensity of immunoreactivity is unaffected (Fig. 1, *Alz-50* lanes). These results also verify the specificity of the alkaline phosphatase, and control for the absence of PHF degradation during the phosphatase incubation.

The TG/MC Antibodies Are Markers for AD Pathology and React with Cultured Cells in Mitosis

Immunocytochemical analyses with the PHF antibodies were conducted using routine formalin-fixed autopsy tissue from histopathologically confirmed AD and age-matched normal cases. The observations are based on studies of 16 AD and 16 age-matched normals, except for
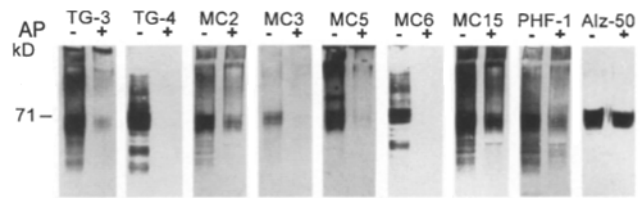

Figure 1. Effects of alkaline phosphatase treatment on the immunoreactivity with the TG/MC antibodies and purified PHF. Samples of the native immuno-affinity purified PHF preparation (−) and preparations incubated with alkaline phosphatase (*AP*) (+) were loaded at equivalent amounts of protein into SDS gels. After electrophoreses and electrophoretic blotting, nitrocellulose strips were stained with the indicated PHF antibodies. The staining with TG-4, MC2, MC15, PHF-1, and Alz-50 was visualized colorimetrically, and the remainder were detected by ECL (exposure time 30 s). Pretreatment of PHF preparations with alkaline phosphatase decreased TG/MC immunoreactivity with PHF, suggesting that the antibodies react with phosphorylated epitopes in PHF.

TG-3, which has been used to examine tissue from more than 60 cases, and Alz-50 and PHF-1 which have been very extensively used for studies of AD. The antibodies were also used in indirect immunofluorescence staining of human neuroblastoma cells (MSN), and the fluorescence was analyzed by dual confocal laser microscopy (Fig. 2).

Like the previously characterized antibodies, Alz-50 and PHF-1, all the TG and MC antibodies discriminate strongly between normal and AD brain. In normal brain, there is either no staining (TG-3, MC3, and MC5), some faint process and occasional cellular staining (TG-4, MC6, MC15), or staining of cellular nuclei (MC2). In AD brain, all the antibodies react intensely with the classical neurofibrillary lesions, namely, NFT, the neuritic elements of senile plaques, and the neuropil threads (Fig. 2).

When applied to the MSN neuroblastoma cells the TG/MC antibodies show pronounced reactivity with cells in mitosis (Fig. 2), but very little to no reactivity with interphase cells. Cells in mitosis were distinguished from interphase cells by the pattern of chromosomal staining with propidium iodide, a dye that displays intense fluorescence of condensed chromosomes but a more pale and diffuse staining of interphase nuclei. Unlike the antibodies recognizing phospho-epitopes, staining with the sequence directed antibody Alz-50 is uniformly distributed throughout the cytoplasm of all cells, with no increase in mitotic cells.

The TG/MC Antigens Do Not Colocalize with the Microtubule Spindle Apparatus or Chromosomes in Mitotic Cells

Progression through mitosis can be monitored by the dynamic phase-specific changes in the microtubular network visualized with anti-tubulin antibodies. We explored this means for defining the precise temporal and spatial distribution of TG and MC immunofluorescence in mitotic MSN cells. Double immunofluorescence staining with the TG/MC antibodies and an anti-β-tubulin antibody showed that TG/MC immunofluorescence is most prominent in mitotic metaphase (Fig. 3 *A*, shown for β-tubulin [*red*] and
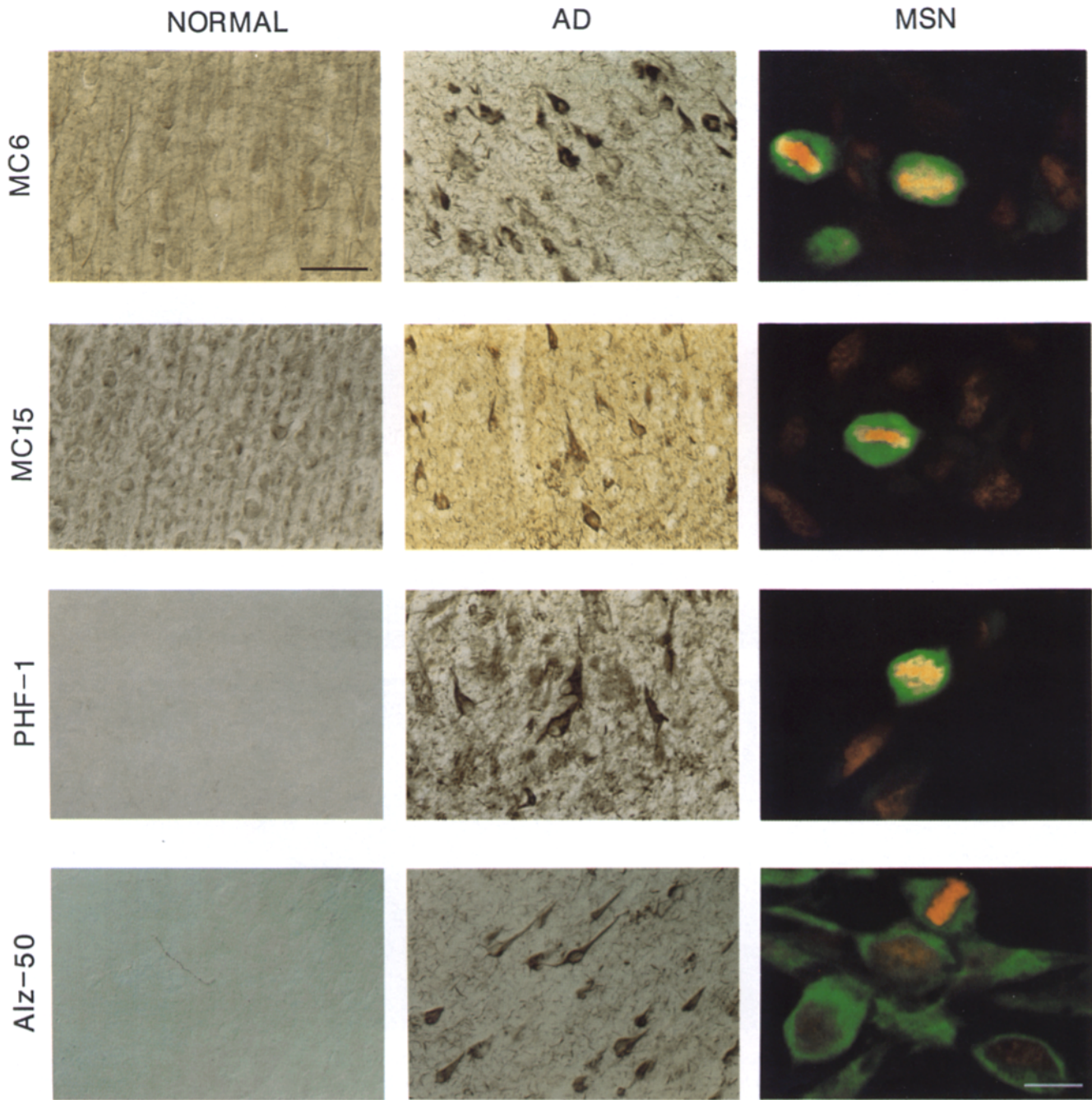
NORMAL   AD   MSN

TG-3

TG-4

MC2

MC3

MC5
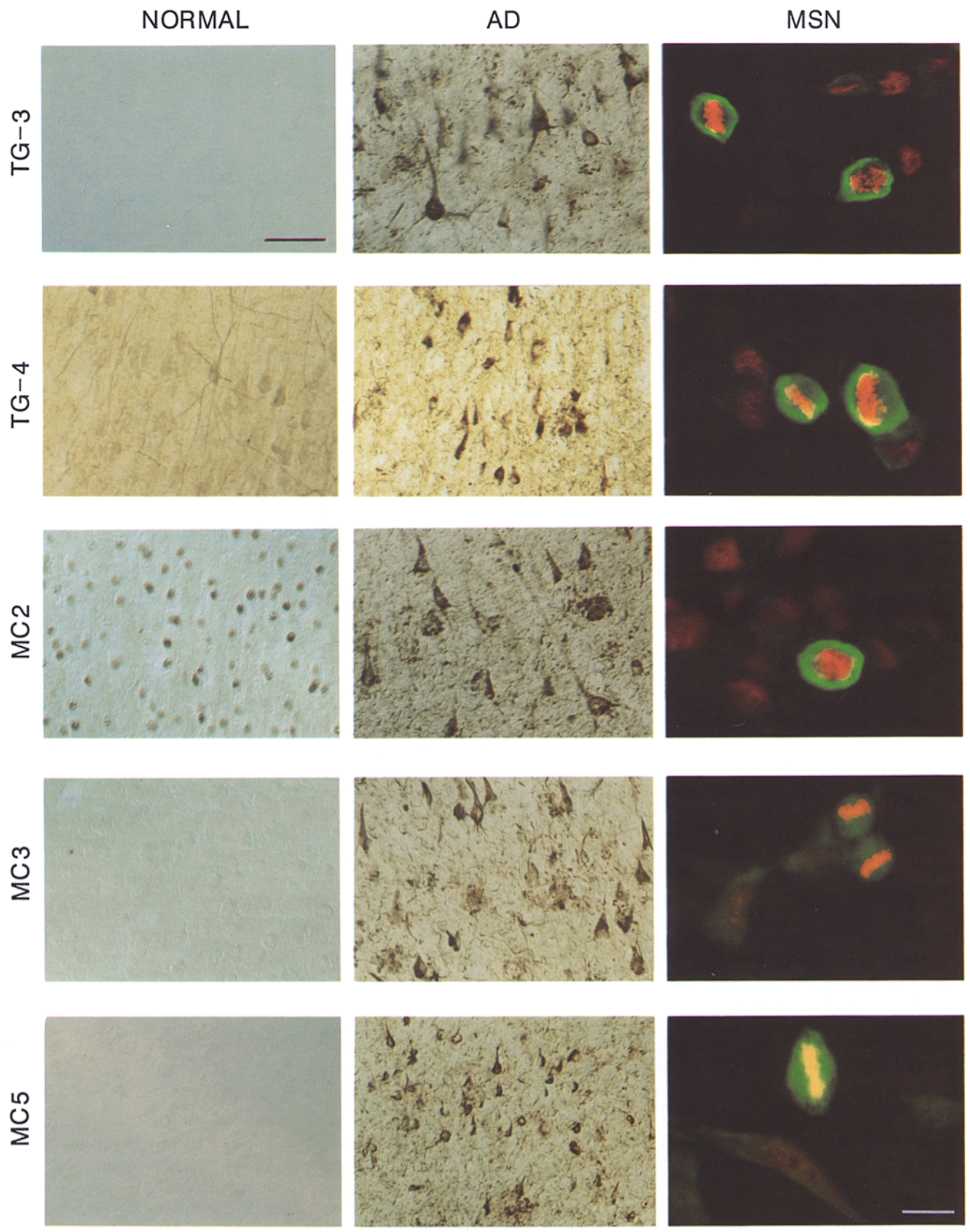
Figure 2. Immunocytochemical staining of human brain tissue, and immunofluorescence staining of MSN neuroblastoma cells with the TG/MC antibodies. Formalin-fixed tissue sections from the hippocampus of normal and AD cases were stained with the indicated antibodies. Light micrographs were generated using Nomarski optics to indicate the presence of tissue in those panels with negative staining. The TG/MC antibodies stain neurofibrillary lesions in AD brain tissue (*middle*), but have little to no reactivity with normal human brain (*left*). The bar in the light microscopy pictures is 20 μm. Double immunofluorescence staining was conducted with MSN cells grown on glass coverslips, using the TG/MC antibodies (*green*) and propidium iodide (*red*) as a counter stain for chromosomal material (*right*). Data were analyzed by dual confocal laser microscopy. The TG/MC antibodies display immunofluorescence in M phase cells but not in interphase cells. The bar in the fluorescence micrographs represents a range of 8–10 μm.

TG-3 [*green*]). Triple fluorescence staining with the TG/MC antibodies, β-tubulin antibody, and propidium iodide, shows localization of TG/MC-immunofluorescence in the cytoplasm surrounding the spindle apparatus, with no staining of any of the structural components of the mitotic microtubule organizing centers, and no staining of chromosomes (shown with TG-3 [*green*], tubulin [*red*], and propidium iodide [*blue*] Fig. 3 *B*). In contrast, the pattern of immunofluorescence with the PHF-1 antibody in mitotic cells differs from that of the TG/MC antibodies, both
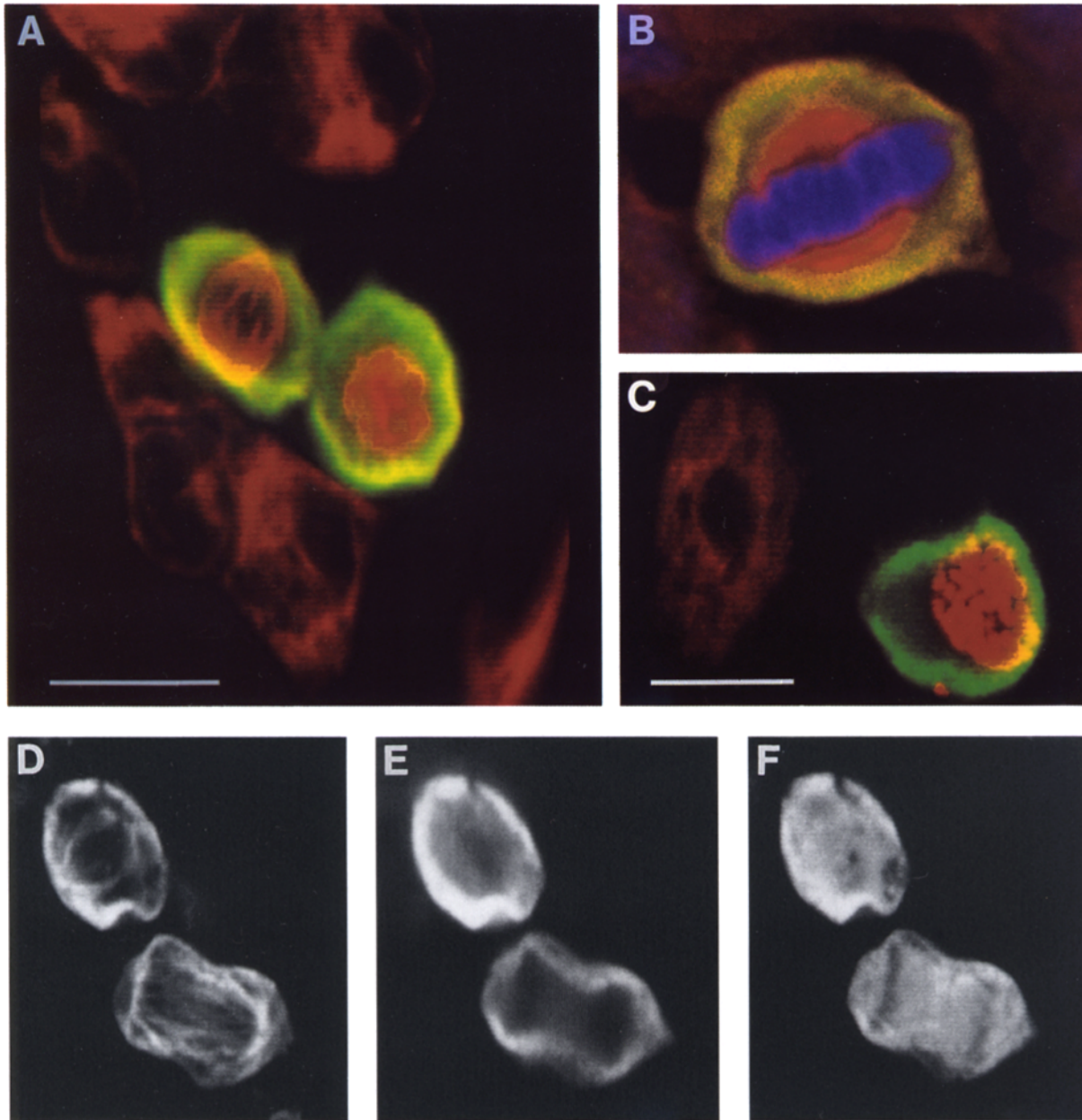
Figure 3. Temporal and spatial distribution of TG/MC immunofluorescence in mitotic cells. (*A*) Double staining of MSN cells in metaphase: MSN neuroblastoma cells were subjected to double immunofluorescence staining with the TG-3 antibody and an antibody against β-tubulin. TG-3 staining detected with anti–mouse IgM-FITC (*green*) does not colocalize with the staining of the tubulin antibody as visualized with anti–mouse IgG-rhodamine (*red*). Only cells in metaphase are stained with TG-3, whereas surrounding interphase cells are not. (*B*) Triple staining of MSN cells in metaphase: these cells were stained with TG-3 (*green*), the tubulin antibody (*red*) and propidium iodide (*blue*), to show the lack of colocalization of TG-3 immunofluorescence with the microtubule spindles or the metaphase chromosomes. The magnification for this micrograph is 1.6-fold that in *A*. (*C*) Hela cells double stained with TG-3 and propidium iodide. TG-3 staining (*green*) is found only in the mitotic Hela cell (*right*), but not in the adjacent interphase cell (*left*). (*D–F*) Cells were triple stained with the tubulin antibody (shown in *D*), TG-3 (shown in *E*), and PHF-1 (shown in *F*), to illustrate the differences in distribution of the TG-3 and PHF-1 antigens in metaphase (cell on upper left of each panel) and in anaphase (cell on lower right of each panel). TG-3 staining is localized to a peripheral region of the cytoplasm in both mitotic cells, but the intensity of staining is reduced in anaphase relative to metaphase. In contrast, PHF-1 immunofluorescence is distributed throughout the body of the mitotic cells, and is not reduced in anaphase. The magnification for panels *D-F* is the same as in *A*. Bars: (*A* and *C*) 10 μm.

in subcellular distribution and in timing. These differences are best illustrated when cells are triple stained with PHF-1, TG or MC antibody, and β-tubulin antibody (Fig. 3, *D–F*). While TG/MC immunofluorescence is confined to the cytoplasm peripheral to the spindle apparatus and chromosomes (Fig. 3 *E*), PHF-1 immunofluorescence occurs throughout the mitotic cell overlapping with the spindles and chromosomes (Fig. 3 *F*). When segregation of

daughter chromosomes occurs in anaphase, immunofluorescence with the TG and MC antibodies is greatly diminished (Fig. 3 E), whereas PHF-1 immunofluorescence remains unaltered (F).

TG/MC Antibodies Are M Phase Specific in Different Cell Types and Species

To rule out the possibility that the mitotic profile of TG/MC immunofluorescence is a phenomenon unique to MSN cells, we examined cells of other lineages and species for similar immunoreactivity. Identical results were obtained with HeLa cells, human lymphocytes, human B cells, human astrocytes, and mouse and rat fibroblasts. Fig. 3 C illustrates the specific immunofluorescence with TG-3 in a Hela cell with condensed chromosomes, and the lack of fluorescence associated with a neighboring interphase nucleus. These data suggest that the epitopes recognized by the TG and MC antibodies belong to a family of conserved mitotic antigens.

Immunoreactivity with the TG/MC Antibodies Is Elevated in Nocodazole-arrested Cells

To establish whether immunoreactivity with the TG/MC antibodies is activated specifically in mitosis, we made use of nocodazole, a microtubule depolymerizing agent that arrests cells in metaphase. The effects of nocodazole were assessed by immunofluorescence (data not shown), ELISA, and immunoblotting. Nocodazole treatment produced an increase in the total number of cells in metaphase as indicated by double staining with propidium iodide (Fig. 4). Exponentially growing cultures had an average of 15% of the cells in M phase and after nocodazole this number rose to 85%. The number of cells stained with the TG/MC antibodies increased concurrently with the number of cells in M phase (Fig. 4). Whole cell lysates from control and nocodazole-treated cultures were assayed by ELISA for total cellular immunoreactivity with the TG/MC antibodies (Table I). A significant increase in immunoreactivity with TG-3, TG-4, MC2, MC3, MC5, MC6, and MC15, is observed with nocodazole-treated cells relative to the controls. On the other hand, total cellular immunoreactivity with PHF-1 and Alz-50 in the ELISA remains unchanged (Table I).

Nocodazole treatment produced an increase in TG and MC reactivity with certain antigens on immunoblots (Fig. 5). In general, marked increases in immunoreactivity with one or two specific antigens was seen, but phospho-epitopes recognized by some antibodies appear more ubiquitous. For example, TG-3 recognizes a 105-kD antigen that is not recognized by any other TG or MC antibody, whereas a 125-kD mitotic antigen is recognized by TG-4, MC2, MC15, and MC6, and a 33–35-kD antigen is recognized by MC5 and MC15.

Surprisingly, none of the TG/MC antibodies react with the 55–57-kD tau proteins which are readily detected with Alz-50 and PHF-1 (Fig. 5). We have confirmed these findings using heat stable fractions in which the tau proteins are characteristically enriched (data not shown). Moreover, the reactivity of the PHF-1 antibody with tau decreases upon treatment with nocodazole, as does the apparent molecular weight of tau in SDS-PAGE (Fig. 5),

suggesting that tau becomes dephosphorylated under these conditions. These results concur with the lack of a quantitative increase (or slight decrease) in PHF-1 immunoreactivity in ELISA. On the other hand, the metaphase-associated increases in TG and MC immunofluorescence, are corroborated by quantitative increases in total cellular immunoreactivity with the antibodies in ELISA, and increased immunoreactivity with specific antigens on immunoblots.

TG/MC Reactivity in Mitotic Cells Correlates with an Increase in Mitotic Kinase Activity But Not cdk5 Activity

The progression of mitotic cell division is driven by a cascade of protein phosphorylation mediated by the cdc2 kinase/cyclin B complex whose activity peaks in metaphase (reviewed by Nurse, 1990; Maller, 1991). While the level and activity of cdc2 in dividing cells is high, the kinase is barely detectable in fully differentiated cells such as neurons of adult brain (Hayes et al., 1991; Meyerson et al., 1992). Instead, postmitotic neurons have been found to contain another cdc2-related cyclin–dependent protein kinase called cdk5 (Hellmich et al., 1992; Lew et al., 1992; Tsai et al., 1993). Although cdk5 is expressed in all tissues, it is active only in differentiated neurons (Lew et al., 1994; Tsai et al., 1994). The kinase has been reported to phosphorylate tau in vitro producing PHF epitopes (Bauman et al., 1992; Hisanaga et al., 1993; Paudel et al., 1993), and is therefore a more reasonable candidate for PHF phosphorylation than is the cdc2 kinase.

Our findings above show abundant expression of PHF phospho-epitopes in cycling mammalian cells of various tissue types, none of which are expected to have any cdk5 activity. The appearance of the TG/MC phospho-epitopes in these cells in metaphase synchronizes with the optimal activity of the mitotic cdc2 kinase, and their reduced appearance in anaphase coincides with the inactivation of the cdc2 kinase. We therefore hypothesized that mitotic kinases may be responsible for the production of the TG/MC phospho-epitopes. To verify this, we assayed the activities of the mitotic kinase cdc2 and cdk5 in lysates from control and nocodazole-treated MSN cells.

An antibody recognizing a COOH-terminal sequence and specific for the p34 cdc2 kinase, and a cdk5-specific polyclonal antibody were used to immunoprecipitate the respective kinases for activity assays. Reciprocal cross blotting of the cdc2 and cdk5 immunoprecipitates revealed no detectable cdk5 immunoreactivity in the cdc2 precipitate from control or nocodazole-treated cells, and no cdc2 contamination of the precipitated cdk5 kinase from either samples (Fig. 6 A). In addition, mitotic kinase activity was precipitated using an agarose conjugate of the yeast protein, p13suc1, which forms a stable functionally active complex with mitotic kinases (Brizuela et al., 1987; Draetta et al., 1987). Kinase activities were assayed using histone H1 as exogenous substrate, and incorporation of labeled phosphate into the histone bands in the gel were quantitated using a Phosphoimager. H1 kinase activity was increased 10-fold in p13suc1 and 2-fold in cdc2 precipitates obtained from nocodazole-treated cells in comparison with precipitates isolated from control cells (Fig. 6 B). In
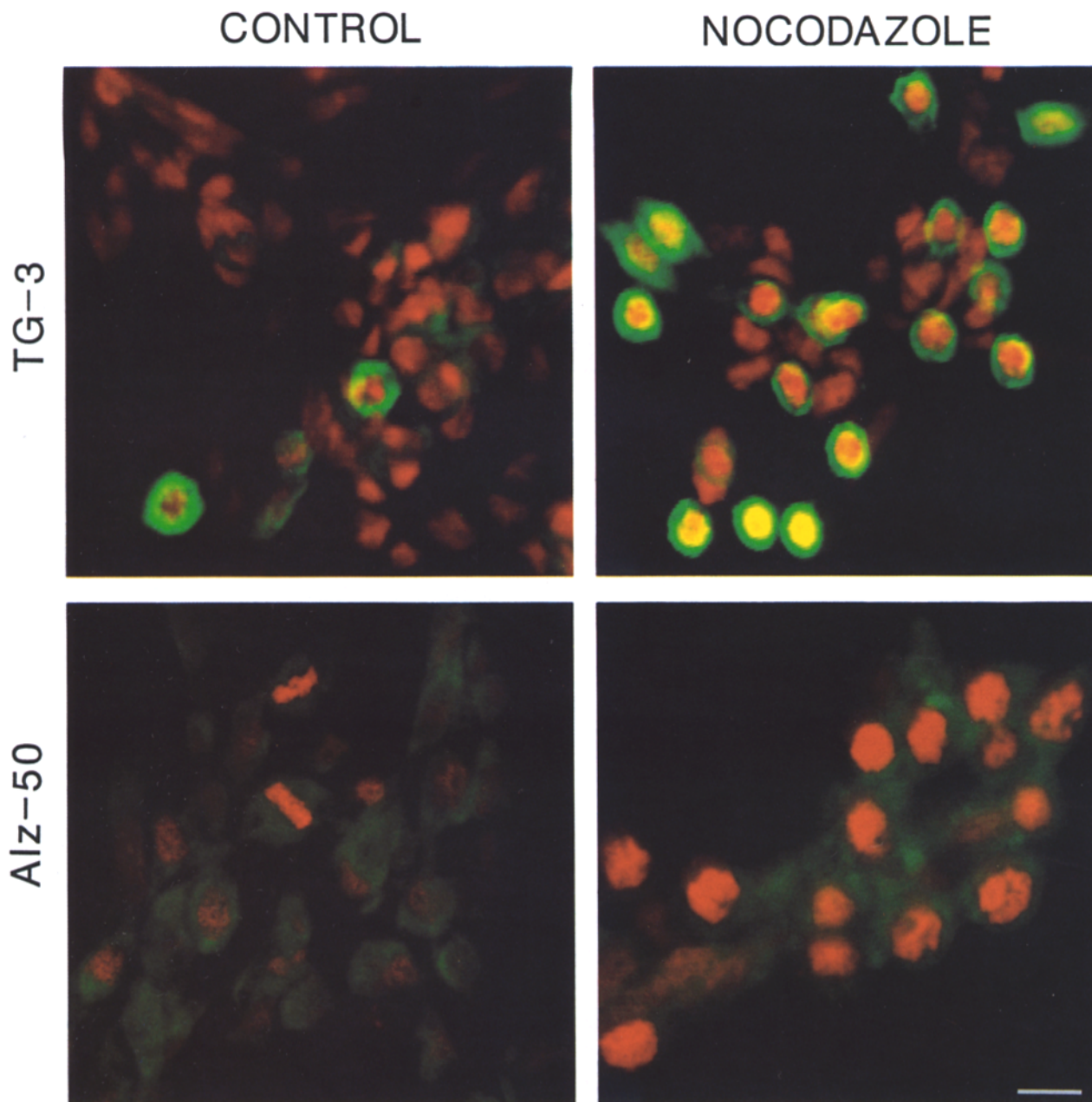
CONTROL   NOCODAZOLE

Figure 4. Effects of nocodazole on TG-3 immunofluorescence staining in MSN cells. Asynchronous cultures of MSN cells (*control*) and those treated with nocodazole were double stained with TG-3 (*green, top* panels) and propidium iodide (*red*). Nocodàzole increased the number of cells in M phase, and concomittantly increased the number of cells stained with TG-3. Additional samples were double stained with Alz-50 (*green, bottom*) and propidium iodide (*red*) and showed no increase in Alz-50 staining accompanying the nocoda-zole-induced increase in M phase cells. Data were analyzed by confocal laser microscopy. Bar, 10 µM.

contrast, there was no detectable activity in the cdk5 ki-nase precipitates from either control or nocodazole-treated samples. The failure to detect cdk5 kinase activity in these samples is not due to the cdk5 antibody used for precipitation, because the same antibody does precipitate active cdk5 kinase from human brain tissue (Fig. 6 *A*). In-stead, the lack of measurable cdk5 kinase activity in these neuroblastoma cells is consistent with the data from Tsai et al. (1993), showing an absence of cdk5 activity in neuro-blastoma cells despite high cdk5 protein levels. These ki-nase activity assays support the suggestion that the appear-

ance of the TG and MC epitopes in MSN neuroblastoma cells correlates with increased mitotic kinase activity, but not with cdk5 activity.

The Monoclonal Antibody MPM-2, a Marker for Mitosis, Reacts with NFT, Senile Plaques, and Neurons in AD

The correspondence between the appearance of PHF phospho-epitopes and increased mitotic kinase activity in dividing cells, led to speculation that a similar increase in

Antibody	Control	Nocodazole	P value
	mean ± SEM	mean ± SEM	
TG-3	2.10 ± 0.18	4.24 ± 0.11	0.000
TG-4	1.72 ± 0.31	4.25 ± 0.28	0.003
MC2	3.83 ± 0.49	9.36 ± 0.90	0.003
MC3	1.26 ± 0.18	2.47 ± 0.46	0.022
MC5	1.38 ± 0.19	1.93 ± 0.52	0.107
MC6	1.89 ± 0.28	4.93 ± 0.97	0.003
MC15	4.84 ± 0.60	10.94 ± 0.49	0.002
PHF-1	4.00 ± 0.13	3.82 ± 0.23	0.716
Alz-50	1.69 ± 0.19	1.60 ± 0.29	0.576

Total cell lystates were prepared from control and nocodazole treated MSN cultures, and serially diluted into microtitre ELISA plates. Immunoreactivity with the TG/MC antibodies was assayed using isotype-specific secondary antibodies conjugated with HRP and ABTS substrate. The units for activity are arbitrary. The data represent means for a total of six determinations in the case of TG-3, TG-4, and PHF-1, and three determinations for the remaining antibodies, with standard errors of the mean. Differences between control and nocodazole treated specimens were evaluated using a paired *t* test, and *P* values are presented for the significance of the differences. Total cellular immunoreactivity with the TG/MC antibodies was increased following treatment of MSN cells with nocodazole.

mitotic kinase activity may be responsible for production of these mitotic phospho-epitopes in AD. If a mitotic mechanism is involved in AD pathology, then other markers for mitosis would be found in AD brain tissue. We examined the staining of routinely fixed autopsy human brain tissue with the monoclonal antibody MPM-2 that was raised against mitotic Hela cells (Davis et al., 1983), and recognizes a phospho-epitope that is highly conserved in mitotic proteins of all eukaryotic species (Vandre et al., 1984, Vandre et al., 1986; Keryer et al., 1987; Engle et al., 1988; Wordeman et al., 1989; Tombes et al., 1991; Vandre et al., 1991). No detectable staining is observed with this antibody in hippocampal or temporal cortical brain sections from 12 normal individuals. On the other hand, the MPM-2 antibody displays widespread staining of NFT and neuritic plaques in hippocampal and cortical tissue sections from 12 AD cases (Fig. 7 A). In addition to the staining of these lesions in AD, the MPM-2 antibody reacts with large numbers of hippocampal pyramidal cells displaying a punctate pattern in the cytoplasm. Similar staining of neurons is not observed in normal brain tissue. To verify that the MPM-2 antibody is specific for mitotic cells, we stained MSN cultures with the antibody and found that
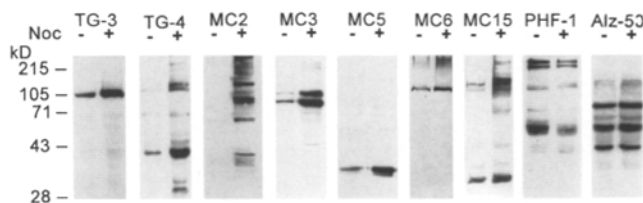

Figure 5. The effects of nocodazole on the immunoreactivity of the TG/MC antibodies with MSN proteins in immunoblots. Detergent-extracted supernatants from control (−) and nocodazole-treated (+) MSN cell cultures were immunoblotted with the TG/MC antibodies. The staining with MC15 was detected colorimetrically, and the staining with the remaining antibodies was detected by ECL (exposure time 10–30 s). The TG/MC antibodies show marked increases in immunoreactivity with proteins in M phase extracts relative to control.
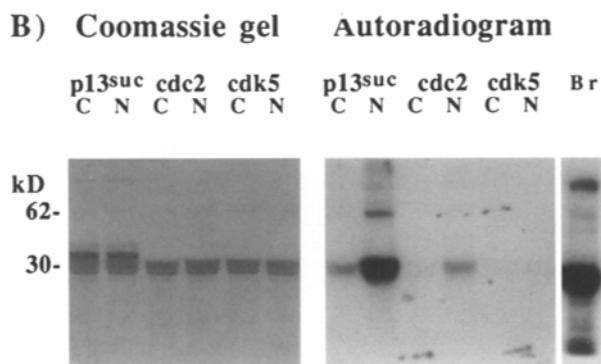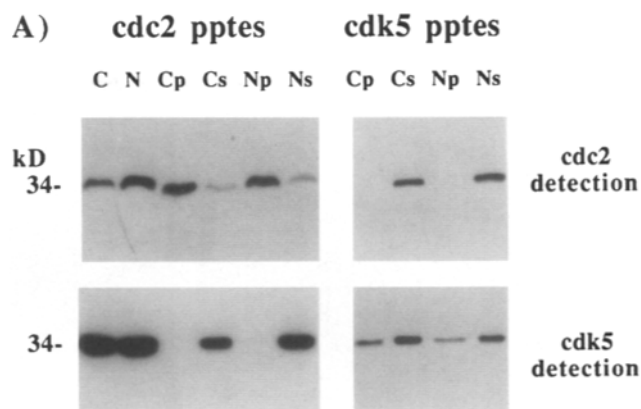

Figure 6. (A) Reciprocal immunoprecipitation and cross blotting with cdc2 and cdk5 antibodies and extracts from control and nocodazole-treated MSN cells. Supernatants from control (C) MSN cells and those incubated with nocodazole (N) containing equal amounts of protein were subjected to immunoprecipitation with the COOH-terminal polyclonal cdc2 antibody (left) and an anti-cdk5 polyclonal antibody (right) as described in methods. In the two upper panels the cdc2 detection antibody is an anti-pstaire monoclonal antibody, and in the bottom panels the detection antibody is an anti-cdk5 monoclonal antibody. The first two lanes marked C and N in the left panels represent the starting supernatants from control and nocodazole treated cells respectively. Cp and Np refer to the precipitates from control or nocodazole cells, and Cs and Ns represent the supernatants remaining after each precipitation. The results indicate that the cdc2-immunoprecipitate is not contaminated with any co-precipitated cdk5, and that the cdk5 immunoprecipitate is free from any co-precipitating cdc2. (B) Histone H1 kinase assay with p13suc1-precipitates, cdc2 immunoprecipitates, and cdk5 immunoprecipitates from control (C) and nocodazole-treated MSN cells (N). Supernatants from control and nocodazole-treated cells were incubated with GST-p13suc1-agarose beads to enrich for mitotic kinases. The p13suc1 precipitates along with aliquots of the cdc2 and cdk5 immunoprecipitates shown in A, were incubated with histone H1 and γ^{32}P-ATP, and the phosphorylated mixtures were resolved by electrophoreses. The gel was stained with Coomassie blue (left), dried, and subjected to autoradiography (right). The relative amounts of phosphate incorporation into the histone bands were quantitated using phosphoImager. The histone H1-phosphorylating activity of cdc2 immunoprecipiated from M phase extracts was greatly increased relative to the control. On the other hand, cdk5 immunoprecipitates from M phase extracts showed no increased phosphorylating activity. The last lane labeled Br is a kinase reaction with cdk5 immunoprecipitate from normal human brain, and controls for the ability of the cdk5 polyclonal antibody to precipitate active cdk5.
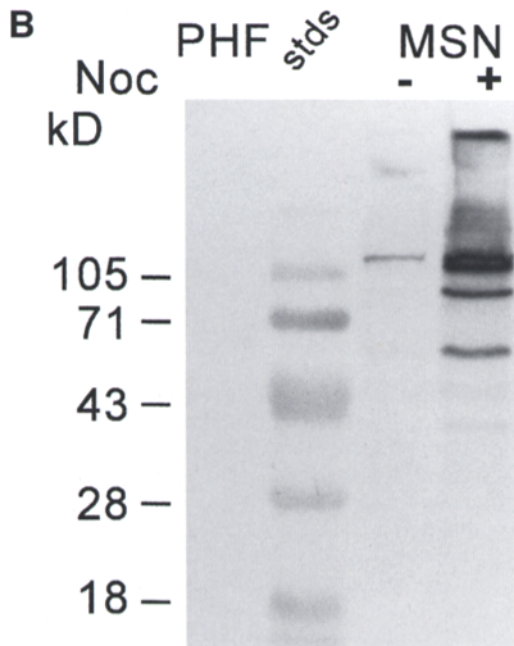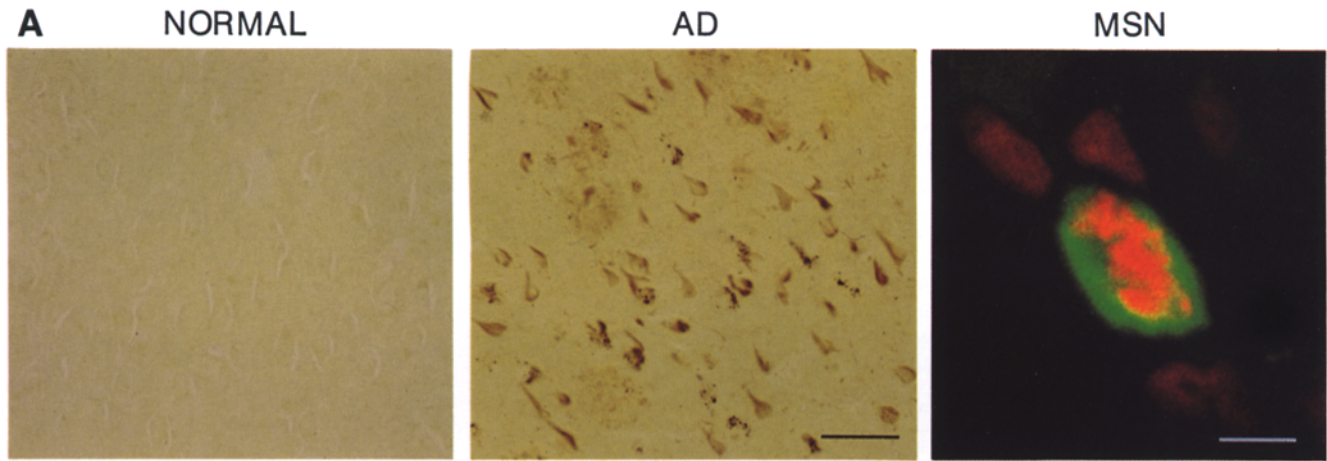
A NORMAL   AD   MSN

B
Noc PHF stds MSN
kD   − +

105 —
71 —
43 —
28 —
18 —

Figure 7. (*A*) Staining of human brain and MSN cells with the MPM-2 monoclonal antibody. Sections of hippocampal tissue from normal and AD cases were stained with the MPM-2 antibody. MPM-2 reacts with NFT, neurites of senile plaques, and neurons in AD, but has no detectable reactivity in normal brain. The bar shown in the light micrographs represents 20 μm. Double staining experiments were conducted with the MSN cells using MPM-2 (*green*) and propidium iodide (*red*) to show that MPM-2 is specific for cells in M phase. Fluorescence data were analyzed by confocal laser microscopy. Bar = 10 μm. (*B*) Immunoreactivity of MPM-2 with PHF and MSN lysates. Immunoaffinity purified PHF preparations and detergent-extracted supernatants from control (−) and nocodazole-treated (+) MSN cells were subjected to electrophoreses and electrophoretic blotting. For comparison, the same amounts of protein were loaded onto this gel as were loaded for studies with the TG/MC antibodies in Figs. 1 and 5, respectively. The blot was stained with the MPM-2 antibody, and staining was detected by ECL, with an exposure time of 30 s. Although MPM-2 displayed increased reactivity with M phase MSN cell extracts relative to control, no MPM-2 reactivity was detected with the PHF sample after exposure with ECL reagent for 15 min.

it reacted exclusively with cells undergoing mitosis (Fig. 7 *A*). On immunoblots MPM-2 showed a dramatic increase in immunoreactivity with a number of proteins in nocodazole-treated cell lysates relative to the control lysates (Fig. 7). MPM-2 does not react with the immunoaffinity purified PHF preparation, suggesting that it recognizes other phosphoproteins in AD brain that are not detectable in normal brain.

Discussion

The temporal coincidence of the occurrence of phospho-epitopes recognized by seven new AD-specific monoclonal antibodies with optimal mitotic kinase activity in cycling eukaryotic cells, has led us to hypothesize that mitotic kinase activity produces these phospho-epitopes in AD pathology. This hypothesis is supported by evidence obtained with the monoclonal antibody, MPM-2, which was raised against mitotic HeLa extracts and shows abundant reactivity with neurofibrillary pathological structures in AD brain tissue, but no reactivity in normal brain.

The confinement of AD-specific phospho-epitopes to M

phase of eukaryotic cell division is of value in predicting which protein kinase produces these epitopes in intact cells. Evidence suggests that the MAP kinases, GSK-3 kinase, and the nonmitotic cyclin dependent kinases are not activated in M phase, and may be excluded from having a role in PHF epitope formation in dividing cells. From amongst the protein kinases that have been implicated in PHF phosphorylation in vitro, only the mitotic kinases, p34 cdc2 and cdk2 are activated in M phase, and are therefore likely to be involved in the production of the PHF epitopes. Mitotic kinases have also been implicated in the formation of the MPM-2 epitope (Verde et al., 1990; Yamashita et al., 1990; Tombes et al., 1991) which is elevated in M phase, and in AD brain.

The suggestion that mitotic kinases may produce mitotic phospho-epitopes in brain presents a puzzle, because mitotic kinases are abundant in proliferating neurons but become undetectable upon terminal differentiation (Hayes et al., 1991; Meyerson et al., 1992; Freeman et al., 1994). However, cdc2 kinase immunoreactivity has been described in NFT in AD brain tissue using a spectrum of cdc2-specific antibodies (Wood et al., 1993; Liu et al., 1995). The

latter of these studies (Liu et al., 1995) also reported a novel PHF-associated 33-kD kinase reactive with NH_2- and COOH-terminal cdc2 antibodies but lacking reactivity with anti-pstaire antibodies. Antibodies directed at COOH-terminal cdc2 sequences are the only available reagents entirely specific for cdc2 (Draetta and Beach, 1988). An additional study employing COOH-terminal, cdc2-antibodies found elevated levels of p34 cdc2 in extracts from AD brain in comparison with normal brain (Ledesma et al., 1992). The striking association of the cdc2 kinase with NFT in AD brain supports a role for this kinase in the disease pathology. It is unclear why the TG/MC and MPM-2 epitopes, which are present in most M phase cells in culture, are totally undetected in nonneuronal proliferating glial and endothelial cells in brain. A possible explanation is that these epitopes in dividing cells and NFT may be differentially sensitive to the method of fixation of brain tissue, an idea reminiscent of tau epitopes which are undetectable in neurons after formalin fixation, but are clearly evident in NFT in formalin-fixed tissue from AD brain (reviewed by Kosik and Greenberg, 1994). An intriguing adjunct to the suggested role of mitotic kinases in AD, is the phosphorylation of the amyloid precursor protein (APP) by cdc2, with maximal phosphorylation at the G2/M phase of the cell cycle (Suzuki et al., 1994). It is believed that inappropriate processing of the APP protein in AD may be crucial to the massive deposition of insoluble β-amyloid in the brains of AD patients (Kang et al., 1987; Haas et al., 1992). Although phosphorylation of APP by the cdc2 kinase has not been reported in AD brain tissue, this posttranslational event has been suggested to affect the proteolytic processing of the APP protein.

Our finding of the TG/MC phospho-epitopes in HeLa cervical carcinoma cells, B and T cells, and fibroblasts, i.e., cells without functionally active cdk5 kinase (Lew et al., 1994; Tsai et al., 1994), suggests that cdk5 may not be essential for formation of these epitopes. Histone H1 kinase assays also demonstrate that cdk5 is not active in nocodazole-treated neuroblastoma, and is therefore unlikely to be involved in the formation of PHF epitopes in M phase. While our data illustrate the significance of mitotic kinases in AD they do not exclude the possibility that other kinases like cdk5 may function either concomitantly or in series to produce other phospho-epitopes in AD.

Despite the colocalization of the PHF-1 and TG/MC phospho-epitopes in NFT in AD, it appears that the biochemical mechanisms regulating the turnover of these phospho-epitopes may be different. While the TG/MC phospho-epitopes are quantitatively increased in mitotic cells, the PHF-1 epitope in tau becomes dephosphorylated during mitosis. This finding is consistent with the increased ability of tau to bind to microtubules when it is dephosphorylated (Lindwall and Cole, 1984; Drechsel et al., 1992). It is however, in contrast to the data of Pope et al. (1994) who showed that PHF-1 immunoreactivity with tau from mitotic cells is enhanced relative to tau from interphase cells. Our studies also indicate that the TG and MC antibodies show little reactivity with hyperphosphorylated MSN tau which we previously showed (Vincent et al., 1994) to be strongly reactive with PHF-1, NP8, and the T3P antibody of Lee et al. (1991). Since the TG/MC phospho-epitopes appear to be evolutionarily conserved in mitotic cells, their antigens may correspond to previously described mitotic proteins. Although some of these proteins seem similar to the mitotic phosphoproteins recognized by the MPM-2 antibody in mitotic MSN cells, MPM-2 does not react with our purified PHF preparation on blots, raising the possibility that the staining of NFT with MPM-2 may be associated with yet another independent group of antigens. Thus in addition to tau, other as yet unidentified aberrantly phosphorylated proteins may be present in AD brain. Further studies aimed at the identification of the TG/MC and MPM-2 antigens in AD brain would greatly clarify this picture. Just as the occurrence of the MPM-2 epitope in proteins is indicative of mitotic kinase activity regardless of what proteins the epitope is found in during mitosis, our data point to a functional relationship between mitotic kinase activity and the formation of the AD-specific TG/MC phospho-epitopes. It is not unreasonable to expect that misappropriate activation of mitotic kinases in mature neurons might lead to production of mitotic phospho-epitopes in proteins quite different from those in cycling cells, because the available protein substrates in mature neurons are quite different. Thus the TG/MC and MPM-2 antigens in AD may not necessarily be the same antigens found in cultured cells in M phase. We suggest that, irrespective of the identities of the TG/MC and MPM-2 antigens in AD brain, mitotic kinases appear to be the most reasonable candidates for the production of the TG/MC and MPM-2 phospho-epitopes.

Cell cycle proteins are progressively being implicated in cellular processes that encompass the entire repertoire of cellular responses to external and internal stimuli (Williams et al., 1992; Williams and Smith, 1993). Programmed cell death has recently been hypothesized to result from aberrant cell cycle control leading to "abortive mitosis" (Ucker et al., 1991; Lee et al., 1993). Certain studies have shown a lack of involvement of the cdc2 kinase in apoptosis (Norbury et al., 1994; Oberhammer et al., 1994) and others support activation of cdc2 (Steinmann et al., 1991; Meikrantz et al., 1994; Shi et al., 1994). Although it is not known whether neuronal death in AD proceeds by apoptosis, necrosis, or some as yet unidentified mechanism (Dickson, 1995; Lassman et al., 1995), the cdc2 kinase or other mitotic kinases may be a convergent point for any one of the mechanisms cited above. Recent reports of the occurrence of S phase markers, p105 (Masliah et al., 1993) and Ki67 (Smith and Lippa, 1995) in NFT are consistent with the idea of misregulated expression of proliferation antigens in AD. A systematic analysis of mitotic kinases and their associated proteins would help determine whether production of mitotic phospho-epitopes in AD is mediated by mitotic posttranslational mechanisms or other cellular pathways.

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