Variations in *in vivo* phosphorylation at the proline-rich domain of the microtubule-associated protein 2 (MAP2) during rat brain development

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Microtubule-associated protein 2 (MAP2) is an *in vitro* substrate for MAP kinase. Part of the phosphorylation occurs at the C-terminal microtubule-binding domain of the molecule which contains a cluster of putative consensus sites for MAP kinase on a proline-rich region. A peptide with the sequence RTPGTPGTPSY, located at this region of the molecule, is efficiently phosphorylated by MAP kinase *in vitro*. An antibody (972) raised against this non-phosphorylated peptide has been used to test for *in vivo* phosphorylation at the proline-rich domain of the MAP2 molecule. The reaction of purified MAP2 with antibody

972 diminishes after *in vitro* phosphorylation by MAP kinase and is enhanced after *in vitro* dephosphorylation by alkaline phosphatase. A fraction of brain MAP2 isolated by iron-chelation affinity chromatography appears to be phosphorylated *in vivo* at the site recognized by antibody 972. There is some variation in the phosphorylation of MAP2 at the proline-rich region throughout rat brain development. MAP2C is more highly phosphorylated in the developing rat brain, whereas high-molecular-mass MAP2 is more extensively phosphorylated in the adult rat brain.

INTRODUCTION

Microtubule-associated protein 2 (MAP2) is a major component of the neuronal cytoskeleton [1–3]. In mammalian brain, there are several forms of MAP2 that arise from alternative splicing [3,4]. These include high-molecular-mass proteins known as MAP2A and MAP2B (with apparent molecular masses around 270 kDa determined by denaturing gel electrophoresis [5]) and low-molecular-mass forms referred to as MAP2C and MAP2D (with apparent molecular masses around 70 kDa determined by denaturing gel electrophoresis [6,7]). MAP2C consists of the N- and C-terminal domains of MAP2A/B joined together and lacking the middle intervening sequence. The C-terminal segment of the molecule contains a proline-rich region followed by three or four conserved microtubule-binding sequences [5–7].

MAP2C is expressed in developing mammalian brain and it is down-regulated during brain maturation, whereas MAP2A/B is more prominent in the adult brain [3]. MAP2C has a widespread distribution in brain, being present in neuronal cell bodies, dendrites and axons as well as in glial cells [3]. In contrast, MAP2A/B is selectively localized in dendrites and neuronal cell bodies [3,8–10]. In addition to its association with microtubules, MAP2 is co-localized with actin and associated with actin microfilaments, membrane organelles and the post-synaptic density in dendritic spines [11,12].

MAP2A/B (simply referred to as MAP2) is one of the best *in vitro* substrates for a variety of protein kinases [13–19] and phosphatases [20,21]. Extensive *in vitro* phosphorylation of MAP2 favours microtubule disassembly [13,22–24]. In fact, a fraction of MAP2 isolated from adult rat brain contains up to 46 phosphates per molecule and binds less efficiently to microtubules than another fraction of MAP2 which contains 16 phosphates per molecule [25]. However, completely dephosphorylated MAP2 cannot efficiently interact with microtubules [26]. Phosphoryl-

ation also controls the association of MAP2 with actin microfilaments [27,28].

Interestingly, a correlation between the phosphorylation state of MAP2 and several forms of neuronal plasticity has been noticed, leading to the proposal of a physiological role for MAP2 phosphorylation and dephosphorylation in triggering cytoskeletal changes in response to certain extracellular signals [29–34]. It is, however, extremely difficult to observe *in vivo* subcellular and molecular events that may be linked to adaptative changes in brain. The development of reagents such as antibodies that can discriminate between distinct phosphorylated forms of a protein may be useful in this respect. Here we have focused on the phosphorylation of MAP2 at the proline-rich region of the C-terminal domain of the molecule. This domain contains eight Pro-X-Ser/Thr-Pro motifs which may be targets for MAP kinases *in vitro*. One of these sites has been used to raise a monospecific polyclonal antibody that binds to MAP2 in a phosphorylation-sensitive manner. Using this immunochemical approach we have analysed the *in vivo* phosphorylation of MAP2 in rat brain extracts at three developmental stages: embryonic day 19, postnatal day 1 and postnatal day 21.

MATERIALS AND METHODS

Protein preparation

Brains were obtained from cows or Wistar rats of different ages. To prepare MAP2, brains were homogenized in 0.1 M Mes, pH 6.5, containing 1 mM MgCl₂, 2 mM EGTA, 0.1 M NaCl, 1 mM phenylmethanesulphonyl fluoride (PMSF), 10 µg/ml pepstatin, 10 µg/ml leupeptin and 10 µg/ml aprotinin (in some cases, homogenization was performed in the presence of 1 µM okadaic acid and 50 mM NaF as phosphatase inhibitors). Homogenates were centrifuged at 100000 g for 1 h at 2 °C. Pellets were

discarded and the supernatants (brain cytosolic fractions) were collected. In some cases, supernatant were treated with alkaline phosphatase (see below). Brain cytosolic fractions were brought to 1 M NaCl and 10 mM dithiothreitol (DTT), boiled for 10 min and centrifuged at 100000 *g* for 30 min at 2 °C. Heat-resistant proteins present in the resulting supernatants were used as a source for MAP2 purification.

Heat-resistant cytosolic proteins were applied to a Sepharose CL-4B (Pharmacia) column (40 ml) previously equilibrated with buffer (10 mM Pipes, pH 6.9, 0.2 M NaCl and 1 mM DTT). Purified MAP2A/B was eluted as one of the first peaks from this column as previously described by Herzog and Weber [35].

Microtubule protein from brain cytosol was prepared by the procedure of Karr et al. [36].

The peptide P with the sequence RTPGTPGTPSY (comprising residues 1616–1626 of MAP2 according to the sequence published by Lewis et al. [5]) was synthesized on an automatic solid-phase peptide synthesizer (type A430A; Applied Biosystems) and purified by reversed-phase h.p.l.c. on a Nova Pak C18 column (Waters). The corresponding phosphorylated peptide was obtained by solid-phase phosphorylation and oxidation [37].

Purified MAP kinase (p44^{mapk}, ERK1) was purchased from UBI (Lake Placid, NY, U.S.A.; Cat. no. 14-102).

Antibodies

Polyclonal antibody 972 was raised in rabbit against the synthetic peptide P (see above) coupled to keyhole limpet haemocyanin using glutaraldehyde as cross-linker. Peptide coupling and immunization were performed as described by Ulloa et al. [38]. Immunoglobulins were purified from sera by (NH₄)₂SO₄ precipitation. The specific reaction of antibody 972 with the P peptide was checked by dot-blot and competition assays as indicated by Ulloa et al. [38]. Polyclonal antibody 10 was raised in a mouse immunized with purified rat brain MAP2. The specificity of the reaction was checked by an immunoblotting assay. It recognizes mainly MAP2A/B. Rabbit polyclonal antibody 660, raised against bovine brain MAP2, was also used to recognize MAP2A/B and MAP2C. Monoclonal antibody MPM2, which binds to a phosphothreonine-proline-containing epitope [39], was the generous gift of Dr. J. Kuang and Dr. P. N. Rao (MD Anderson Cancer Center, Houston, TX, U.S.A.).

Protein and peptide phosphorylation

The phosphorylation of purified MAP2 (10 µg) with MAP kinase (40 ng of a preparation which transfers 0.6 nmol of phosphate/min and 1 mg of kinase into myelin basic protein) was performed for 4 h at 37 °C in 20 mM Hepes, pH 7.4, containing 2 mM EGTA, 10 mM MgCl₂, 1 mM PMSF, 1 mM DTT, 10 µg/ml pepstatin, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 2 µM okadaic acid and 100 µM [γ -³²P]ATP (Amersham). Phosphorylation was stopped by the addition of SDS sample electrophoresis buffer. Phosphorylated protein was analysed by SDS/PAGE and autoradiography of dried gels exposed to Kodak X-Omat films. To determine the stoichiometry of phosphorylation of MAP2, the phosphorylation assays were performed at increasing ATP concentrations and counts incorporated into MAP2 were determined.

Phosphorylation of the P peptide by MAP kinase was carried out in the same buffer as was used for MAP2 protein. Phosphorylation was stopped by boiling for 2 min in the presence of 1% trifluoroacetic acid. Phosphorylated peptide was isolated by reversed-phase h.p.l.c. on a Nova Pak C18 column and the radioactivity associated with the eluted peptide was determined by Cerenkov radiation counting.

Phosphatase treatment

Samples (50 µg of protein) were incubated with 3.2 units of alkaline phosphatase (Boehringer-Mannheim GmbH; cat. no. 1097 075) in dephosphorylation buffer containing 50 mM MgCl₂, 100 mM Tris/HCl pH 8.5, 1 mM PMSF, 10 µg/ml pepstatin, 10 µg/ml leupeptin and 10 µg/ml aprotinin for 2 h at 37 °C. After dephosphorylation, samples were brought to 1 M NaCl and 10 mM DTT, boiled for 5 min and centrifuged at 100000 *g* for 15 min. Supernatants containing dephosphorylated heat-resistant proteins were collected.

Limited proteolysis of MAP2

Purified MAP2, previously phosphorylated *in vitro* by MAP kinase, was partially digested with *Staphylococcus aureus* V8 protease as previously described [30,40]. Phosphopeptides were analysed by gel electrophoresis and autoradiography of dried gels on to Kodak X-Omat films. Among the resulting phosphopeptides, the 54 kDa peptide was eluted from the gel and sequenced with an Applied Biosystems gas-phase sequencer (model 470A) equipped with an on-line phenylthiohydantoin amino acid analyser (model 120A). Alternatively, peptides derived from digested MAP2 were analysed by Western blotting.

Iron-chelation affinity chromatography (i.c.a.c.)

Columns (100 µl) were prepared with chelating Sepharose Fast Flow (Pharmacia Fine Chemicals, Uppsala, Sweden) [41,42]. Briefly, the gel bed was washed with 4 vol. of water, treated with 4 vol. of 50 mM FeCl₃ and washed again with water. The column was then equilibrated in binding buffer (0.1 M Mes, 0.2 M NaCl and 1 mM DTT, pH 5.7). Purified MAP2 (200–300 µg) was loaded on to the i.c.a.c. column, which was washed with binding buffer and eluted by a stepwise pH gradient composed of the following solutions: 100 mM Tris/HCl, 1 M NaCl and 1 mM DTT at pH 7.8, pH 8.0, pH 8.4 and 8.6. The most strongly bound protein and the chelated iron were eluted by washing with 200 mM EDTA. Collected fraction were analysed by electrophoresis.

Electrophoresis

SDS/PAGE was carried out by the procedure of Laemmli [43] on 5% or 8% polyacrylamide gels. Gels were stained with either Coomassie Brilliant Blue or imidazole/zinc salts [44]. Gel densitometry was performed on a Molecular Dynamics densitometer model 300A equipped with Image Quant software version 3.0.

Western-blot analysis was performed by the method of Towbin et al. [45]. To test for antibody reaction, the ECL Amersham kit was used. Autoradiograms were also quantified by densitometry as described above.

RESULTS AND DISCUSSION

In vitro phosphorylation of MAP2 by MAP kinase

Incubation of purified bovine brain MAP2 with pure MAP kinase p44^{mapk}, ERK1) results in modification of the protein as shown in Figure 1(a). In the presence of 1 mM ATP and after 4 h of incubation at 37 °C, nearly 6 mol of phosphate/mol of MAP2 are incorporated (Figure 1b). This phosphorylation stoichiometry is very similar to that previously described by Hoshi et al. [13]. A higher phosphorylation stoichiometry is observed when phosphatase-treated MAP2 is used as substrate, indicating that some phosphorylation sites are endogenously

Figure 1 *In vitro* phosphorylation of MAP2 by MAP kinase

(a) Purified bovine brain MAP2 (10 μg) was incubated for 30 min with MAP kinase (40 ng of a preparation which transfers 0.6 nmol of phosphate/min and 1 mg of kinase into myelin basic protein) and 100 μM [γ - 32 P]ATP as indicated in the Materials and methods section. Phosphorylated protein was characterized by SDS/PAGE, followed by autoradiography of dried gels exposed to Kodak X-Omat films. Lane 1, Coomassie Blue staining of the gel; lane 2, corresponding autoradiogram. (b) Purified bovine brain MAP2 (10 μg) was incubated for 4 h with p44 MAP kinase (40 ng) and increasing concentrations of [γ - 32 P]ATP: 1 μM, 10 μM, 100 μM, 0.5 mM and 1 mM. Phosphate incorporation into protein was measured. The maximum incorporation was nearly 6 mol of phosphate/mol of MAP2.

Figure 2 Phosphopeptide pattern of *in vitro* phosphorylated MAP2

Purified bovine brain MAP2, previously phosphorylated with MAP kinase, was partially digested with *S. aureus* V8 protease. Phosphopeptides were analysed by gel electrophoresis and autoradiography (a). The positions of prestained molecular-mass standards (Sigma) are indicated on the left. (b) Presumed localization of the major 54 and 42 kDa phosphopeptides (marked with asterisks in a) on the MAP2 molecule according to the scheme proposed by Díaz-Nido et al. [30]. The three squares correspond to the three tubulin-binding motifs of the MAP2 molecule. The sequence determined for the N-terminus of the 54 kDa phosphopeptide is also shown.

phosphorylated in purified brain MAP2, as previously suggested [13,25,26].

Localization of modified residues

Limited proteolysis by *S. aureus* V8 protease of purified bovine brain MAP2 previously phosphorylated *in vitro* with MAP kinase generates several phosphopeptides (Figure 2a). The two largest, with apparent molecular masses of 54 kDa and 42 kDa, are marked with asterisks in Figure 2(a); these phosphopeptides may be derived from the region containing the microtubule-

Figure 3 *In vitro* phosphorylation of P peptide by MAP kinase

(a) Effect of substrate concentration on the incorporation of phosphate into P peptide catalysed by MAP kinase. Different concentrations of P peptide were incubated with MAP kinase in the presence of 0.1 mM [γ - 32 P]ATP for 20 min at 37 °C. The reaction was stopped and the incorporation of phosphate into peptide was determined as indicated in the Materials and methods section. (b) Double-reciprocal plot for the determination of K_m and V_{max} for the phosphorylation of P peptide by MAP kinase. The K_m and V_{max} values obtained are 2 mM and 500 μmol/min per mg respectively.

binding domain of the MAP2 molecule [30,46] as indicated in Figure 2(b). Indeed, the 54 kDa phosphopeptide is located at the C-terminal region of the MAP2 molecule, as its N-terminal sequence is LAKKSE, which corresponds to residues 1475–1480 of the MAP2 molecule. This result agrees well with a previous determination of the sequence of the *in vivo* phosphorylated 54 kDa phosphopeptide derived from MAP2 (J. Díez-Guerra, unpublished work).

Silliman and Sturgill [14] reported that the C-terminal microtubule-binding domain of MAP2 was scarcely phosphorylated, whereas Hoshi et al. [13] found a significant phosphorylation of this MAP2 domain. Our results agree well with those reported by Hoshi et al. [13]. Perhaps the discrepancies between these results and those of Silliman and Sturgill [14] may be due to the presence of different levels of endogenous phosphate in different MAP2 preparations.

The MAP2 molecule contains 18 (Pro)-X-Ser/Thr-Pro motifs which may be considered putative targets for proline-directed protein kinases, particularly MAP kinases, according to the substrate specificity of these enzymes [46–48]. Eight of these motifs are clustered in the C-terminal region of MAP2. Interestingly, one of these sites has the sequence TPGTPGTPSY (comprising residues 1617–1626 of the MAP2 sequence) which is very similar to the sequence SPGSPGTPGS (comprising residues 199–208 of the tau sequence) found at the proline-rich region of the microtubule-associated tau protein [49]. This peptide on tau protein can be phosphorylated *in vitro* by proline-directed kinases including MAP kinases [49]. In the same way, the synthetic peptide referred to as P is efficiently phosphorylated *in vitro* by purified MAP kinase. Figure 3 shows the apparent K_m (2 mM) and V_{max} (500 μmol/min per mg) of the phosphorylation of P peptide by MAP kinase. These values are similar to those reported for the synthetic peptide with the sequence corresponding to the site on myelin basic protein that is phosphorylated by MAP kinase [46].

Characterization of antibody 972 raised against the P peptide

Antibodies to non-phosphorylated synthetic peptides can be used to test for the phosphorylation state of these sequences on their corresponding native proteins [38,50,51]. Thus a polyclonal rabbit antibody (972) was raised to the synthetic P peptide as indicated in the Materials and methods section.

Figure 4 Characterization of the binding of the rabbit polyclonal antibody 972 to MAP2

(a) Reaction of antibody 972 with MAP2 by Western blotting. Adult rat brain microtubule preparation (10 μ g) was subjected to PAGE and either stained with imidazole/zinc (lane 1) or transferred to nitrocellulose. Nitrocellulose paper was incubated with antibody 972 (at a 1:100 000 dilution) and a peroxidase-conjugated secondary antibody. Antibody binding was detected with the ECL Amersham kit and autoradiography. The autoradiogram is shown in lane 2. (b) Competition assay between MAP2 and synthetic P peptide for 972 binding. Samples of 1, 10 and 100 μ M non-phosphorylated P peptide (○) or corresponding phosphorylated peptide (●) were incubated overnight with antibody 972 diluted 1:100 000. These samples were then incubated with 50 ng of purified bovine brain MAP2 previously dotted on to nitrocellulose paper. Control experiments in which antibody 972 was incubated without any peptide were also included. Antibody binding to MAP2 was detected with the ECL Amersham kit and autoradiography. Autoradiograms were quantified by densitometry. The results are means $\pm$ S.D. from three experiments. (c) Reaction of antibody 972 with V8 peptides from MAP2. Purified bovine brain MAP2 was partially digested with *S. aureus* V8 protease (as described in the Materials and methods section and in the legend to Figure 2). The binding of antibody 972 to the resulting proteolytic peptides was assayed by Western blot. The autoradiogram shows the reaction of the 54 and 42 kDa MAP2 peptides with antibody 972.

Figure 5 Decreased binding of antibody 972 to *in vitro* phosphorylated MAP2

Purified rat brain MAP2 was phosphorylated with MAP kinase *in vitro* as indicated in the Materials and methods section. The reactions of purified MAP2 and *in vitro* phosphorylated MAP2 with antibody 972 were compared by Western-blot assay. (a) Imidazole/zinc staining of gel lanes containing purified MAP2 (—) and *in vitro* phosphorylated MAP2 (+). (b) Autoradiograms of the Western blots of purified MAP2 (—) and *in vitro* phosphorylated MAP2 (+) incubated with antibodies 10, MPM2 and 972. Antibody 10 reacts with MAP2 independently of its phosphorylation. Antibody MPM2 reacts more strongly with MAP kinase-phosphorylated MAP2 and antibody 972 reacts more strongly with non-phosphorylated MAP2.

Antibody 972 specifically recognizes MAP2 in a bovine brain microtubule protein preparation by Western-blot assay (Figure 4a). The reaction of antibody 972 with purified bovine brain MAP2 in a dot-blot assay is effectively prevented by preincubation of the antibody with increasing amounts of the non-phosphorylated synthetic P peptide (Figure 4b, open circles). Preincubation with the corresponding phosphopeptide (obtained by phosphorylation and oxidation) is much less efficient at inhibiting the binding of antibody 972 to MAP2 (Figure 4b, closed circles) than preincubation with the non-phosphorylated peptide. Thus 100 μ M phosphopeptide is less inhibitory than 1 μ M non-phosphorylated peptide of the binding of antibody 972

to MAP2 (Figure 4b). These results suggest that antibody 972 has a higher affinity for the non-phosphorylated peptide than for the phosphorylated peptide.

The reaction of antibody 972 with the proteolytic peptides derived from the partial digestion of purified bovine brain MAP2 with *S. aureus* V8 protease has also been checked by Western-blot assay (Figure 4c). Specific reaction of antibody 972 with the above-mentioned 54 and 42 kDa proteolytic peptides is observed.

Diminished reaction of antibody 972 with highly phosphorylated MAP2

In order to analyse whether antibody 972 can discriminate between MAP2 molecules with different degrees of phosphorylation, purified MAP2 was phosphorylated *in vitro* with p44 MAPK kinase. Figure 5 shows that antibody 972 shows weaker binding to *in vitro* phosphorylated MAP2 than to non-phosphorylated MAP2. As controls, an antibody that recognizes MAP2 independently of its phosphorylation state (antibody 10) and another antibody that only binds to phosphorylated MAP2 (monoclonal antibody MPM2) were tested in parallel. The specific reaction of MAP kinase-phosphorylated MAP2 with antibody MPM2 has been previously described [52].

MAP2 appears to be phosphorylated by p44 MAP kinase *in vitro* at the site recognized by antibody 972, and this phosphorylation diminishes the affinity of this antibody for MAP2.

To investigate the interaction of antibody 972 with MAP2 molecules phosphorylated to different extents, purified brain MAP2 was subfractionated by elution at increasing pH values using i.c.a.c.. Ferric ions complexed on chelating agarose selectively adsorb phosphoproteins. The greater the degree of phosphorylation of the protein, the stronger the interaction with the chelated iron, and a buffer with a more basic pH is required to elute the protein from the iron-chelated agarose [41,42].

Figure 6(a) (upper panel) shows the subfractionation of adult rat brain MAP2 by i.c.a.c. Only a small fraction of MAP2 is

Figure 6 Decreased binding of antibody 972 to highly phosphorylated MAP2 isolated from brain

MAP2 was subfractionated according to its degree of phosphorylation by the procedure of García de Añcos et al. [42]. Purified rat brain MAP2 (200–300 μ g) was loaded on to an i.c.a.c. column previously equilibrated in 0.1 M Mes, containing 0.2 M NaCl and 1 mM DTT, and eluted by a stepwise pH gradient with buffer containing 0.1 M Tris/HCl, 1 M NaCl and 1 mM DTT at pH 7.8, 8.0, 8.2, 8.4, 8.6. The chelated iron was eluted with 200 mM EDTA. (a) Elution profile of the protein in the absence (–AP) and presence (+AP) of phosphatase treatment. (b) Reaction of antibodies 10 and 972 with portions of the fractions eluted at pH 7.8 and with EDTA as tested by Western-blot assay.

eluted by washing the column at pH 5.7 and 7.8. At pH 8.0–8.4, most MAP2 is eluted from the column. A small amount of MAP2 is eluted at pH 8.6. Finally, a smaller amount of MAP2, which remains bound to the iron-chelated column even at pH 8.6, can be eluted together with the chelated iron after washing with EDTA. The latter fraction presumably corresponds to highly phosphorylated MAP2 which is strongly bound to the iron-chelated column. To test if this is the case, i.c.a.c. was performed with phosphatase-treated MAP2. A shift in the elution of MAP2 to lower pH values was observed. Most MAPs are now eluted at pH 7.8–8.0 (Figure 6a, lower panel). This indicates that the adsorption of MAP2 to the iron-chelated column indeed depends on the phosphorylation level of the protein, as previously demonstrated for other proteins [41,42]. Similar results were obtained for MAP2 preparations from cow and rat brain.

Antibody 972 reacts much less readily with the MAP2 fraction eluted from the i.c.a.c. column with EDTA than with the MAP2 fraction eluted at pH 7.8 (Figure 6b). No differences in the immunoreactivities of the two MAP2 fractions toward antibody 10 were observed. Thus a fraction of purified MAP2 appears to be highly phosphorylated (because of the strong adsorption to the i.c.a.c. column) and one of its phosphorylated sites seems to correspond to the site recognized by antibody 972 (because of the weak reaction with this antibody).

***In vivo* phosphorylation state of MAP2 at the epitope recognized by antibody 972 during rat brain development**

To analyse the variation in phosphorylation at the site recognized by antibody 972 during rat brain development, heat-resistant cytosolic proteins from brains of rats of different ages were either obtained in the presence of phosphatase inhibitors or were previously treated with alkaline phosphatase. These fractions were subjected to PAGE and analysed by Western-blot assay with antibody 972.

Figure 7 Developmental changes in the immunoreactivity of rat brain MAP2 with antibody 972

Heat-resistant cytosolic proteins from embryonic day 19 (E19), postnatal day 1 (P1) and postnatal day 21 (P21) rat brains were either left untreated (–) or treated (+) with alkaline phosphatase. Portions of these samples containing 10 μ g of total protein were subjected to PAGE, electroblotted on to a nitrocellulose sheet and probed with antibody 972. The positions of MAP2A/B and MAP2C are indicated on the left. Rabbit polyclonal antibody 660 was used as a control (C).

Table 1 Degree of phosphorylation at the site recognized by antibody 972 on MAP2 throughout rat brain development

The fold increase in immunoreactivity for antibody 972 of MAP2A/B and MAP2C after phosphatase treatment is shown. Data correspond to mean values $\pm$ S.D. from three experiments like the one shown in Figure 7 after densitometry of the corresponding autoradiograms. E19, Embryonic day 19; P1, postnatal day 1; P21, postnatal day 21.

Developmental stages of MAP2C	Fold increase	Developmental stages of MAP2A/B	Fold increase
E19	2.3 $\pm$ 0.7	E19	1.3 $\pm$ 0.2
P1	2.1 $\pm$ 0.6	P1	1 $\pm$ 0.6
P21	0.8 $\pm$ 0.3	P21	2.6 $\pm$ 0.4

Figure 7 shows that antibody 972 reacts with both MAP2A/B and MAP2C in these samples. Two extra protein bands are recognized by antibody 972 in the preparation from 21-day-old rat brain. These protein bands presumably correspond to proteolytic fragments derived from MAP2A/B, as MAP2 proteolysis increases with age [53]. Pretreatment with alkaline phosphatase causes an increase in the electrophoretic mobility of MAP2C as well as a sharpening of the protein band.

Pretreatment with alkaline phosphatase increases the immunoreactivity of MAP2C from embryonic and 1-day-old rat brain toward antibody 972, without modifying the reaction of MAP2A/B with this antibody. In contrast, pretreatment with alkaline phosphatase of the brain proteins from 21-day-old rats results in an increase in the immunoreactivity of MAP2A/B with antibody 972, without any significant change in the reaction of MAP2C. No differences in immunoreactivity were observed with antibody 660 which reacts with phosphorylation-independent epitopes on MAP2A/B and MAP2C. The quantification of these results is presented in Table 1. A large increase in the

reaction of the protein with antibody 972 after the pretreatment with alkaline phosphatase may indicate a high degree of phosphorylation of the protein at the region reacting with antibody 972. Accordingly, it appears that MAP2C is more phosphorylated at the site recognized by antibody 972 during early rat brain development, whereas it is essentially dephosphorylated at this site by 21 days after birth. In contrast, MAP2A/B seems to be more phosphorylated at this site in the adult rat brain.

The phosphorylated state of MAP2C can therefore be correlated with the time of axonal growth. In fact, MAP2C has been localized to growing axons in developing neurons [54,55]. Other MAPs are highly phosphorylated at the time of axonal growth. These include MAP1B [38,56–59] and tau [60–65]. Furthermore, some phosphorylated isoforms of these proteins have been specifically localized to growing axons in immunocytochemical studies [56–59,63–66]. More interestingly, all these phosphorylation events have been mapped to Ser/Thr-Pro motifs and are supposed to be catalysed by proline-directed protein kinases. Here we suggest that the phosphorylation of embryonic MAP2C at the epitope for antibody 972 may be included in this scheme. Thus there seems to be phosphorylation of several cytoskeletal proteins including MAP1B, MAP2C and tau by proline-directed protein kinases which is specifically associated with the growth of axons. This may be related to the maintenance of a highly dynamic cytoskeletal organization that is competent for rapid axonal growth. In fact, this type of phosphorylation is reduced at the time of axonal maturation, presumably to stabilize the axonal cytoskeleton [62,66].

The presence of MAP2A/B phosphorylated at the epitope for antibody 972 in the adult rat brain is consistent with previous reports showing a significant degree of phosphorylation in MAP2 isolated from adult rat brain [25,26,31]. MAP2A/B is specifically localized to neuronal cell bodies, dendrites and dendritic spines [8–12]. Phosphorylation and dephosphorylation of MAP2 at these locations may lead to transient postsynaptic cytoskeletal disassembly and reassembly which might contribute to different forms of synaptic plasticity [1,30,32,34].

The fact that MAP2C and MAP2A/B are found maximally phosphorylated at the epitope for antibody 972 at distinct developmental stages (and also possibly at different cellular locations) may indicate that different proline-directed protein kinases are implicated in their phosphorylation. Several proline-directed protein kinases show overlapping substrate specificities [48,49,67] and may play a role in this respect. These include cyclin-dependent kinases such as cdc2 [68,69], cdk4 [70] and cdk5 [70–72], as well as MAP kinases such as p44 MAP kinase (ERK1) and p42 MAP kinase (ERK2) [73,74]. Of particular interest is the fact that p42 MAP kinase seems to be co-localized with MAP2 on dendrites in mature neurons [73]. Furthermore, this enzyme has a high affinity for MAP2 with a K_m of 56 nM [75]. Interestingly, membrane depolarization results in both MAP kinase activation [76] and increased MAP2 phosphorylation [30]. As p42 MAP kinase can be activated in response to certain neurotransmitters [77,78], this enzyme should be considered a candidate for the regulation of MAP2 phosphorylation and cytoskeletal rearrangements in dendrites.

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