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Dephosphorylation of distinct sites on microtubule-associated protein MAP1B by protein phosphatases 1, 2A and 2B

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Rat brain microtubule-associated protein MAP1B has been tested as a substrate for Ser/Thr protein phosphatases (PP). The dephosphorylation reactions were followed by specific antibodies recognizing phosphorylated and phosphorylatable epitopes. One set of phosphorylation sites on MAP1B are referred to as mode I sites, and their phosphorylation is presumably catalyzed by proline-directed protein kinases. These mode I sites are efficiently dephosphorylated by PP2B and 2A but not by PP1. Another set of phosphorylation sites on MAP1B are named mode II sites, and their phosphorylation is possibly due to casein kinase II. These mode II sites are dephosphorylated by PP2A and PP1, the PP2B being ineffective. The selectivity of phosphatases for different sites within the same protein indicates the complexity of the dephosphorylation reactions regulating the functionality of MAP1B in neurons.

Phosphorylation/dephosphorylation; Microtubule-associated protein; Protein phosphatase 1 and 2A; Calcineurin; Proline direct protein kinase (PDPK); Casein kinase II (CKII)

1. INTRODUCTION

The growth and maintenance of neuronal processes depend on microtubules which are mainly composed of tubulin and a group of microtubule-associated proteins (MAPs). It has been suggested that MAPs play a role in the regulation of microtubule assembly and stabilization [1]. In particular a microtubule-associated protein referred to as MAP1B, MAP5 or MAP1X is mainly present in neurons at early developmental stages [2,3], and its phosphorylation has been correlated with neurite extension [4–9]. Interestingly, MAP1B hyperphosphorylation has been found associated with dystrophic neurites and neurofibrillary tangles within brains of Alzheimer's disease patients [10].

Further analyses on the phosphorylation of MAP1B have indicated the existence of at least two major modes of MAP1B phosphorylation [11]. These are the result of the modification of MAP1B by distinct protein kinases and can be recognized by the use of different antibodies to phosphorylation-sensitive epitopes [17]. Mode I-phosphorylated MAP1B is abundant in newborn and early postnatal rat brain, whereas it is hardly detectable in most adult rat brain regions, with the exception of the olfactory bulb, where axonal growth persists into adulthood. In contrast, mode II-phosphorylated MAP1B is also present in adult rat brain. Preliminary results indicate that a proline-directed protein kinase (PDPK) may be involved in mode I phosphorylation while mode II of MAP1B phosphorylation may be catalyzed by casein kinase II [11,17].

Thus, phosphorylation of MAP1B, especially on mode I sites, should be considered as a dynamic process. The mode I phosphorylation seems to be important for axonal growth, whereas the corresponding dephosphorylation may accompany axonal maturation, as suggested by Riederer et al. [12]. Consequently, it is important to know not only the protein kinases involved in these modifications but also the protein phosphatases that could dephosphorylate MAP1B. Different phosphatases have been described in mammalian tissues, including brain, and have been classified into phosphatases (PP) 1, 2A, 2B and 2C [13–15].

In this work we have analyzed the action of PP1, PP2A and PP2B on phosphorylated MAP1B (pMAP1B) at mode I and mode II sites that are recognized by specific antibodies.

2. MATERIALS AND METHODS

2.1. *Antibodies*

Antibodies to MAP1B used in this study include mouse monoclonal (IgM) antibody 125 (obtained in our laboratory), mouse monoclonal

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(IgM) antibody 150 (a generous gift from Dr. C. Alberto and Dr F Moya, University of Alicante, Spain), rabbit polyclonal antibody 842, raised against the synthetic peptide ASTYSYETSD (corresponding to residues 2,050 2,059 of the MAP1B sequence according to Noble et al. [16]) and rabbit polyclonal antibody 531, raised against the synthetic peptide SPAKSPSLSPSPPSP (corresponding to residues 1,247–1,261 of the MAP1B sequence according to Noble et al. [16]). These antibodies have previously been characterized by Ulloa et al [11 17]

2.2. *Protein preparation*

Microtubule protein from 5-day-old rat brain cytosol was prepared according to the procedure of Karr et al [18] From this preparation, MAPs with no associated kinase nor phosphatase activities were obtained as described by Díaz-Nido et al. [6]

The catalytic subunits of protein phosphatase 1 ($PP1_c$) and protein phosphatase 2A ($PP2A_c$) were isolated from rabbit skeletal muscle [19,20]. Both preparations were homogeneous as detected by sodium dodecyl sulfate (SDS) gel electrophoresis. The activity of $PP1_c$ and $PP2A_2$ was measured using [^{32}P]-phospholabeled phosphorylase 'a' as substrate [21]. Calcineurin (PP2B) was purified to apparent homogeneity (29 U/mg) from bovine brain [22]. PP2B activity was determined using [^{32}P]-phospholabeled inhibitor-1 [23] according to the method of Yang et al [24]. One unit of phosphatase dephosphorylates 1 nmol of substrate in 1 min under the assay conditions.

Calmodulin was prepared from porcine brain [25].

Protein was determined according to Read and Northcote [26] using bovine serum albumin as a standard.

2.3. *Dephosphorylation of brain MAPs by purified phosphatases*

Aliquots (500 μl) of a brain MAP preparation at a protein concentration of 1.2 mg/ml in buffer 20 mM PIPES buffer, pH 6 9, 0.5 mM EGTA were preincubated at 30°C for 1 min. Dephosphorylation reactions were started by the addition of purified PP1c or PP2Ac at final concentrations of 10 U/ml in 50 mM Tris-HCl buffer, pH 7.5, 0.1 mM EDTA, 10 mM DTT, or purified PP2B at a final concentration of 3 U/ml in 40 mM Tris-HCl buffer, pH 7.0, 0.4 mM DTT, 0 2 mM $CaCl_2$, 1 mM $MnCl_2$ containing 12 μM calmodulin at 30°C. The reactions were stopped after 3, 10 and 45 min incubation by the addition of SDS sample buffer, mixing, and freezing in liquid nitrogen. All reactions contained 5 mM benzamidine, 1 mM *o*-phenanthroline and 0.2 mM PMSF as protease inhibitors.

2.4 *Gel electrophoresis and immunoblotting*

Proteins were separated on SDS-polyacrylamide gels (SDS-PAGE) according to the procedure of Laemmli [27] using 4–10% gradient polyacrylamide gels, with the modifications previously described [17]

Protein electroblotting was performed as described by Towbin et al. [28] with a modified transfer buffer (20% (v/v) methanol, 25 mM Tris-HCl, 200 mM glycine, pH 7.0 and 0.1% SDS). Transferred proteins were stained with 0.3% Ponceau-S in 3% trichloroacetic acid for 15 min. After blocking the membranes with 2% bovine serum albumin in 20 mM sodium phosphate, pH 7 4, 140 mM NaCl containing 0.1% Tween 20 during 1 h at room temperature, the blots were incubated with primary antibodies overnight at 4°C. Detection of bound antibodies was performed by incubation with a second peroxidase-conjugated antiserum (Daho-Dtuwark) at the manufacturer's recommended dilution. Detection was performed using the ECL detection system (Amersham UK). After washing, with phosphate-buffered saline containing 0.1% Tween 20, the labeled proteins were characterized by autoradiography of the dried blots exposed to Kodak X Omat films and quantified by densitometric scanning using a Densitometer model 200A from Molecular Dynamics.

3. RESULTS

3.1. *Dephosphorylation of phosphorylated MAP1B (pMAP1B) on mode I sites*

The mode I of MAP1B phosphorylation can be identified by the use of two antibodies [17], one recognizing

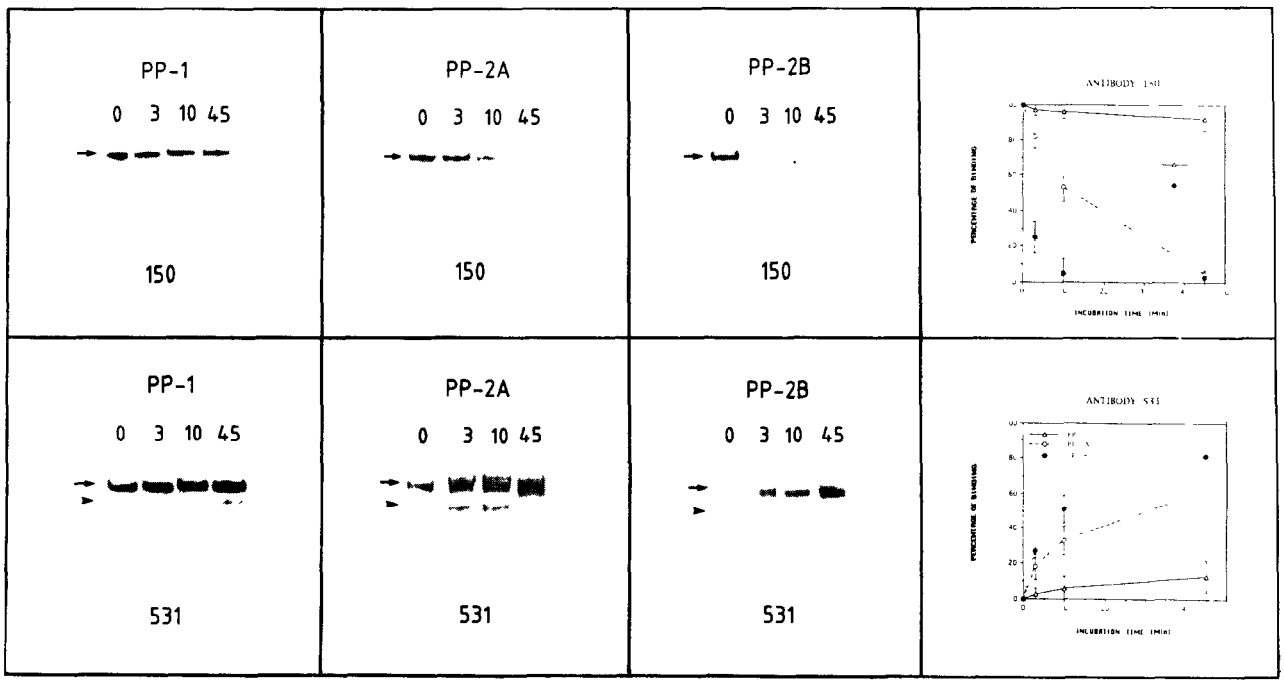

Fig. 1. Analysis of pMAP1B dephosphorylation at 150 and 531 epitopes. Left. Autoradiograms of immunoblots with antibodies 150 (upper panels) and 531 (lower panels) of a MAPs preparation treated with $PP1_c$ (left), $PP2A_c$ (center) or PP2B (right) for 1, 3, 10, or 45 min Arrows (→) indicate the MAP1B position and the arrowheads mark the p260 proteolytic fragment of MAP1B Right. Kinetics of pMAP1B dephosphorylation at 150 and 531 epitopes. Quantification of the immunoreactivity of MAP1B toward antibodies 150 and 531 after dephosphorylation with $PP1_c$, $PP2A_c$ or PP2B. Bars indicate the S D. values of the mean, from different experiments.

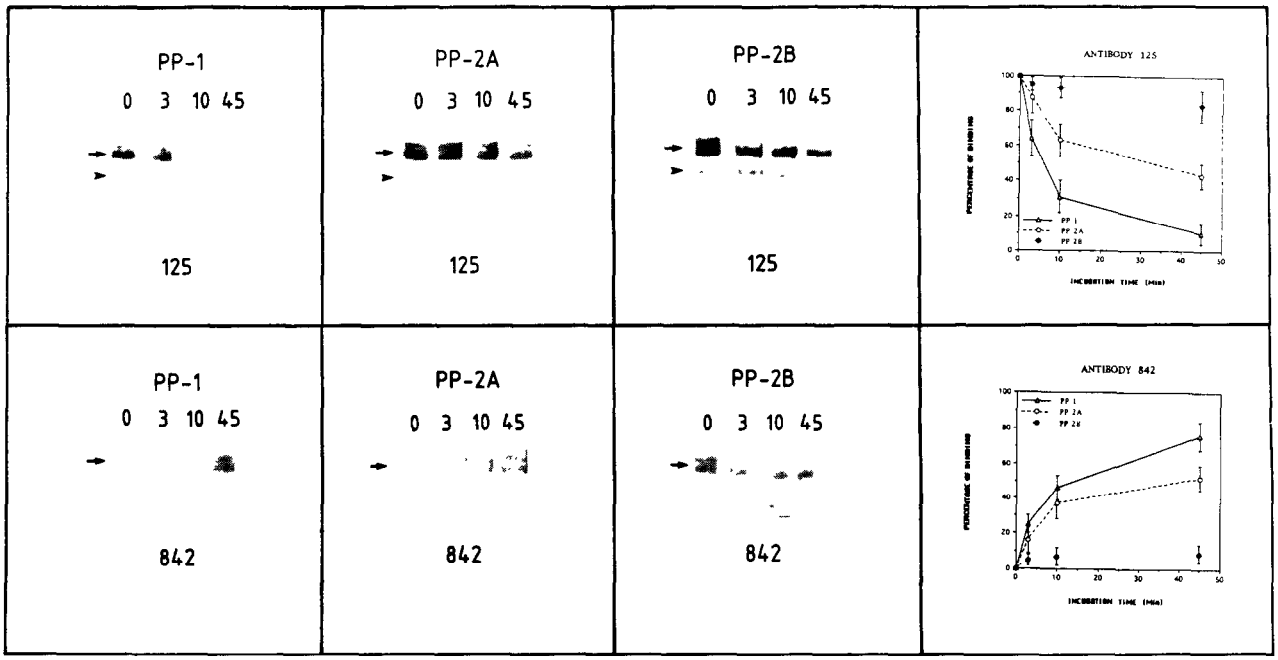

Fig. 2. Analysis of MAP1B dephosphorylation at 125 and 842 epitopes Left. Autoradiograms of immunoblots with antibodies 125 (upper panels) and 842 (lower panels) of a MAPs preparation treated with $PP1_c$ (left), $PP2A_c$ (center) or PP2B (right) for 1, 3, 10, or 45 min. Arrows (→) indicate the MAP1B position and the arrowheads mark the p260 proteolytic fragment of MAP1B. Right. Kinetics of pMAP1B dephosphorylation at 125 and 842 epitopes. Quantification of the immunoreactivity of MAP1B toward antibodies 125 and 842 after dephosphorylation with $PP1_c$, $PP2A_c$ or PP2B. Bars indicate the S.D. values of the mean, from different experiments

a phosphorylated epitope (antibody 150), and another reacting with a phosphorylatable epitope when it is unphosphorylated (antibody 531). We have used these antibodies to test for the dephosphorylation of pMAP1B with purified phosphatases $PP1_c$, $PP2A_c$ and PP2B. Phosphatase treatment was performed for different times until 45 min to measure the rate of dephosphorylation and the final extent of it (Fig. 1). Incubation of pMAP1B with $PP2A_2$ leads to an increase in the reaction of MAP1B with antibody 531 and a decrease in the reaction of MAP1B with antibody 150. The effect of PP2B is even more marked and an increase in the overall electrophoretic mobility of phosphatase-treated MAP1B was also found. No effect is observed with $PP1_c$. It is apparent that dephosphorylation of pMAP1B at the 150 epitope is almost complete in 3 min with PP2B. No variations in the immunoreactivity of MAP1B for these antibodies are observed when MAPs are incubated in the absence of purified phosphatases (data not shown).

3.2. *Dephosphorylation of pMAP1B on mode II sites*

To test for the dephosphorylation of MAP1B on mode II sites, a similar approach has been followed. In this case, antibody 125, which recognizes a phosphorylated epitope that can be phosphorylated in vitro with casein kinase II [11] and antibody 842, which binds to an unmodified epitope which is also phosphorylatable by casein kinase II [17], were employed. These two epitopes are located apart on the MAP1B molecule, as detected by limited proteolysis with calpain in which two main fragments are generated. The larger phosphorylated 260 kDa proteolytic fragment (p260), which contains the N-terminal domain and the epitope for antibody 125, is also usually present in MAP1B preparation (Fig. 2). The smaller fragment contains the epitope for antibody 842 and the C-terminal end [17].

Fig. 2 shows that $PP2A_c$ and $PP1_c$ can dephosphorylate pMAP1B at these sites, whereas PP2B is ineffective. Again, no variations in the immunoreactivity of MAP1B for antibodies 125 and 842 were observed in the absence of phosphatases (data not shown).

MAP1B could be resolved, by gel electrophoresis under certain conditions, into two different isoforms with different electrophoretic mobilities [17]. The phosphoisoform with a slower mobility is modified by the mode I of phosphorylation whereas the other (faster) one is only phosphorylated by the mode II. In Fig. 2 (upper panel PP-2B) it is shown that PP-2B treatment results in the disappearance of phosphoMAP1B isoform with the slower electrophoretic mobility but not of the faster migrating form. This result is consistent

with the observed dephosphorylation of mode I-phosphorylated MAP1B by PP2B.

4. DISCUSSION

Distinct phosphorylation sites on the MAP1B molecule are differentially susceptible to in vitro dephosphorylation with purified protein phosphatases. Thus, the sites constituting the epitopes for antibodies 150 and 531 (mode I sites), which are presumably phosphorylated by proline-directed protein kinases [17], are readily dephosphorylated by PP2A and PP2B, and PP1 having no effect. In contrast, the sites constituting the epitopes for antibodies 125 and 842, which are presumably phosphorylated by casein kinase II [11,17] are dephosphorylated by both PP2A and PP1, PP2B being ineffective. It should be mentioned that strict quantitative comparisons of specific dephosphorylation rates cannot be made. This particularly holds for PP2B, which was assayed with a substrate different from that used for PP1 and PP2A. Nevertheless, the ability of PP2A to dephosphorylate both mode I and mode II sites, that of PP2B to dephosphorylate mode I sites, as well as that of PP1 to dephosphorylate mode II sites, can be clearly established.

These results are compatible with previous data on the specificity of protein phosphatases toward synthetic peptide substrates. Thus, the catalytic subunit of PP2A is more effective than PP1 in the dephosphorylation of several synthetic peptides phosphorylated by p34 cdc2, which is a proline-directed protein kinase [29]. Interestingly, the oligomeric structure of the PP2A phosphatases is quite important for the efficient dephosphorylation of these substrates, suggesting a regulatory role for associated subunits. Similar results have been obtained upon studying the PP2A-catalyzed dephosphorylation of microtubule-associated tau protein at the tau-1 epitope [30], which is a site phosphorylated by proline-directed protein kinases including cdc2 kinase [31,32]. In addition, histone H1 phosphorylated by the cdc2 kinase was also best dephosphorylated by PP2A [33]. Furthermore, PP2A has been shown to dephosphorylate synthetic peptides phosphorylated by casein kinase II [34]. However, in this case, the primary structure of the phosphorylation sites is important in determining the dephosphorylation rates. This would explain why some sites phosphorylated by casein kinase II are only slowly dephosphorylated by PP2A [35,36].

Further research is required to understand the relative roles played by kinase and phosphatase activities in controlling the state of phosphorylation of MAP1B in different neuronal compartments during development. Thus, MAP1B phosphorylated at the 150 epitope is confined to the distal, growing portions of developing axons [9] whereas MAP1B phosphorylated at the 125 epitope shows a widespread distribution including axon and dendrites of developing and mature neurons (Ulloa et al., submitted).

It is not known whether an increase in phosphatase activity and/or a decrease in kinase activity is/are responsible for the net dephosphorylation on MAP1B at the 150 epitope which is observed during axonal maturation [9,17]. This dephosphorylation seems to affect the mode I-phosphorylated sites on the MAP1B molecule, as it is correlated with the disappearance of the slower-migrating MAP1B isoform on SDS-PAGE [12,17]. A defect in protein dephosphorylation by PP2A has also been suggested to play a role in the hyperphosphorylation of neuronal proteins phosphorylated by proline-directed protein kinases in Alzheimer's disease [30].

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