Protein phosphatase 2A is associated in an inactive state with microtubules through 2A1-specific interaction with tubulin

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Protein phosphatase (PP) 2A1, a trimer composed of A-, B- and C-subunits in the PP2A family, has been regarded as a principal form localizing at microtubules (MT), but PP2A2, the dimer of A- and C-subunits, has not. Substantiating the claim, the present work shows that the PP2A1 but not PP2A2, both isolated from bovine extract, largely associated with the purified preparation of MT. Furthermore, PP2A1 was found to bind purified tubulin polymerized by taxol. The presence of MT associated proteins with purified tubulin hardly affected the binding of PP2A1 to the tubulin. In addition, PP2A1 activity towards glycogen phosphorylase, a probably unphysiological but good substrate, was similarly inhibited by MT proteins and purified tubulin, which accounts for $\geqslant 85\%$ of MT proteins, with their IC_{50} of about 0.15 mg/ml. In contrast, the inhibition of PP2A2 was about 40% with 1 mg/ml MT proteins and 20% with 0.8 mg/ml tubulin, consistent with its weak association with MT. Therefore, the association with and resultant inhibition by MT proteins of PP2A1 is largely effected by the binding of PP2A1 to tubulin molecule. Moreover, PP2A1 isolated from MT has higher affinity for polymerized MT proteins than has PP2A1 from the postmicrotubule supernatant. The MT PP2A1 has also higher sensitivity to the inhibition by tubulin and MT proteins than has the supernatant PP2A1 (IC_{50}: 0.1–0.2 mg/ml vs. 0.3–0.6 mg/ml), demonstrating the importance of its association with polymerized tubulin.

Key words: enzyme regulation, protein dephosphorylation, signal transduction, subcellular location.

INTRODUCTION

Microtubules (MT) are ubiquitous cellular structure involved in a variety of essential functions of the cell (reviewed in [1–4]). MT structures are composed of the polymerized tubulin $\alpha\beta$ dimers and MT associated proteins (MAPs). The characteristic properties of MT, which are known as dynamic instability and involve rapid transition between polymerization and depolymerization of tubulin dimers, are at least in part regulated by the phosphorylation state of the MAPs and tubulin. For example, tau stabilizes MT by binding to tubulin polymers but the phosphorylation of tau decreases its binding affinity toward MT and thereby lowers its stabilizing effect [5,6]. Conversely, oncoprotein 18, when phosphorylated, is prevented from binding to tubulin and thus weakened in its destabilizing activity [7–9].

The phosphorylation state of proteins is determined by the balance of the activities of protein kinases and protein phosphatases. Among the latter group of enzymes several protein phosphatases have been shown or thought to localize and function at particular intracellular sites (reviewed in [10,11]). Proteins termed targeting subunits/proteins which localize type 1 protein phosphatase or type 2B protein phosphatase have been identified so far. Type 2A protein phosphtase (PP2A) which had been regarded as cytoplasmic protein was recently shown to colocalize with MT in intact cells [12]. PP2A is composed of three groups of subunits termed A, B and C subunits and considered to be present as trimeric form *in vivo* (reviewed in [13,14]). PP2A1, previously demonstrated to be a trimeric form [13,15–18], includes Bα or Bβ subunit in addition to A and C subunits [14,19,20]. PP2A2 is the dimer of A and C subunits which is regarded as the core structure of PP2A [13,14]; it is not totally certain whether the AC form is present or absent *in vivo*.

PP2A is regarded as a candidate for the phosphatases dephosphorylating a number of MT proteins including tau [21–23] and oncoprotein 18 [24,25] which have opposing function as described above. A protein kinase termed mitogen-activated protein kinase which phosphorylates the above two MT proteins and regulates their function [9,26] is also thought to be dephosphorylated and thereby inactivated by PP2A [27,28]. Therefore PP2A would affect the functions of MTs in various manners, depending on which of the substrates including the three proteins it dephosphorylates. Several species of PP1 are shown to be localized to certain subcellular compartments and thereby activated toward the substrate present therein, whereas PP2B is thought to be inactive when targeted to postsynaptic densities [10,11]. As for PP2A on MTs, selective activation and/or inactivation may occur among PP2A molecules on MTs, allowing the activated PP2A to specifically dephosphorylate the particular substrate which needs to be regulated in the intracellular milieu.

Immunoreactivity against Bα and Bβ subunits on or close to MT structures is observed, respectively, in CV1 cells [12] and Purkinje cells [29]. Accordingly, PP2A1 with a certain special form of B subunit is localized to MT. Tau is shown to bind PP2A1 (both BαAC and BβAC) [23], but it is not known whether it is tau or another MT protein that targets PP2A to MT. Neither is it certain that PP2A2 (AC) is incapable of associating with MT. Furthermore, it is almost entirely unclear how MT proteins modulate the activity of PP2A, although tubulin is reported to affect the PP2A2 activity towards aryl phosphates such as paranitrophenylphosphate [30]. The present study shows that PP2A is associated in a form that is inactive towards phosphorylase a with MT proteins through 2A1-specific interaction with tubulin. The localization of PP2A1 in the regulated state on polymerized tubulin may provide a basis for

Abbreviations used: CL, cold labile; CS, cold stable; MAP, MT associated protein; MT, microtubule; PP2A, protein phosphatase 2A.

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the subsequent and selective modulation of particular PP2A1 molecules by MAPs.

MATERIALS AND METHODS

Materials

Glycogen phosphorylase b and phosphorylase kinase were purchased from Boehringer and Sigma, respectively. Taxol was from Wako Chemicals (Osaka, Japan). Biogel-6PDG and phosphocellulose P11 were from Bio–Rad and Whatman (Maidstone, Kent, U.K.), respectively. Mono-Q HR5/5 column was from Pharmacia.

Buffers

The following solutions were routinely used in the assay of PP2A and the preparation of MT proteins: buffer A1: 0.1 M Mes pH 6.4, 0.5 mM $MgCl_2$, 1 mM EGTA; buffer A2: the same as buffer A1 except that $MgCl_2$ was used at 1 mM; buffer A3: 0.1 M Mes pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA; buffer B: 50 mM Tris/HCl pH 7.4, 0.2 % 2-mercaptoethanol, 0.1 mM EDTA, 1 mg/ml BSA; buffer C: 20 mM Tris/HCl pH 7.4, 0.1 % mercaptoethanol. The pH was adjusted at 8 °C for buffer A1 and at ambient temperature (20–30 °C) for buffer A3, buffer B and buffer C. When the buffers were diluted with water and then used, those are expressed as $n\times$ the buffer ($0.25\times$ buffer A1 for four-fold diluted buffer A1).

Protein preparations

MT proteins were prepared from bovine brain by two cycles of temperature-dependent assembly and disassembly [31]. Briefly, bovine brain was homogenized in buffer A1 and the homogenate was centrifuged at 10000 ***g*** for 1 h. The supernatant (referred to as extract) was saved, made 1 mM and 25 % respectively, in ATP (employed instead of GTP) and glycerol, incubated at 37 °C for 40 min and centrifuged at 100000 ***g*** at 30 °C for 45 min. The resultant supernatant (postmicrotubule supernatant) was saved and used for the isolation of PP2A as described below. The pellet which contained the polymerized MT proteins was resuspended in buffer A1 containing 1 mM ATP but no glycerol, the suspension centrifuged at 100000 ***g*** for 45 min at 4 °C. The supernatant was subjected to assembly and then disassembly once more. Conventional preparation of MT proteins in the last supernatant referred to as cold labile (cl) fraction of MT proteins and the MT proteins in the final precipitates which do not become depolymerized at 4 °C without glycerol are called cold stable (cs) fraction of MT proteins [32].

For inactivation of the phosphatases in the MT fractions the fractions were made 50 mM and 1 mM in NaF and EDTA, respectively, incubated at 4 °C overnight and dialysed against buffer A3 with three changes of the buffer. The treatment reduced the phosphatase activity to 10 % or less of that in untreated fractions. The preparations of MT proteins were all stored at −70 °C.

Tubulin was purified from the cl MT proteins by two methods, phosphocellulose chromatography [33] and taxol precipitation with NaCl [34]. In short, the cl MT proteins were desalted by gel filtration on Biogel-6PDG equilibrated in 20 mM Mes, 0.2 mM EGTA, 0.5 mM $MgCl_2$ pH 6.7 (4 °C) and chromatographed on phosphocellulose column in the above buffer. Tubulin in the flow-through fraction was dialysed against buffer A3 and stored at a concentration of 1 mg/ml at −70 °C. When tubulin was purified with taxol precipitation, cl MT proteins (7.2 mg) were incubated at 37 °C for 30 min in a 1 ml solution containing $0.6\times$ buffer A1, 30 μM taxol and 0.5 M NaCl and the incubated solution was centrifuged at 22000 ***g*** for 40 min. Tubulin in the precipitate was resuspended in 300 μl of buffer A3, dialysed against the buffer A3 with one change of the buffer and stored at 4–8 mg/ml at −70 °C. The supernatant (850 μl) containing MAPs was also dialysed against buffer A3 and stored at −70 °C. Tubulin with the phosphatase activities less than half the amounts of those in the above preparation was prepared by one more precipitation of the purified-tubulin with taxol in the presence of NaCl. The extensively purified tubulin was resuspended and dialysed as above.

PP2A1 and PP2A2 were isolated from the three subcellular fractions. Those fractions were the extract, to which ATP and glycerol were added for the polymerization of MTs as above, the postmicrotubule supernatant and the cl fraction of MT proteins. Aliquots (1 ml) of the three fractions were dialysed against buffer C for 4 h. The dialysates were each made 2 ml by the addition of buffer C and applied to an FPLC Mono-Q column. PP2A was eluted from the column as described in Figure 1. The chromatography was also used for the studies to ascertain which forms of PP2A associate with MT, as described in the Results section. The PP2A1 (1 ml) isolated from the three subcellular fractions and the PP2A2 (1 ml) from the extract and the postmicrotubule supernatant were dialysed against buffer C containing 60 % glycerol and stored at −70 °C.

Protein was measured by the method of Bradford [35] using bovine serum albumin ($A_{280\,nm}$ at 1 % = 6.5) as standard. PAGE was carried out in the presence of SDS according to Laemmli [36].

Assay of PP2A

This was carried out as described in [37], except that the concentrations of ^{32}P-labelled phosphorylase were 0.05–0.3 mg/ml in the assay. Briefly, the reaction was initiated by the addition of ^{32}P-labelled phosphorylase (200–4000 cpm/pmol, 5 μl) to PP2A diluted with buffer B (5 μl). When the effect of MT proteins or tubulin was examined, the phosphorylase (5 μl) was added to the solution made up of buffer B-diluted PP2A (10 μl) and buffer A3 (5 μl) with or without the proteins. After incubation at 30 °C for 2–40 min, the released ^{32}Pi separated from trichloroacetic acid-precipitated phosphorylase was quantified for radioactivity. The controls where PP2A was omitted were routinely conducted so that the added PP2A activities were calculated by subtraction in which the endogenous activities in the control were deducted from the total activities composed of the added PP2A and the endogenous phosphatases. One unit of phosphatase activity was defined as that amount which catalysed the release of 1 nmol of phosphate/min.

Assay of binding of PP2A to MT proteins

The cs fraction of MT proteins (15 μg) in buffer A1 were incubated at 37 °C for 10 min and used for preparation of the assembly solution comprising $0.3\times$ buffer A1, 30 % glycerol, 1.5 mg/ml of the preincubated cs MT proteins and PP2A (450–800 mU/ml) in a total volume of 10 μl. The assembly solutions (2 ml) were saved on ice and the remaining solution was kept at ambient temperature for 30 min and centrifuged at 22000 ***g*** for 35 min. The resultant supernatants were removed (7–8 μl) and the pellets were dissolved in buffer B to give a final volume of 8 μl. The dissolved pellets and the supernatants were assayed at 1:100–200 final dilution for phosphatase activity at which dilution MT proteins do not inhibit the activities of the PP2A examined in this work as described in the Results section.

Taxol purified tubulin (8 μg) was incubated at 37 °C for 30 min in $0.87\times$ buffer A3 containing 25 μM taxol with or

without MAPs (13.5 μg) in a total volume of 8 μl. Thereafter PP2A1 (10–30 mU) in 2 μl of buffer C was included and the incubation was continued and carried out for 7 min at 37 °C and for a further 8 min at ambient temperature. The suspensions were centrifuged at 22000 ***g*** for 35 min for precipitation of the polymerized tubulin. The supernatants (10 μl) were removed and the pellets were roughly suspended in buffer C to give a final volume of 10 μl. Next, 10–30 μl of 0.5 × buffer B was added to the resuspended pellets as well as the supernatants and the two centrifugational fractions were kept on ice for about 3 h with occasional vortexing sufficient to dissolve the pellets. The supernatant and pellets were assayed at a 80-fold final dilution for PP2A1 activity.

RESULTS

PP2A1 but not PP2A2 strongly associates with MT proteins through its interaction with polymerized tubulin

The chromatography of the brain extract on Mono-Q column and subsequent assay of the eluate for the activity towards phosphorylated glycogen-phosphorylase, a probably unphysiological but good substrate for mammalian PP2A, revealed two

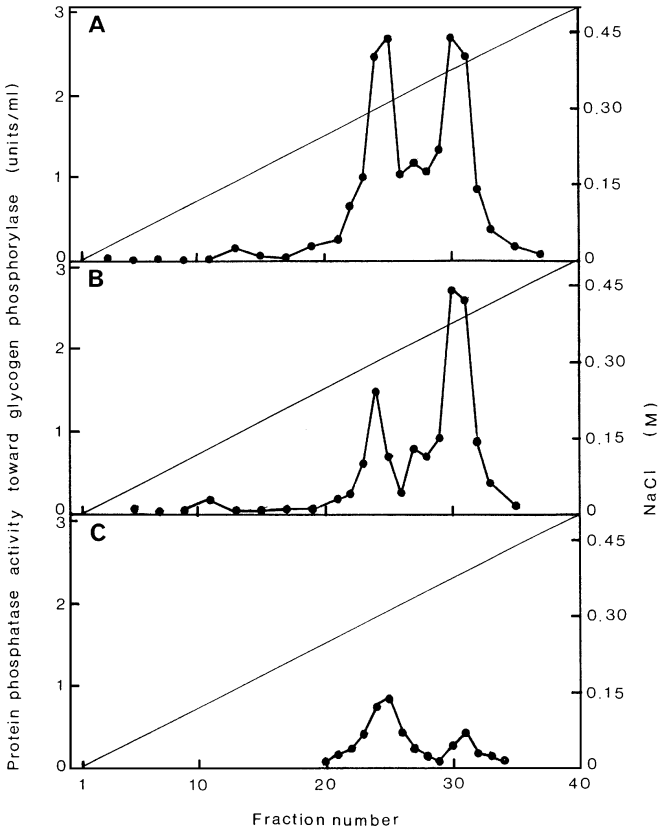

Figure 1 Selective coprecipitation of PP2A1 with MTs in bovine brain extract

The brain extract (**A**), the postmicrotubule supernatant (**B**) and the cl fraction of MT proteins (**C**) were dialysed and the dialysates containing protein phosphatase activity (5.94, 3.12 and 1.62 units in A, B and C, respectively) were applied to an FPLC Mono-Q HR5/5 column. The Mono-Q column was developed by a linear gradient from 0 to 0.5 M NaCl in a total volume of 20 ml. The flow rate was 1 ml/min and fractions of 0.5 ml were collected. Further details are given in the Materials and methods section. PP2A1 and PP2A2 were located by measurement of phosphatase-activity toward ^{32}P-labelled phosphorylase. The fine oblique line shows the NaCl gradient.

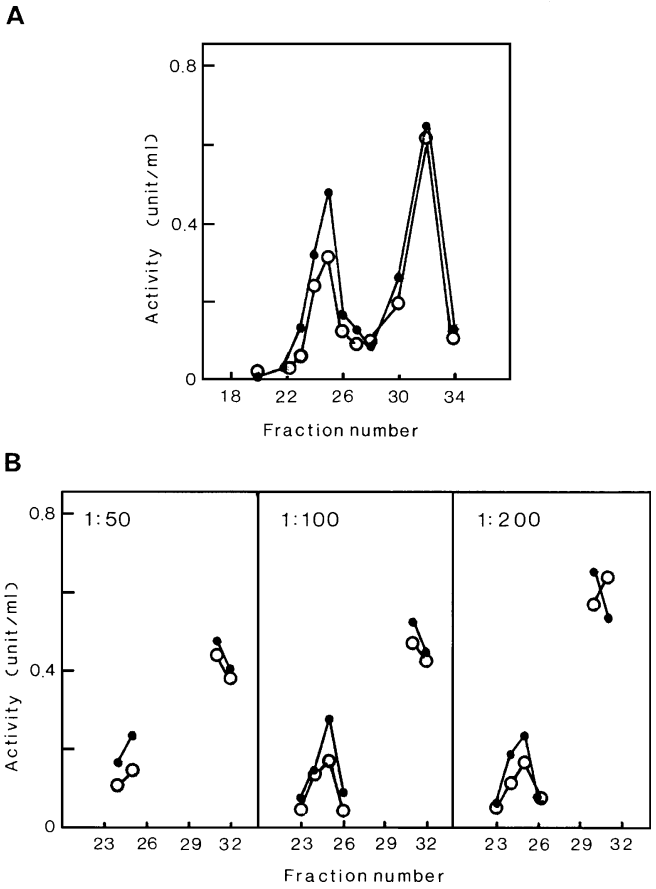

Figure 2 Effect of dilution on PP2A activity in Mono Q fractions

The brain extract and the postmicrotubule supernatant were each (0.75 ml) dialysed and chromatographed on Mono-Q as described in Figure 1. The activities of PP2A1 and PP2A2 were located by assay at a 1:50 final dilution within 2–3 h after the elution (**A**) and measured again at 1:50, 1:100 or 1:200 final dilution using the fractions stored at 4 °C overnight (**B**). The activities from the extract and the postmicrotubule supernatant are indicated by the closed circles and the open circles, respectively.

major peaks of the activity (Figure 1A). The second peak activity was found at 0.37 M NaCl (Figure 1A), 50% inhibited by okadaic acid at 0.5 nM (not shown) and thus judged to be PP2A2 from the NaCl concentration required for the elution from the Mono-Q and the sensitivity to the inhibition by okadaic acid [18,38,39]. The first peak found at 0.31 M NaCl, was judged to be PP2A1 from the NaCl concentration for the elution and the sensitivity to okadaic acid [18,38,39]. PP1 or PP2A0 reported to elute at 0.25 M NaCl or less [38,40] was hardly detected possibly because of loss of the unstable enzymes and/or omission of the activation procedures used for these enzymes [40]. When the postmicrotubule supernatant was chromatographed under the conditions identical with those used in Figure 1A, the total activity in the peak of PP2A1 was found to be about 40% of that of PP2A1 isolated from the brain extract. There was no significant difference in the amounts of activity between PP2A2 peaks obtained from the extract and the postmicrotubule supernatant (Figure 1B). The cl fraction of MT proteins purified from the extract by two cycles of assembly and disassembly was also examined by chromatography on Mono-Q column. PP2A1 was found to be present in amounts about 2.5 times as high as PP2A2 (Figure 1C). The PP2A1/PP2A2 activity ratios were estimated to

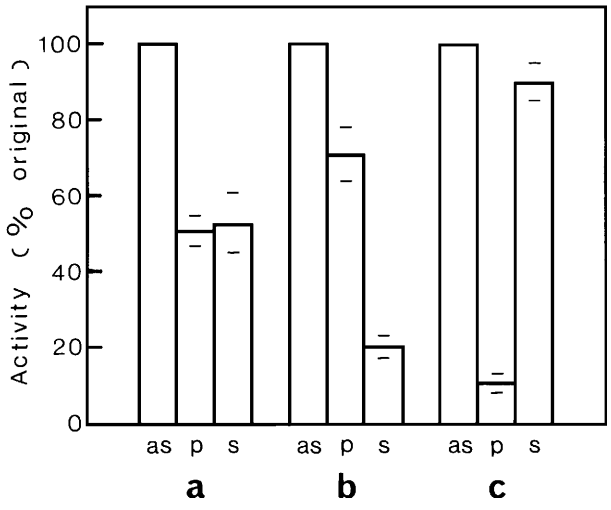

Figure 3 PP2A1 isolated from the MT fraction and the postmicrotubule supernatant associate with MTs, showing different affinity

PP2A1 from the postmicrotubule supernatant (4.59 ± 0.63 mU) (**a**), PP2A1 isolated from the cl fraction of MT proteins (5.73 ± 0.74 mU) (**b**), or PP2A2 from the above supernatant (7.66 ± 0.20 mU) (**c**) was tested for its ability to associate with the cs MT proteins as described in the Materials and methods section and controls in which PP2A was omitted (not shown) were also conducted. The phosphatase activities in the assembly solutions (as), pellets (p) and supernatants (s) in the controls were subtracted from those in the respective fractions in the tests. The activity in the control was about 10% of that in the test. Thus obtained activities of the added PP2A are expressed as recoveries to the fractions (percentages of the activities in the original assembly solutions). The values are means ± S.E.M. ($n = 4$).

be 0.98 for the extract, 0.30 for the postmicrotubule supernatant and 2.8 for the cl MT proteins. Assays of PP2A which were carried out at 1:50, 1:100 and 1:200 final dilution of the Mono-Q fractions (Figure 2) indicated that no substances in the fractions significantly interfered with the assays by inhibiting or stimulating the activities of PP2A1 under the conditions employed, although the PP2A1 activities decreased rapidly during the store. These results taken together suggested that PP2A1 had higher affinity for MT than had PP2A2.

Hence, isolated PP2A1 and 2A2 were examined for their ability to associate with MT. There are two ways to prevent inactivation of PP2A during the incubation for polymerization of MT proteins. The first is the use of the cs fraction of MT proteins which is known to assemble even at 4 °C and in the absence of glycerol. The second is to omit both GTP and ATP which are ordinarily employed to promote the polymerization but known to potently inhibit PP2A. Thus cs MT proteins were incubated at 37 °C with neither glycerol nor the nucleoside triphosphates, prior to the inclusion of PP2A and thereafter the incubation of PP2A with the preincubated MTs was carried out at ambient temperature for 30 min in the presence of glycerol (Figure 3). The subsequent centrifugation revealed that 51% and 53% of the postmicrotubule supernatant-PP2A1 in the original assembly solutions were found in the precipitates and the supernatants, respectively. The PP2A1 isolated from the cl MT fraction more greatly associated with MT proteins: 71% was in the pellets while only 20% in the supernatants. PP2A2 largely (90%) remained in the supernatants, precipitated only slightly (11%) and thus associated with MT much more poorly than did PP2A1 from either the two subcellular fractions. The assays were performed with 100–200 fold finally diluted PP2A1 and consequently in the presence of MT proteins at 0.0075–0.015 mg/ml at

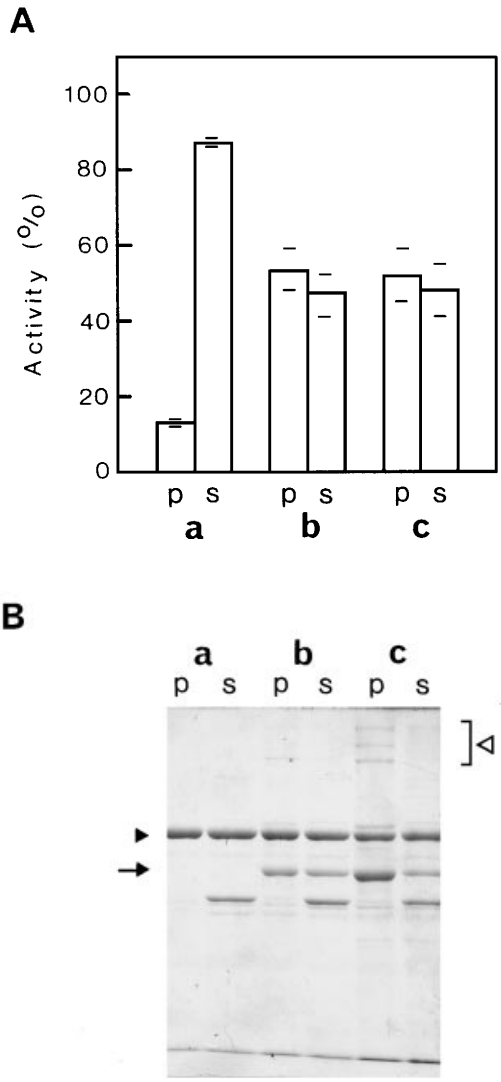

Figure 4 PP2A1 coprecipitates with purified tubulin

(**A**) PP2A1 was examined for its ability to bind taxol-purified tubulin. PP2A1 isolated from the extract was incubated in the absence (a) or the presence of MT proteins (b, purified tubulin; c, purified tubulin in addition to MAP) which had been polymerized prior to the inclusion of PP2A1 as described in the Materials and methods section. Controls omitting PP2A1 were conducted (not shown) and the added PP2A1 in the dissolved pellets (p) as well as the supernatant (s) were calculated by subtraction as described in Figure 3. Distributions (percentages of total) of the added PP2A1 activities are shown and the values are means ± S.E.M. ($n = 2$). (**B**) Aliquots (7.5 μl) of the pellets and the supernatants in (a), (b) and (c) in (**A**) were analysed by SDS/PAGE. The arrow denotes tubulin and the open arrow head indicates the high molecular mass MAP. The closed arrow head indicates BSA in buffer B added to the two fractions. PP2A1 were in too low amounts to be visible on the gel.

which concentrations MT proteins do not affect the activities of PP2A1.

For identification of proteins in the MT fraction which were responsible for the association of PP2A1, tubulin, the major constituent of MT proteins, was first examined for its ability to bind to PP2A1. When purified tubulin is polymerized, incubated with PP2A1 (of the extract origin) and thereafter sedimented, 54% of the total (supernatant and pellet) PP2A1 activity was found with the precipitated tubulin and 46% was in the supernatant (Figure 4Ab). The rather low distribution to the

Table 1 Effect of MT proteins and tubulin on PP2A activities

Activities of PP2A1 and PP2A2 which were both isolated from the extract were separately measured with cl MT proteins, tubulin (total activity) or without additions. The tubulin was purified by chromatography on phosphocellulose and the MT proteins were dialysed against buffer A3 before use. Activities of phosphatases in the cl MT proteins and the preparations of tubulin in the assays without the added PP2A (endogenous activity) were also determined. Activities of the added PP2A1 and PP2A2 were calculated by subtraction where the endogenous phosphatase activities in the presence of tubulin or MT proteins were deduced from the total (added and endogenous) phosphatase activities. Values in parentheses are the added PP2A activity expressed as a percentage of that measured under control conditions, i.e. without MT proteins or tubulin.

	Protein phosphatase activities (munits/ml)		
Additions	Endogenous	Added PP2A1	Added PP2A2
None	0	97.5 (100)	79.2 (100)
cl MT proteins at 1 mg/ml	37.1	14.4 (15)	48.4 (61)
Tubulin at 0.25 mg/ml	8.83	41.4 (42)	70.0 (88)

pellets is probably due to incomplete precipitation of tubulin itself because the amount of the sedimented tubulin was, under the conditions, also about half of the total (Figure 4Bb). The absence of tubulin greatly reduced the distribution of PP2A1 activity to the precipitates (Figure 4Aa). When MAPs were also included in the polymerization, the MAPs were mostly found in the pellets but almost none in the supernatant (Figure 4Bc). The inclusion of MAPs did not increase the distribution of PP2A1 to the pellets at all (Figure 4Abc). The PP2A1 was assayed at 80-fold final dilution and thus in the presence of tubulin at 0.01 mg/ml where tubulin does not inhibit the PP2A1 activity.

The interaction of PP2A1 with tubulin inhibits PP2A1 activity toward phosphorylase

The association described above raises the possibility that MT proteins may have a regulatory effect on the function of PP2A1 or PP2A2. Accordingly, the cl fraction of MT proteins which is hard to polymerize at 30 °C without glycerol was examined for its effect on the activities of the PP2A. Tubulin amounts to $\geqslant 85\%$ (by weight) of the MT proteins; hence, phosphocellulose-purified tubulin was tested for its effect on the PP2A as well (Table 1). Since the phosphatase activity towards phosphorylase is detected in appreciable amounts in the MT proteins, unless those are treated with NaF, and in small but significant amounts in the tubulin preparation; the added PP2A activities calculated by subtraction were compared as shown in Table 1. Thus PP2A1 and PP2A2 (both isolated from the extract) were found to be inhibited by 85% and 39%, respectively, by the presence of cl MT proteins at 1 mg/ml. The purified tubulin at 0.25 mg/ml decreased PP2A1 activity by 58% but only slightly reduced PP2A2 activity (by 12%).

The decrease in the total (added PP2A and the endogenous) activities is brought about by reduction in the activities of the added PP2A activity but not the endogenous phosphatases. This is because the inactivation of the endogenous phosphatases by either heat or NaF treatment of the MT proteins decreased only the total activities but produced no change of the inhibition of the added PP2A1 (not shown and see Figure 6).

The inhibition by 0.2 mg/ml cl MT proteins of added PP2A1 (of the extract origin) was 47% (not shown) and similar to that elicited by tubulin at 0.25 mg/ml (Table 1). Tubulin at various concentrations was thus examined for its ability to affect the activity of PP2A1 (of the MT fraction origin). As shown in

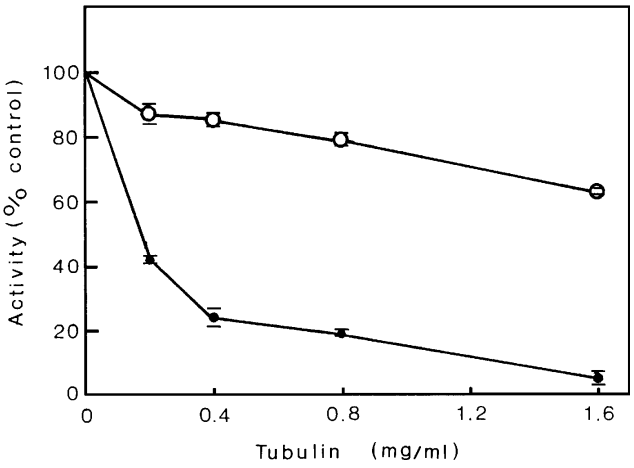

Figure 5 Potent inhibition of PP2A1 by purified tubulin

PP2A1 (21.5 ± 1.9 mU/ml) isolated from cl MT proteins and PP2A2 (18.8 ± 2.1 mU/ml) from the postmicrotubule supernatant were assayed separately for their activities in the presence of taxol-purified tubulin at the indicated concentrations. The endogenous phosphatase activities in the purified-tubulin were also measured. The added PP2A1 (closed circles) as well as PP2A2 (open circles) activities were calculated by subtraction as in Table 1 and are expressed as a percentage of that measured without tubulin. The values are means $\pm$ S.E.M. ($n = 3$–5).

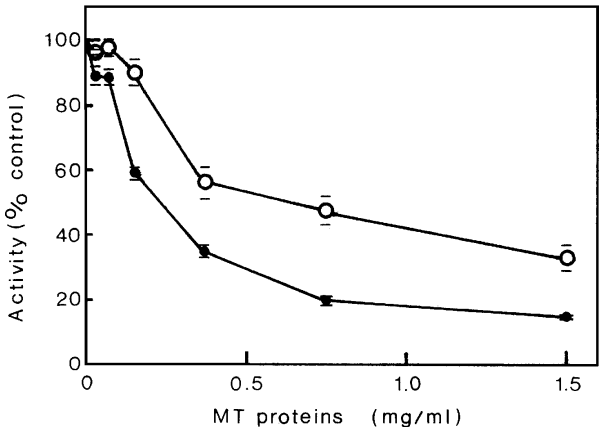

Figure 6 PP2A1 from MTs is more sensitive to the inhibition by MT proteins than is PP2A1 from the postmicrotubule supernatant

PP2A1 isolated from MT proteins (49.8 ± 4.5 munits/ml, closed circles) or the postmicrotubule supernatant (62.8 ± 5.0 munits/ml, open circles) was assayed in the presence of cl MT proteins at the indicated concentrations. The cl MT fraction was treated with NaF and EDTA and thus contained very low phosphatase activity. The added PP2A1 activities were expressed as a percentage of that measured without the MT proteins. The values are means $\pm$ S.E.M. ($n = 2$–3).

Figure 5, taxol-purified tubulin at 0.2 mg/ml gave 58% inhibition for PP2A1 but only 13% for PP2A2, consistent with the inhibitory effect of tubulin purified by phosphocellulose (Table 1). Taxol-purified tubulin at 0.8 mg/ml reduced the activities of PP2A1 and PP2A2 by 81% and only 21% respectively. Thus tubulin was fairly similar to cl MT proteins in inhibition of PP2A1 but slightly less potent in inhibition of PP2A2 than were cl MT proteins (Table 1 and Figure 5). The purified tubulin at 1.6 mg/ml inhibited PP2A1 nearly completely (by 95%). Tubulin further purified by one more precipitation with taxol in the

presence of NaCl to remove the endogenous phosphatase produced similar inhibition (not shown).

PP2A1 isolated from MT proteins has higher affinity for MT proteins as well as purified tubulin than has PP2A1 from postmicrotubule supernatant

Since PP2A1 isolated from the cl fraction of MT more largely cosedimented with the polymerized MT proteins than did PP2A1 isolated from the postmicrotubule supernatant (Figure 3) and because of the repeated detection of the significantly higher sensitivity of the MT PP2A1 to the inhibition by tubulin than that of PP2A1 isolated from the extract (58 % inhibition by tubulin at 0.25 mg/ml obtained for the extract PP2A1, Table 1, versus 58 % inhibition at 0.2 mg/ml for the MT PP2A1, Figure 5), PP2A1 isolated from the MT fraction and PP2A1 from the postmicrotubule supernatant were examined for their sensitivity to the inhibition by cl MT proteins. As depicted in Figure 6, 50 % inhibition was observed at 0.2 mg/ml of cl MT proteins for the MT phosphatase and at 0.6 mg/ml for the supernatant PP2A1. The MT proteins at 1.5 mg/ml, where the inhibition nearly reached a plateau, decreased the activity of the MT PP2A1 by 86 %.

The difference in the inhibition between PP2A1s of the two origins hardly diminished as the concentrations of cl MT proteins decreased from 1.5 to 0.15 mg/ml. It is therefore highly improbable that the difference was entirely brought about by the phosphatases copurified with the supernatant PP2A1, which were distinct from PP2A1 and totally insensitive to the inhibition by tubulin (Figure 6). Purified tubulin was found to 50 % inhibit the MT PP2A1 and the supernatant PP2A1 at 0.1 mg/ml and 0.3 mg/ml, respectively (not shown).

DISCUSSION

The present work shows that tubulin is very likely to target PP2A1 to MT and thereby modulate the activity of the phosphatase. PP2A1 but not PP2A2 largely binds to the polymerized MT proteins *in vitro* (Figure 3) in good agreement with the proposal by Sontag et al. that a heterotrimeric form of PP2A (BαAC), but not the AC dimer, is the form bound to MT [12]. Tau is shown by the authors to bind to BαAC, BβAC and AC (albeit more weakly) forms and is a candidate protein associating PP2A with MT [23]. This work demonstrates further that the isolated PP2A1 cosediments with purified tubulin independently of MAP (Figure 4). Accordingly, the association of PP2A1 with MT is at least in part effected by tubulin which is most likely to bind B subunit of PP2A1 and thus MAP may not be indispensable to the association. Tau probably additionally regulates the PP2A1 associated with MT.

Tubulin and MT proteins potently inhibit the activity of PP2A1, but not PP2A2, towards glycogen phosphorylase a (Table 1 and Figure 5). In addition, PP2A1 isolated from the cl fraction of MT is 50 % inhibited by tubulin and MT proteins, respectively, at 0.1 and 0.2 mg/ml, while PP2A1 from the postmicrotubule supernatant is, respectively, at 0.3 and 0.6 mg/ml (Figure 6 and not shown). Besides, the MT PP2A1 shows higher recovery to the polymerized MT pellets than does the supernatant PP2A1 (Figure 3).

Therefore, PP2A1 isolated from cl MT proteins has higher affinity for tubulin and MT proteins than has PP2A1 from the postmicrotubule supernatant, consistent with its co-isolation with the MT fraction during the repeated assembly and disassembly (Figures 1 and 2). The molecular basis for the difference in the affinity is not known at present. Some MAPs which may increase the affinity may be copurified with the MT PP2A1. Another possibility is that two major forms of PP2A1, for example BαAC and BβAC, may be present in the bovine extract, which is employed in this work, although BαAC form is the major PP2A1 in CV1 cell [28]. In support of this, the immunoreactivity against A, Bα, Bβ and C subunits reveals that Bα and Bβ subunits, each capable of associating with MT structures [12,29], are found with appreciable intensity in rat brain [29].

Tubulin inhibits the activity of PP2A1 towards phosphorylase, a good but probably unphysiological substrate for PP2A on MTs, but may stimulate the activity toward *in vivo* substrate as observed for type 1 phosphatase [10]. Irrespective as to whether the effect of tubulin is inhibitory or stimulatory, it is obvious that tubulin is very likely to play a principal role in regulation of the activity and localization of PP2A1 on MT. The MT PP2A1 may be largely tubulin-bound and under the control of tubulin *in vivo* since it is strongly affected by tubulin at 0.1–0.2 mg/ml, well within the concentration in the cell. Interaction of the PP2A1 with MT proteins may not only regulate its activity but also play roles in the stabilization of its trimeric structure since the conversion of PP2A1 to PP2A2 during the dialysis prior to the Mono-Q chromatography appeared to occur less appreciably in the extract than in the postmicrotubule supernatant (preliminary results). PP2A1 of the supernatant origin with the lower affinity may be subject to moderate regulation by tubulin.

PP2A1 from cl MT proteins is not inhibited totally identically by purified tubulin and MT proteins (Figures 5 and 6), nor is PP2A2 (Table 1 and Figure 5). Hence, some MAP such as tau may modulate the action of tubulin on the PP2A1 by interacting with its subunits. Phosphorylation of the PP2A1-MT complexes is another possible mechanism to modulate the effect of tubulin. The modulatory mechanisms may allow particular PP2A1-molecules on MT to selectively dephosphorylate limited proteins among the many substrates and/or may contribute to the cell cycle changes observed in the overall activity of PP2A1 associated with MT [12].

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