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Interaction of maize (*Zea mays*) protein phosphatase 2A with tubulin

Olubunmi S. Awotunde[1*], Katarzyna Lechward[2*], Katarzyna Krajewska[1], Stanisław Żołnierowicz and Grażyna Muszyńska[1✉]

[1]*Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warszawa, Poland;*
[2]*Intercollegiate Faculty of Biotechnology, Medical University of Gdańsk, Gdańsk, Poland*

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Immunological and biochemical evidence has been obtained for an interaction of maize protein phosphatase 2A (PP2A) holoenzyme with tubulin. Tubulin co-purifies with maize seedling PP2A. Affinity chromatography of the maize PP2A preparation on immobilized tubulin revealed two peaks of phosphorylase *a* phosphatase activity. In one of the peaks, the catalytic (C) and constant regulatory (A) subunits of PP2A were identified by Western blotting. The subunits (C and A) of PP2A were co-immunoprecipitated from maize seedlings homogenate by an anti-α-tubulin antibody.

The interaction of plant PP2A with tubulin indicates a possible role of reversible protein phosphorylation in the dynamic structure of plant cytoskeleton.

Tubulin polymerizes into long chains or filaments that form microtubules, hollow fibers which serve as a skeletal system for living cells. Microtubules (MT) have the ability to shift through various conformations, which enables a cell to undergo mitosis. Mitotic movements of chromosomes are usually coupled to the elongation and shortening of the microtubules to which they are bound. The length of kinetochore-associated MT changes by incorporation or loss of tubulin subunits, principally at their chromosome-bound ends (Hunt & McIntosh, 1998). The rapid transitions between polymerization and depolyme-

[*]O.S. Awotunde and K. Lechward contributed equally to this work.

[✉]Corresponding author: Grażyna Muszyńska, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, A. Pawińskiego 5A, 02-106 Warszawa, Poland; e-mail: muszynsk@ibb.waw.pl

Abbreviations: MT, microtubule; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; A, constant regulatory subunit of PP2A; C, catalytic subunit of PP2A; tau, the axonal microtubule-associated protein.

rization of tubulin subunits are at least in part regulated by the phosphorylation state of tubulin. The control of MT dynamics during the cell cycle transitions is determined by the balance between cdc kinases and phosphatases that directly or indirectly oppose each other's action on target molecules (Tournebize *et al.*, 1997). The trimeric form of bovine protein phosphatase 2A (PP2A) composed of a catalytic (C), structural (A) and variable (B) subunit (for a review see Janssens & Goris, 2001; Lechward *et al.*, 2001) has been regarded as a cytoplasmic protein and was shown to colocalize with MTs in intact cells (Sontag *et al.*, 1995). Furthermore, this phosphatase was found to bind tubulin and its activity was inhibited by tubulin (Hiraga & Tamura, 2000). Mutations in genes encoding PP2A subunits resulted in defects of yeast and *Drosophila* MT function (Kinoshita *et al.*, 1996; Evans & Stark, 1997; Snaith *et al.*, 1996). The control of MT dynamics was studied using specific inhibitors of PP2A in *Xenopus* egg extracts. It was found that PP2A is involved in controlling MT length because inhibition of its activity by okadaic acid results in the formation of long MT. Thus, PP2A is required to maintain the short steady-state length of MTs in mitosis by regulating the frequency of transitions between growth and shrinkage of MTs (Tournebize *et al.*, 1997). The activity of microtubule-associated PP2A is differentially regulated during the cell cycle (Sontag *et al.*, 1995). PP2A is believed to be able to dephosphorylate tubulin (Sontag *et al.*, 1999) and a number of MT-associated proteins including tau (Sontag *et al.*, 1999; Merrick *et al.*, 1996) and oncoprotein 18/stathmin (Cassimeris, 2002). Tau (the axonal microtubule-associated protein) stabilizes MT by binding to tubulin polymers but the phosphorylation of tau decreases its binding activity toward MT and thereby lowers its stabilizing effect (Merrick *et al.*, 1996). In contrast, oncoprotein 18, when phosphorylated, is prevented from binding to tubulin and thus its microtubule destabilizing activity is weakened (Cassimeris, 2002). These findings indicate that interactions between the PP2A and MT proteins, especially tubulin and tau, might modulate MT assembly.

In plant cells, MTs have a number of specialized roles, including participation in cell division and differentiation processes. Due to the existence of the cell wall, differentiation in plants is regulated not by cell migration or changes in the shape of the cells, but by the definition of the plane of cell division and the direction of cell elongation, processes in which MTs are involved. In this sense, MTs are at the basis of the morphogenetic processes occurring in plant cells (Uribe *et al.*, 1998). Like in animal cells, plant MT arrays change during the cell cycle and MT behaviour is thought to be influenced by protein phosphorylation. The morphology of primary roots of *Arabidopsis thaliana* and the organization of cortical MT are affected by inhibitors of protein kinases and serine-threonine phosphatases (Baskin & Wilson, 1997). Specific inhibitors of PP2A in the nanomolar range arrest root hair growth, severely affect the shape of cells within the elongation zone and inhibit root growth (Smith *et al.,* 1994). Further studies have demonstrated that exposure of alfalfa cells to endothal (ET), a cell-permeable inhibitor of PP2A, resulted in a disturbance of preprophase band formation, increase in the number of nuclei with prophase MT assembly, premature polarization of the spindle and abnormal phragmoplast maturation (Ayaydin *et* al., 2000). It has been also shown that the PP2A regulatory subunit A of *A. thaliana* functions as a positive regulator of the PP2A holoenzyme, increasing its activity towards substrates involved in organ elongation and differential cell elongation responses (Deruère *et al.,* 1999). The above results show that plant PP2A may be involve in organization of MTs.

In maize three tubulin isotypes are associated with different groups of cells, throughout the distinct states of cell differentiation (Uribe *et al.,* 1998). Also, the presence in

maize of PP2A holoenzymes generally resembling those of animal cells (Awotunde *et al.*, 2000) prompted us to investigate whether the plant cytoskeleton structure is associated with a reversible phosphorylation system.

MATERIALS AND METHODS

Chemicals. Tubulin was from Sigma. CNBr-activated Sepharose 4B and protein A-Sepharose were from Pharmacia Biotech. Protein standards for electrophoresis, acrylamide, bis-acrylamide, *N,N,N′,N′*-tetramethylethylenediamine (TEMED) and sodium dodecyl sulfate (SDS) were from Bio-Rad. Miracloth (quick filtration material for gelatinous grindates) was purchased from Calbiochem, and filter paper 3MM from Whatman. All other reagents were of analytical grade.

Antibodies. Monoclonal anti-α-tubulin antibody (mouse IgG1 isotype) was from Sigma. Antibodies against a peptide from the N-terminus of the Cα subunit (MDEKVFTKELDQWIEQLNEC), and against an internal peptide of the Aα subunit (DTPMVRRAAASKLGEFAKVL) of mammalian PP2A were kindly donated by Dr. B.A. Hemmings from the Friedrich Miescher-Institut, Basel, Switzerland.

Mass spectrometry of internal tryptic peptides. PP2A from maize seedlings was purified as described previously (Awotunde *et al.*, 2000). The enzymatically active material was pooled and concentrated by using a Centricon 30 concentrator (Amicon Inc., Beverley, MA, U.S.A.), and the proteins were separated on a 12% polyacrylamide gel. A slice of the gel, containing the protein of interest, was lyophilized and sent for analysis at the Beckman Centre, Stanford University Medical Centre (Palo Alto, CA, U.S.A.). The protein was subjected to digestion with trypsin, then the resulting peptides were separated by reversed-phase HPLC and analyzed by mass spectrometry.

Plant material and partial purification of PP2A. Maize (*Zea mays*, var. Mona) seedlings were grown as described before (Awotunde *et al.*, 2000). Three-day old maize seedlings, being in an intensive elongation phase, with a dynamic structure of MT, were used. The etiolated apical parts of the seedlings were harvested, immediately frozen in liquid nitrogen and kept at –80°C. Frozen maize seedlings (20 g) were ground with glass beads in liquid nitrogen in a mortar and homogenized in 60 ml of solution (2 mM EDTA, pH 7.0, 2 mM EGTA, pH 7.0, 0.1%, v/v, 2-mercaptoethanol, 2 mM benzamidine, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 1 mM PMSF and 250 mM sucrose). The homogenate was centrifuged at 14 000 × ***g*** for 15 min. The supernatant obtained was filtered through Miracloth and mixed with 5 ml of DEAE-cellulose suspension previously equilibrated in buffer A (20 mM Tris/HCl, pH 7.5, 0.1 mM EDTA, pH 7.0, 0.2%, v/v, 2-mercaptoethanol, 1 mM PMSF). After 30 min of stirring, unbound material was discarded by filtration and the resin was washed with 25 ml of buffer A, followed by 25 ml of buffer B (buffer A plus 50 mM NaCl). The DEAE-cellulose-bound proteins were eluted with buffer C (buffer A plus 300 mM NaCl). The first 6 ml of the eluate was removed and from the next 42 ml protein was precipitated with ammonium sulfate. The pellet collected between 30 and 60% saturation of ammonium sulfate was suspended in 10 ml of buffer D (buffer A plus 10%, v/v, glycerol), dialyzed against the same buffer for 16 h followed by centrifugation at 60 000 × ***g*** for 60 min. The supernatant was applied onto a tubulin- Sepharose 4B column previously equilibrated with tubulin buffer.

Reconstitution and immobilization of tubulin. Tubulin (5.0 mg) was reconstituted by addition of 900 μl of distilled water at 37°C and gentle shaking for 5 min. The reconstituted tubulin was diluted further in 1 ml of cold tubulin buffer (0.1 M Mes, pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 2.5 M glycerol). The tubulin solution was incubated

on ice for 40 min with homogenization every 10 min and centrifuged for 40 min at 25 000 × ***g*** at 4°C. The supernatant was collected, kept on ice and the protein content was determined.

Tubulin, 3 mg diluted in tubulin buffer to the concentration of 1 mg/ml, was mixed with swollen CNBr-Sepharose (3 ml) for 2 h at room temperature. Unbound protein was washed away with 10 times gel volume of tubulin buffer. The remaining reactive groups were blocked in 0.1 M Tris/HCl buffer, pH 6.5 for 2 h. The gel with immobilized tubulin was washed with tubulin buffer, and used for affinity column chromatography.

Immunoprecipitation of proteins from maize seedlings homogenate by anti-tubulin antibody. Frozen maize seedlings (10 g) were ground with glass beads in liquid nitrogen, homogenized with 27 ml of homogenization buffer (10 mM Tris/HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20). The suspension was centrifuged twice at 14 000 × ***g*** for 10 min at 4°C. The obtained supernatant (1 ml containing 2 mg of protein) was incubated with 5 μl of anti-α-tubulin antibody for 2 h with rotation at 4°C. Then, the immunocomplex was coupled for 1 h to 100 μl of Protein A-Sepharose (PAS) previously equilibrated with homogenization buffer. The resulting immunoprecipitate was washed and subjected to SDS/PAGE and Western blotting as described previously (Nick *et al.,* 1995).

SDS/PAGE. The slab gel system of Laemmli (Laemmli, 1970) was applied. Routinely, 10% polyacrylamide gels were used.

Immunodetection of PP2A subunits and determination of protein phosphatase activity (using ^{32}P-labelled phosphorylase *a* as a substrate) were performed as previously described (Awotunde *et al.,* 2000).

Protein was measured either by recording the absorbance at 280 nm or according to Bradford (Bradford, 1976) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

In searching for a role of plant PP2A, the first attempt was to check whether maize PP2A interacts with other protein(s) of known physiological role. Therefore, a protein co-purifying with maize PP2A holoenzyme was analyzed.

We found out that the protein which co-purifies with PP2A belongs to the tubulin family. This result was obtained by mass spectrometry, through comparison of the peptide pattern of the tryptic digest with proteins in the data base (not shown). In order to answer whether tubulin interacts specifically with maize PP2A, immunological and biochemical approaches were undertaken. To demonstrate rigorously that maize seedling PP2A interacts with tubulin, proteins interacting with tubulin were immunoprecipitated from maize homogenate by anti-α-tubulin antibodies. In the resulting immunocomplex the presence of PP2A subunits was monitored by Western blotting (Fig. 1). The presence of the constant regulatory subunit of PP2A (A), molecular mass 66 kDa, and the catalytic subunit (C) of molecular mass 38 kDa was detected after immunoprecipitation (Fig. 1, lane 2, panels A and B, respectively). In the immunoprecipitate, tubulin was also detected by Western blotting with anti-α-tubulin antibodies (not shown). The above results indicate that an oligomeric form (dimer or trimer) of maize PP2A is associated with tubulin. Recent studies of PP2A from bovine brain have shown that the trimeric form, but not the dimeric one, strongly associates with MT proteins through its interaction with tubulin (Hiraga & Tamura, 2000). Also, the activity of the trimeric form of PP2A is more strongly inhibited by MT proteins and purified tubulin (Hiraga & Tamura, 2000). These results are in good agreement with the proposal of Sontag *et al.* (1995) that the trimeric PP2A is the form bound to MT.

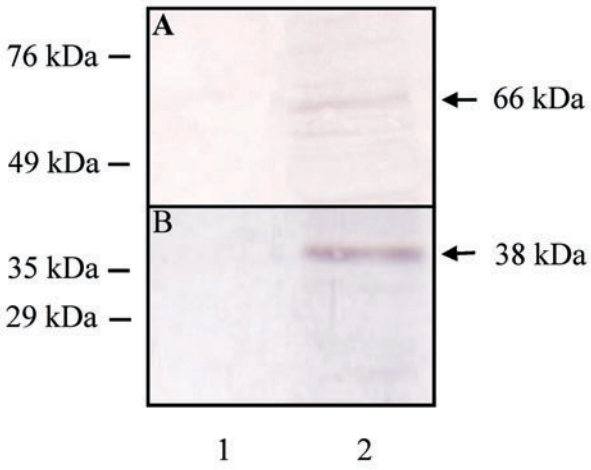

Figure 1. Immunodetection of PP2A subunits in maize protein extract after immunoprecipitation with anti-α-tubulin antibody.

Western blotting of proteins with antibodies against the A (panel A) and C (panel B) subunits. Lanes 1, homogenate with immobilized protein A without anti-α-tubulin antibody (control); lanes 2, homogenate with immobilized protein A and anti-α-tubulin antibody (immunoprecipitated proteins). Molecular masses of markers and of the identified proteins are marked on the left and on the right, respectively.

To support the results of immunoprecipitation concerning maize PP2A – tubulin interaction, a biochemical approach was utilized. Commercially available tubulin immobilized on CNBr-activated Sepharose was used for an affinity binding study of maize seedling PP2A. From the total phosphatase activity present in the enzymatic preparation two peaks (I and II) of maize phosphorylase *a* phosphatase activity were eluted, by linear gradient of salt, from a tubulin-Sepharose 4B column in fractions 4–6 (peak I) and 12–14 (peak II), at 0.13 and 0.31 M NaCl, respectively (Fig. 2). For the identification of PP2A in the eluted peaks of activity, immunodetection with antibodies directed against the catalytic (C) and structural (A) subunits was performed. These antibodies reacted with two proteins of 66 kDa and 38 kDa present in peak I (Fig 3, panels A and B, respectively). These molecular masses are in agreement with the masses of maize seedling PP2A subunits (Awotunde *et al.*, 2000). The protein phosphatase activity as well as the immunodetection of the catalytic and structural subunits of PP2A in peak I provide evidence that a maize PP2A isoenzyme is able to interact with tubulin. In fractions 12–14 exhibiting phosphatase activity, and also in fraction 9 without such activity, no immunoreactivity against the subunits of PP2A was detected (Fig. 3, panels A and B, respectively). Because protein phosphatase type 1 (PP1) and PP2A are the only enzymes in eukaryotic tissues with significant activity toward dephosphorylation of phosphorylase *a* (MacKintosh & Cohen, 1989), the activity of the enzyme eluted in peak II suggests that this phosphatase may belong to the PP1 class. The suggestion of the binding of PP1 to tubulin is supported by data indicating that the function of PP1 may be associated with MT dynamics (Tournebize *et al.*, 1997). The results of inhibitor studies suggest that alfalfa PP1 (Ayaydin *et al.*, 2000), like *Xenopus* PP1 (Tournebize *et al.*, 1997), is required for MT dynamics during mitotic transition. Moreover, changes in cell morphology mediated by altered MT arrays

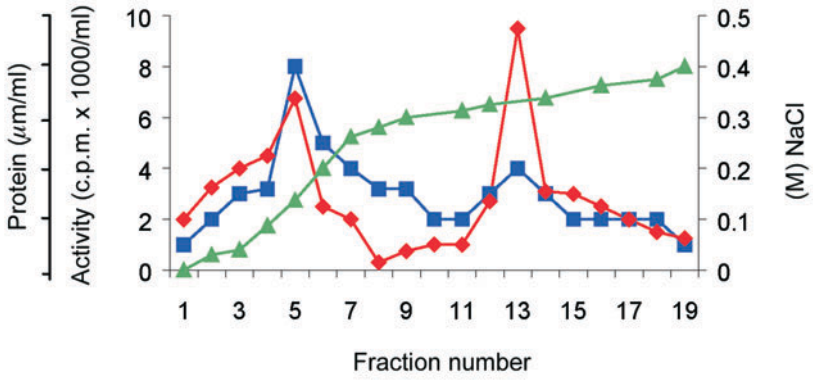

Figure 2. Elution profile of protein phosphatase activity from tubulin-Sepharose 4B column.

Phosphatase activity was measured using ^{32}P-labelled phosphorylase *a* as substrate: ◆, activity; ■, protein; ▲, NaCl.

caused by tubulin depolymerization were observed in roots of *A. thaliana* in the presence of specific and potent PP1 and PP2A inhibitors (okadaic acid and calyculin A). These compounds arrested root hair growth, severely affected the shape of cells within the elongation zone and inhibited root growth (Smith *et al.*, 1994).

It is know that microtubules play a major role in plant morphogenesis (for a review see Shibaoka, 1991). The orientation of cortical MTs in the cell is believed to modulate the direction of cellulose microfibril deposition in the cell wall, which ultimately controls the direction of expansion of a growing cell. Therefore, conditions that perturb the stability and orientation of cortical MTs are expected to affect the final shape of growing cells (Smith *et al.*, 1994), and PP2A and PP1 may function in controlling the mitotic and morphogenic events in plant cell.

It should be pointed out that the interaction of PP2A with tubulin described here was observed in etiolated maize seedlings being at the intensive cell elongation phase. The changes in plant cell expansion are mediated by reorganization of cortical MT due to the activity of specific microtubule associated proteins. One of the proteins is expressed exclusively upon induction of maize cell elongation (Nick *et al.*, 1995). This 50 kDa putative microtubule-associated protein (MAP) can be detected also in etiolated coleoptiles of rice, where cell elongation can proceed only in darkness. This suggests that, in Graminean coleoptiles, expression of the 50 kDa protein can be a marker for cell elongation (Nick *et al.*, 1995). Therefore, it is possible that in plant cell elongation, complexes of PP2A with MAP may modulate the reorganization of the cytoskeleton either by interaction with other proteins or by regulation of the phosphorylation state of the complex.

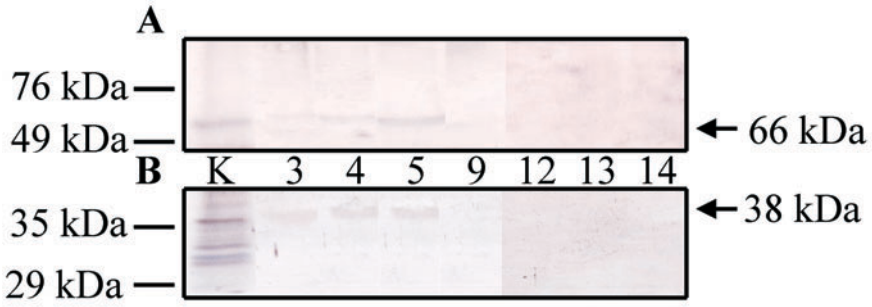

Figure 3. Immunodetection of PP2A subunits after chromatography on tubulin-Sepharose 4B column.

Western blotting of proteins with antibodies against PP2A subunit A (panel A), and against PP2A subunit C (panel B). Lanes K, positive control, maize protein extract after immunoprecipitation with anti-α-tubulin antibody. Lanes: 3, 4, 5, 9, 12, 13, 14, corresponding fractions eluted from the column with immobilized tubulin. Positions of mass standards and of the identified proteins are marked on the left and on the right, respectively.

The present results demonstrate that plant PP2A, like the mammalian enzyme, interacts specifically with tubulin. Molecular cloning of catalytic subunits of PP2A has revealed that it is one of the evolutionarily most conserved proteins. The identity between the plant, human and yeast amino-acid sequences is 79–82% (MacKintosh *et al.*, 1990; Ariño *et al.*, 1993). Also, the sequence of the main components of all MTs, α- and β-tubulin, has been highly conserved throughout evolution (Uribe *et al.*, 1998). These results support the suggestion that the mechanisms regulating essential cellular functions are similar for all eukaryotic cells.

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