

# Phosphofructokinase from *Dictyostelium discoideum* Is a Potent Inhibitor of Tubulin Polymerization<sup>†</sup>

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**ABSTRACT:** We identified the nonallosteric phosphofructokinase from the slime mold *Dictyostelium discoideum* as a potent protein factor that inhibits the rate of polymerization of tubulin at a molar ratio of 1 molecule to about 300 tubulin dimers for half-maximal action ( $IC_{50} = 32$  nM). This effect was (i) assessed by turbidity measurements, pelleting of microtubules, and electron microscopy, (ii) observed when tubulin assembly was induced by taxol as well as by GTP in the presence of microtubule-associated proteins or glutamate, and (iii) specific as it was not produced by the phosphofructokinase from rabbit muscle. Also in contrast to the latter, neither tubulin nor microtubules modified the catalytic activity of the slime mold isozyme. Immunoelectron microscopy provided further evidence that *D. discoideum* phosphofructokinase physically interacts with tubulin, leading to the formation of aggregates. The process seems to be reversible since microtubules eventually formed in the presence of the inhibitor with concomitant reduction of tubulin aggregates. Limited proteolysis by subtilisin showed that the hypervariable C-termini of tubulin is not involved in the interaction with the enzyme. The possible physiological relevance of this novel function of *D. discoideum* phosphofructokinase different from its glycolytic action is discussed.

Microtubules are one of the three major components of the eukaryotic cytoskeleton, involved in several vital functions such as mitosis, intracellular transport, secretion, cell shaping, and motility. These structures are protein polymers composed of  $\alpha\beta$  heterodimeric tubulin subunits. Microtubules and tubulin subunits coexist in living cells in a rapid exchange between them, known as dynamic instability, that implies alternative periods of polymerization and depolymerization (reviewed in refs 1 and 2). This unusual property is related to the utilization of GTP in microtubule assembly, and is modulated by a large family of proteins called microtubule-associated proteins (MAPs)<sup>1</sup> that work as positive regulators of tubulin polymerization. So far, only a few cellular factors efficiently acting as negative controls of microtubule dynamics have been identified, namely, the small

cytosolic phosphoprotein stathmin (3–6) that inhibits tubulin polymerization, and a kinesin-related protein (7) that promotes microtubule depolymerization during mitotic spindle assembly. Tubulin polymerization can also be negatively and positively affected by a number of drugs. Examples are plant alkaloids, long known inhibitors of microtubule assembly (reviewed in ref 8), or taxol, which strongly enhances both microtubule formation and stability (8, 9). The microtubules produced by taxol-induced assembly are not significantly different from those formed in the absence of this agent (10); so that, taxol is conventionally used to induce polymerization of tubulins in vitro.

In addition to the above-mentioned implications of microtubules in cellular processes, several glycolytic enzymes have been found to interact in vitro with cytoskeletal proteins, such as actin, tubulin, and microtubules, the interaction leading to a change in catalytic activity (reviewed in refs 11 and 12). In particular, phosphofructokinase (PFK) (13), aldolase (14), glyceraldehyde-3-phosphate dehydrogenase (15), and lactate dehydrogenase (16) were shown to be inhibited by tubulin. A possible involvement of metabolic regulation was therefore suggested (11–13) for the binding of enzymes to structural elements. Some of these associations also result in an effect on the cytoskeletal network, as reported for PFK (17, 18) and glyceraldehyde-3-phosphate dehydrogenase (19), which causes bundling of microtubules. It is still unknown whether these interactions operate in vivo. However, PFK and several other glycolytic enzymes, such as aldolase and pyruvate kinase, have been identified in axonal transport (20, 21), hence supporting the hypothesis of an association between these enzymes and structural constituents of the cytoplasm (20). In the case of PFK, the

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<sup>1</sup> Abbreviations: MAP, microtubule-associated protein; PFK, phosphofructokinase, EC 2.7.1.11; RmPFK and DdPFK, the phosphofructokinases from rabbit muscle and *Dictyostelium discoideum*, respectively; MES, 2-(N-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; MTs, microtubules.

inhibition reported (13) for the M-type isozyme from rabbit muscle (RmPFK) is mediated by the binding of the inactive dissociated forms of the enzyme to tubulin and microtubules, thus shifting the equilibrium between active tetramers and inactive dimers and monomers.

PFK is well-known to be a paradigm of an allosteric enzyme whose activity is modulated by a variety of metabolic signals, and is considered to be fundamental for the control of glycolysis (22, 23). It is also known that PFK from several sources is able to polymerize into higher than tetrameric forms with increasing enzyme concentration, a change that greatly alter its allosteric regulation (reviewed in ref 24). To further investigate how the reported (13) association of PFK with tubulin and taxol-grown microtubules is related to the allosteric and associative properties of the enzyme, we have examined the possibility of interaction between these cytoskeletal components and a nonallosteric PFK isozyme, that from the slime mold *Dictyostelium discoideum* (DdPFK) (25). This isozyme lacks any regulatory mechanism other than substrate concentration, and is also devoid of a concentration-dependent activity being a stable tetramer (25). We observed that DdPFK activity was insensitive to either tubulin or microtubules. However, and also in contrast to RmPFK, the slime mold isozyme was found to be a novel and potent inhibitor of tubulin polymerization which works at substoichiometric enzyme concentration. This action has been characterized, and evidence for the physical interaction of DdPFK with tubulin is provided. A possible physiological role for this inhibitory effect on the dynamics of tubulin in the slime mold is suggested.

## MATERIALS AND METHODS

**Materials.** Nucleotides, phosphoric esters, auxiliary enzymes used in the assay for PFK, taxol, and subtilisin were purchased from Sigma. Colloidal gold conjugated goat anti-rabbit IgG was from British BioCell. Other reagents were obtained from commercial sources and were of the best grade available.

**Enzyme Preparations.** DdPFK was purified as a recombinant protein after expression of its cDNA in yeast as previously described (26). The recombinant enzyme was reported (26) to exhibit the same subunit size, quaternary structure, and kinetic properties as those of the wild-type enzyme. The purified preparation was dialyzed against four changes of buffer A (50 mM MES, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM dithiothreitol, pH 6.8) before use. The resulting preparation had a specific activity of about 40 units/mg. When DdPFK was used with MAP-containing tubulin, it was dialyzed against buffer B (0.1 M MES, 0.5 mM MgCl<sub>2</sub>, 2 mM EGTA, pH 6.4).

Purified RmPFK obtained from Sigma (type III) was centrifuged at 16000g for 15 min at 6 °C. The supernatant was discarded, and the pellet was suspended in buffer A and dialyzed against the same buffer as above. The resulting preparation had a specific activity of about 4 units/mg. Both enzyme preparations were judged to be homogeneous by the criteria of SDS-PAGE.

**Enzyme Assay.** PFK activity was determined in an assay mixture that unless otherwise indicated contained 50 mM HEPES, 100 mM KCl, 5 mM MgCl<sub>2</sub>, pH 7.0, 0.15 mM NADH, 5 mM P<sub>i</sub>, 0.1 mM cAMP, 1 mM NH<sub>4</sub><sup>+</sup>, 1 mM

MgATP, 1.2 units of aldolase, 10 units of triosephosphate isomerase, 1 unit of glycerophosphate dehydrogenase, and 5–20  $\mu$ L of either DdPFK or RmPFK preparation in a total volume of 1 mL. After 5 min, the reaction was started by adding 1 mM fructose-6-P and was followed by measuring the absorbance change at 340 nm in a Perkin-Elmer Lambda 5 spectrophotometer at 37 °C. Auxiliary enzymes were desalted as described (27). One unit of activity is defined as the amount that catalyzes the conversion of 1  $\mu$ mol of substrate/min under these conditions.

**Tubulin Preparation.** Tubulin was purified from fresh bovine brain according to the modified Weisenberg procedure of Williams and Lee (28), except that Sephadex G-25 substituted for Sephadex G-50 in the gel filtration chromatography and that the final MgCl<sub>2</sub> precipitation step was omitted. The pooled Sephadex G-50 fractions were dialyzed overnight against 1 M sucrose and stored at –80 °C. Before use, tubulin was dialyzed overnight against buffer A without dithiothreitol and centrifuged at 100000g for 30 min at 6 °C to remove aggregated protein. However, neither taxol-induced polymerization of tubulin nor the effect of DdPFK on this process was modified if the tubulin preparation was used without previous dialysis. Purified tubulin was essentially free of MAPs, as judged by SDS-PAGE. When MAP-containing tubulin was used, it was prepared from rat brain by two cycles of assembly and disassembly as described by Shelanski et al. (29) and used immediately. The presence of MAPs of high molecular mass in the preparation was visualized on SDS-PAGE gels.

**Tubulin Polymerization Assay.** Tubulin samples with or without PFK were diluted in buffer A to a final volume of 1 mL and final concentrations as indicated in the text. After allowing the temperature of the mixture to equilibrate at 37 °C, polymerization was initiated by adding 20  $\mu$ M taxol and followed by measuring the turbidity at 350 nm in the spectrophotometer. Tubulin polymerization was also assessed by pelleting the assembled microtubules. To this purpose, samples from the tubulin polymerization mixtures were taken at the times indicated in the text and centrifuged at 16000g for 30 min at 25 °C, and supernatants and resuspended pellets in equivalent volumes of buffer A were subjected to SDS-PAGE on 10% polyacrylamide gels according to Laemmli (30). Gels were stained with Coomassie brilliant blue R-250 (31). Band intensities from stained gels were quantified with a color image scanner (Seiko Epson G520A). When taxol-stabilized microtubules were used, tubulin polymerization was carried out for at least 30 min. Other incubations of tubulin and microtubules with PFK were also carried out at 37 °C. In some experiments, tubulin polymerization was induced by 1 mM GTP in the presence of 1 M glutamate, 1 mM mM MgCl<sub>2</sub>, 1 mM EGTA, pH 6.6. Assembly of MAP-containing tubulin was carried out as above, except that buffer B was used for dilution and that polymerization was initiated by adding 1 mM GTP.

**Digestion of Tubulin by Subtilisin.** Limited digestion of tubulin (1.2 mg/mL) to release the C-terminal tail was carried out with subtilisin at 1:100 (w/w) in buffer A at 30 °C (32, 33). The reaction was terminated after 30 min by addition of 1 mM PMSF, and digestion was verified by SDS-PAGE (32). Taxol-induced polymerization of digested tubulin (1 mg/mL) was performed in buffer A at 30 °C in the absence or the presence of different concentrations of DdPFK and

Table 1: Effect of Tubulin and Microtubules on PFK Activity<sup>a</sup>

	relative activity (%)	
	DdPFK	RmPFK
control	100 ± 5	100 ± 5
+tubulin	118 ± 12	28 ± 9
+MTs	105 ± 19 <sup>b</sup>	26 ± 5 <sup>b</sup>
after sedimentation of PFK + MTs		
supernatant	95 ± 12 <sup>b</sup>	51 ± 5 <sup>b</sup>
pellet	<5 <sup>b</sup>	49 ± 5 <sup>b</sup>

<sup>a</sup> DdPFK and RmPFK at a concentration of 0.2 mg/mL each were incubated in the absence (control) or the presence of 2 mg/mL of either tubulin or taxol-stabilized microtubules (MTs) for 30 min at 37 °C. Sedimentation of MTs in the presence of PFK was carried out at 16000g for 25 min at 25 °C. <sup>b</sup> Enzyme activity in the supernatant and pellet is relative to that measured in the presence of MTs before sedimentation. Results show the mean ± SE of three to six determinations.

assessed as above. No proteolysis of DdPFK was observed during polymerization, as indicated by SDS–PAGE.

**Electron Microscopy.** For negative staining, a drop (10  $\mu$ L) from the tubulin polymerization mixture with or without DdPFK was placed on a collodion–carbon-coated grid for 60 s. The solution was then removed, and the grid was rinsed in water and stained with one drop of 2% uranyl acetate. The excess uranyl acetate was removed from the grid with filter paper. For immunogold electron microscopy, samples (50  $\mu$ L) taken from a mixture of tubulin with DdPFK were fixed with glutaraldehyde and treated according to the procedure of De la Torre et al. (34), except that after treatment with antibodies against DdPFK (25) diluted 1:1000, the grids were incubated with 10  $\mu$ L of 5 nm colloidal gold conjugated goat anti-rabbit IgG diluted 1:30 and stained with 2% uranyl acetate. Grids were examined in a JEOL 1010 electron microscope at 40000 $\times$  and 80000 $\times$  magnifications.

**Other Methods.** Protein concentration in preparations of PFK and taxol-stabilized microtubules was measured by the method of Bradford (35) with bovine  $\gamma$ -globulin as standard and in tubulin preparations by the absorbance at 276 nm using an extinction coefficient of  $1.03 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  (36). The IC<sub>50</sub> value was calculated from the highest slopes of turbidity curves. Molar concentrations of DdPFK tetramer and tubulin dimer were calculated on the basis of  $M_r$  values of 382 000 (25) and 100 000 (37), respectively. Predictions of secondary structures were obtained from the PHD server (38).

## RESULTS

### PFK Activity in the Presence of Tubulin or Microtubules.

As shown in Table 1, DdPFK activity was not affected by either tubulin or taxol-stabilized microtubules, in contrast to RmPFK, which in agreement with a previous report (13) was inhibited by about 70% by either of these cytoskeletal components. Interestingly, whereas about 50% of RmPFK activity cosedimented with microtubules after centrifugation at 16000g, almost all of the DdPFK activity remained in the supernatant, thus indicating that the later enzyme may not interact with microtubules. As RmPFK was inhibited by microtubules, its relative activity in the supernatant is not equivalent to the quantitative distribution of enzyme protein between both fractions.

**Effect of DdPFK on Tubulin Polymerization.** Figure 1 shows the effect of DdPFK on microtubule formation by

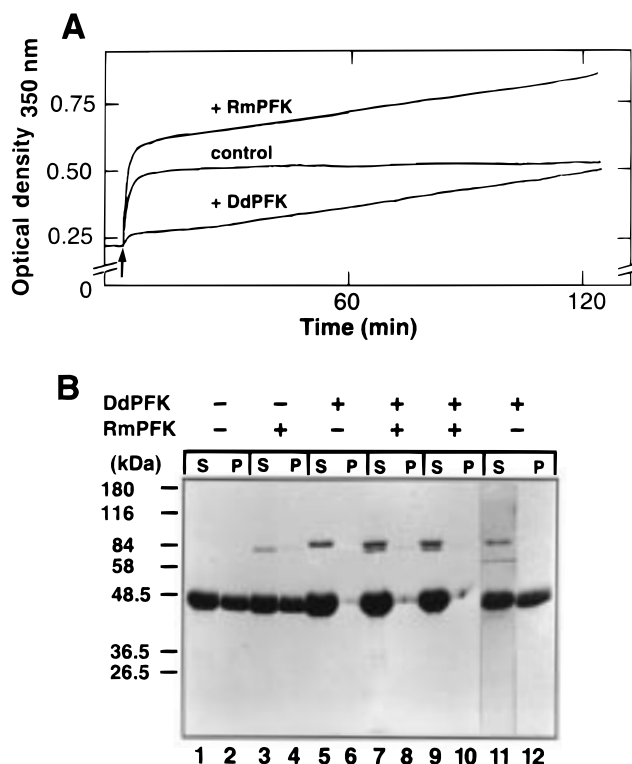


FIGURE 1: Inhibition of tubulin polymerization by DdPFK. Tubulin (2 mg/mL) was incubated for 10 min in the absence (control) or presence of either DdPFK or RmPFK as indicated. Polymerization was initiated by addition of taxol and assessed by measuring turbidity (absorbance at 350 nm) (A) and pelleting of microtubules (B) as described under Materials and Methods. (A) DdPFK and RmPFK were used at a concentration of 0.2 mg/mL each. The arrow indicates the time for taxol addition. (B) DdPFK concentration was 0.2 mg/mL; RmPFK concentration was 0.2 mg/mL (lanes 3, 4, 7, and 8) and 0.4 mg/mL (lanes 9 and 10). Samples were taken from tubulin polymerization mixtures after 30 (lanes 1–10) and 120 (lanes 11 and 12) min incubation and centrifuged at 16000g for 30 min at 25 °C. Aliquots (10  $\mu$ L) of supernatants (S) and resuspended pellets (P) were subjected to SDS–PAGE. Positions of molecular markers (kDa) are shown on the left.

measuring turbidity and sedimentation of the assembled microtubules in comparison with that of RmPFK, which is known (13, 18) not to affect the initial rate of this process under similar polymerization conditions. Addition of taxol to the tubulin solution in the absence of PFK (control) induced polymerization (Figure 1A), as indicated by the rapid increase in turbidity that leveled off after about 10 min. The presence of RmPFK produced no change in the polymerization rate of tubulin, also giving rise to a further and progressive increase in turbidity that has been reported (17) to be related to cross-linking of microtubules. By contrast, addition of 0.2 mg/mL DdPFK strongly inhibited tubulin polymerization, decreasing both its initial rate and the extent of the rapid increase in turbidity. No change in the DdPFK effect was observed if the polymerization assay was carried out either in the presence of fructose-6-P and MgATP at saturating concentrations, 0.2 mM and 0.1 mM (25), respectively, or at a near-physiological salt concentration, such as 100 mM KCl. The inhibitory effect of DdPFK was further evidenced by pelleting experiments. As shown in Figure 1B, the tubulin assembly pattern obtained after polymerization for 30 min in the absence of either of the enzymes (lanes 1 and 2) was not modified when the



polymerization mixture included RmPFK (lanes 3 and 4), part of this enzyme also being visible in the pellet. However, almost all tubulin remained unpolymerized in the presence of DdPFK (lanes 5 and 6), with a very small amount of polymerization products in the pellet and with no DdPFK being apparent in this fraction. The inhibition produced by DdPFK was not neutralized by RmPFK used at the same or double concentration (lanes 7–10). A similar lack of counteraction by the muscle isozyme was observed by turbidity measurements (data not shown). Nevertheless, microtubules eventually formed in the presence of DdPFK if tubulin polymerization was extended to 120 min (lanes 11 and 12). Consistently, turbidity at this time was near that of the control mixture; DdPFK activity remained constant throughout this period (data not shown).

Electron microscopy studies were carried out to identify the nature of the species formed during the extensive polymerization process (at 10 and 120 min) in the presence of DdPFK. As illustrated in Figure 2, whereas microtubules were present in the control sample after 10 min polymerization (Figure 2A), no microtubules were formed in the DdPFK-containing sample after polymerization for the same period and only amorphous tubulin aggregates of various sizes could be visualized (Figure 2C). Most of the aggregated material was present as small (<200 nm) aggregates, though some were considerably larger. However, microtubules were produced in the presence of DdPFK if polymerization was prolonged to 120 min (Figure 2D), and less aggregates were observed after this time. These late-assembled microtubules were identical to those obtained in the control experiment (Figure 2B).

DdPFK inhibited tubulin polymerization in a concentration-dependent manner (Figure 3). The kinetics of the process appear to be complex, no inhibition being observed at 6.2  $\mu\text{g/mL}$  while the initial rate of polymerization decreased about 50% in the presence of 12.5  $\mu\text{g/mL}$ . Nevertheless, from the data shown it seems that half-maximal inhibition is reached at a concentration of DdPFK of 12  $\mu\text{g/mL}$ , corresponding to 32 nM ( $\text{IC}_{50}$ ). Taking into account that the concentration of tubulin dimer was 12  $\mu\text{M}$ , a molar ratio of 1 molecule of the 380 kDa DdPFK tetramer for every 375 tubulin dimers was sufficient for half-maximal inhibition.

As shown in Figure 4, the inhibitory action of DdPFK on microtubule assembly was also exerted under more nearly cellular conditions, such as in the presence of MAPs and polymerization being induced by GTP. Utilization of DdPFK at a 2-fold higher concentration (0.4 mg/mL) further decreased the initial polymerization rate by nearly a factor of 2. Addition of the enzyme to the polymerization reaction once it attained equilibrium did not activate disassembly of microtubules. DdPFK elicited similar effects when MAP-free tubulin was polymerized by 1 mM GTP in the presence of 0.9 M glutamate (data not shown).

Most of the sequences of the known mammalian  $\alpha$  and  $\beta$  tubulins are conserved, except for their acidic C-termini that are hypervariable regions (37, 39, 40). This C-terminal tail ( $M_r < 2000$ ) can be released by limited subtilisin treatment (32, 33, 41), the cleaved protein having an enhanced ability to polymerize into microtubule-like filaments (32, 42–44). Therefore, we checked the effect of DdPFK on subtilisin-digested tubulin to investigate whether the observed reduction of microtubule assembly may reflect a somewhat general

process among the various isoforms, as well as to test the involvement of the C-terminal fragment in the binding of the enzyme. As shown in Figure 5, DdPFK inhibited the polymerization of C-tail free tubulin in a similar way. An  $\text{IC}_{50}$  value of 5  $\mu\text{g/mL}$  was obtained under these conditions. These results are consistent with that MAPs did not interfere with the effect of DdPFK, since the charged C-terminus of tubulin serves as the main site for the binding of MAPs (2).

*Physical Interaction of DdPFK with Tubulin.* Polymerization and pelleting experiments suggested that DdPFK specifically binds tubulin dimers and/or small polymers. These species did not cause significant turbidity enhancement and were not pelleted under conditions at which taxol-stabilized microtubules did (16000g for 30 min; Figure 1). To investigate the interaction of DdPFK with tubulin in the absence of taxol, the samples were centrifuged under conditions (100000g for 30 min) at which only the bound enzyme could be sedimented. As shown in Figure 6, free DdPFK did not pellet (lanes 3 and 4), whereas in the presence of tubulin all of it cosedimented with this protein (lanes 7 and 8), thus evidencing the formation of a complex between DdPFK and tubulin. RmPFK behaved in a similar way (lanes 5, 6 and 9, 10), showing that it was able to associate with tubulin as previously described (13). The binding of DdPFK to tubulin was equally observed when microtubule formation was run in the presence of this enzyme (lanes 11 and 12), and therefore inhibited. Under these conditions, the proportion of tubulin bound to DdPFK was practically similar to that in the absence of taxol (lanes 7 and 8). As expected, tubulin polymerization was not modified by RmPFK [lanes 13, 14 versus the control (lanes 1 and 2)], which cosedimented with microtubules.

Pelleting of taxol-stabilized microtubules in the presence of DdPFK (Figure 7) showed that this enzyme does not bind to microtubules, as all of it remained in the supernatant even when its concentration (0.2 mg/mL) was increased to 0.4 and 0.8 mg/mL. In contrast, RmPFK distributed between supernatant and pellet, indicating that this isozyme interacts with both tubulin and microtubules (13). DdPFK was also found not to cosediment with assembled polymers obtained from C-tail free tubulin (data not shown).

The interaction of DdPFK with tubulin was additionally evidenced by immunoelectron microscopy. Antibodies against this PFK with colloidal gold conjugated IgG, as secondary antibody, were used to visualize the bound enzyme. As shown in Figure 8, the gold particles are clearly seen on the irregular aggregates of tubulin induced by DdPFK in the absence of polymerizing agent. These aggregates were not observed if the enzyme was incubated alone under the same conditions (data not shown) and were similar to those found when DdPFK was present in the polymerization mixture and the sample was taken 10 min after taxol addition (Figure 3C). No binding of the enzyme to microtubules was detected by immunostaining if samples were taken from the same polymerization mixture 120 min after taxol addition (data not shown).

## DISCUSSION

In this study, we identified the nonallosteric DdPFK as a novel and powerful inhibitor of tubulin polymerization. The inhibitory effect occurred independently of whether polym-

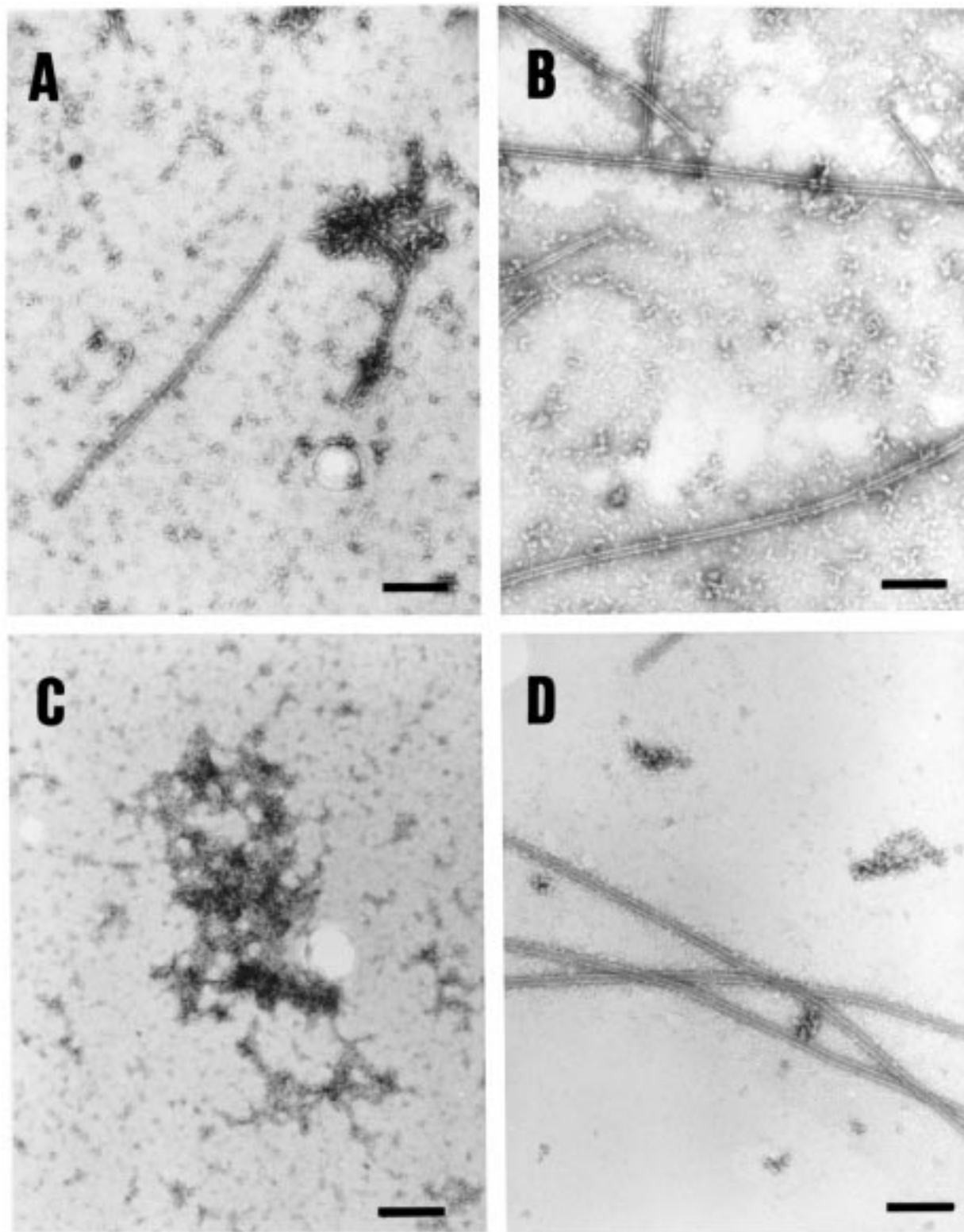


FIGURE 2: Electron microscopy of tubulin polymerization in the presence of DdPFK. Taxol-induced polymerization of tubulin (2 mg/mL) was carried out in the absence (A and B) and in the presence (C and D) of 0.2 mg/mL DdPFK. A drop from the tubulin polymerization mixture was taken 10 (A and C) and 120 (B and D) min after adding taxol, placed on a collodion-carbon-coated grid, and negative stained. Bars = 200 nm.

erization was induced by taxol (Figure 1) or by GTP in the presence of MAPs (Figure 4) or glutamate, and was specific, as it was not produced by the regulatory isozyme RmPFK. Also in contrast to the latter, DdPFK activity was not affected by either tubulin or taxol-stabilized microtubules (Table 1). These isozymes exhibit different dissociation/association

behavior and regulatory properties, although they share a 42% sequence identity (45). Whereas DdPFK appears to be a stable tetramer insensitive to metabolite regulators (25), RmPFK is able to dissociate spontaneously upon enzyme concentration and the presence of specific ligands (22, 23, 46). We suggest that these individual characteristics may

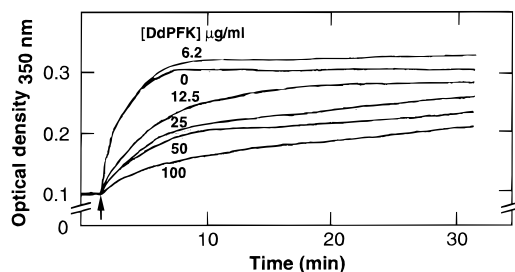


FIGURE 3: Effect of DdPFK concentration on the rate of tubulin polymerization. Tubulin (1.2 mg/mL) was incubated in the absence or presence of various concentrations of DdPFK as indicated. Polymerization was initiated by addition of taxol and followed by measuring turbidity.

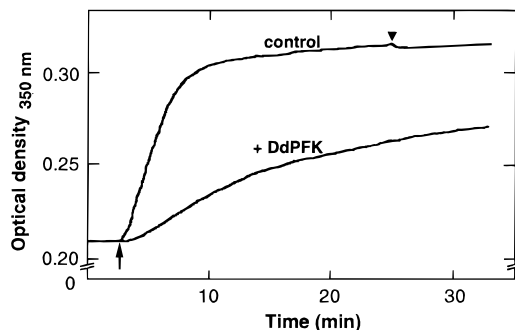


FIGURE 4: Effect of DdPFK on tubulin polymerization in the presence of MAPs. MAP-containing tubulin (2 mg/mL) was incubated in the absence (control) or presence of 0.2 mg/mL DdPFK. Polymerization was initiated by addition of 1 mM GTP and followed by measuring turbidity. DdPFK, at the same concentration, was also added to the control experiment at the time indicated by a black triangle.

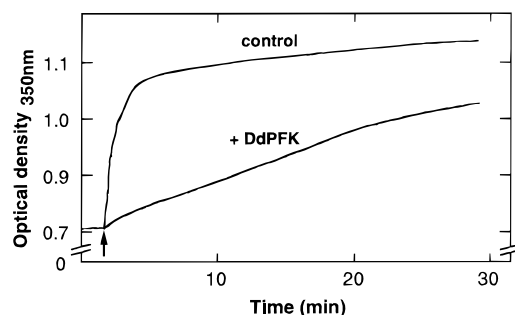


FIGURE 5: Effect of DdPFK on polymerization of subtilisin-digested tubulin. Limited digestion of tubulin (1.2 mg/mL) was carried out as described under Materials and Methods. Polymerization was initiated by addition of taxol and followed by measuring turbidity in the absence (control) and the presence of 0.15 mg/mL DdPFK. In agreement with the reports of Wolff and co-workers (32, 43), subtilisin digestion resulted in a moderate increase in absorbance that leveled off before taxol addition.

contribute at least in part to the remarkably different structure/function consequences that follow the interaction of these kinases with the tubulin/microtubule system. Thus, the insensitivity of the tetrameric DdPFK to tubulin and its lack of binding to microtubules are consistent with the reported (13) finding that only the inactive dissociated forms of RmPFK, probably dimers, associate to tubulin and microtubules. However, both isozymes bind to tubulin. The absence of effect of substrates on the anti-microtubule action of DdPFK, as well as the fact that some allosteric effectors of RmPFK were shown (17) to modulate its association with microtubules, also favors a role for the distinct regulatory

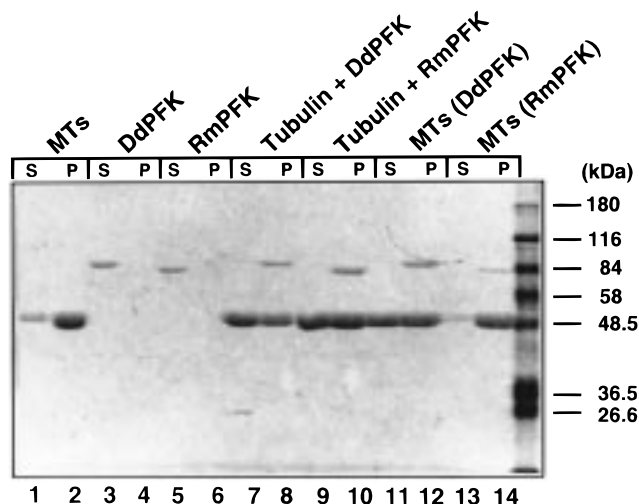


FIGURE 6: Interaction of DdPFK with tubulin. DdPFK (0.2 mg/mL) and RmPFK (0.2 mg/mL) were either incubated separately (lanes 3–6) or mixed with tubulin (2 mg/mL) (lanes 7–10) without taxol as indicated. Taxol-induced polymerization of tubulin was performed in the absence (MTs, lanes 1 and 2) and the presence of either DdPFK [MTs (DdPFK), lanes 11 and 12] or RmPFK [MTs (RmPFK), lanes 13 and 14]; the concentrations of tubulin, DdPFK, and RmPFK were as above. Samples were taken after 30 min incubation and centrifuged at 100000g for 30 min at 37 °C. Aliquots (20 µL) of supernatants (S) and resuspended pellets (P) were subjected to SDS-PAGE.

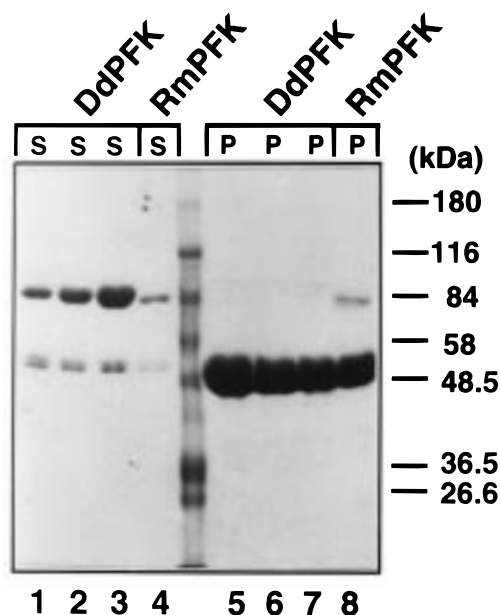


FIGURE 7: Pelleting of microtubules in the presence of DdPFK and RmPFK. Taxol-stabilized microtubules (2 mg/mL) were incubated with either DdPFK at a concentration of 0.2 mg/mL (lanes 1 and 5), 0.4 mg/mL (lanes 2 and 6), and 0.8 mg/mL (lanes 3 and 7) or RmPFK at a concentration of 0.4 mg/mL (lanes 4 and 8). After 30 min, samples were taken and centrifuged at 16000g for 25 min at 25 °C. Aliquots (10 µL) of supernatants (S) and resuspended pellets (P) were subjected to SDS-PAGE. The presence of a small proportion of unpolymerized tubulin dimers is visible in the supernatant fractions.

and structural properties of these PFKs in accounting for their different interactions with tubulin. The molecular bases of these phenomena are unknown yet.

The effect exerted by DdPFK, inhibiting the rate of microtubule formation at a stoichiometry of 1 inhibitor to about 300 tubulin molecules for half-maximal action, could be



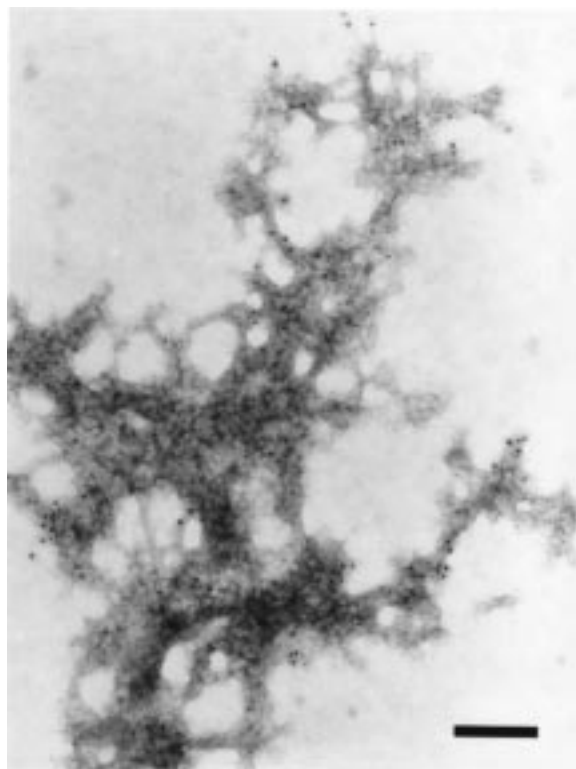


FIGURE 8: Electron microscopy of DdPFK-tubulin complexes. Tubulin (2 mg/mL) was incubated for 15 min with 0.2 mg/mL DdPFK. Samples were taken for immunostaining with anti-DdPFK and gold-conjugated anti-rabbit IgG. The 5-nm diameter gold particles are evident in the aggregates of tubulin. This picture shows one of the biggest aggregates. DdPFK association with smaller aggregates was similarly observed. Bars = 100 nm.

compared to that of some of the strongest natural or synthetic antitumoral drugs that inhibit the self-assembly of tubulin into microtubules, such as vinblastine, vincristine, or KAR-2 (47). However, these compounds operate by different mechanisms that lead to an additional decrease in the extent of polymerization. Among the newly identified proteins that behave as negative regulators of microtubule dynamics (3, 7), stathmin also acts by inhibiting tubulin polymerization in a noncatalytic manner, albeit reducing the formation of microtubules at steady state (4) and exhibiting a much lower affinity and higher molar ratio (3, 5). We compared the sequence of DdPFK with that of the 149 amino acid stathmin from rat brain (48) and found only a 16% identity between them, with identical residues sparsely distributed. The predicted secondary structures showed three  $\alpha$ -helix regions common to both proteins, at DdPFK positions 221–231, 247–257, and 275–287, corresponding to stathmin 47–57, 78–88, and 113–125, respectively, that exhibited sequence identities of 45%, 9%, and 54%, respectively (data not shown). Taken together, these data do not seem to be significant enough to infer any molecular base for the certain similarity in function shown by these proteins, at least in the absence of further information on three-dimensional structure.

Direct evidence that DdPFK physically interacts with tubulin was obtained by sedimentation experiments (Figure 6) and immunoelectron microscopy (Figure 8). This further indicates that the enzyme is responsible for the observed antimicrotubule effect. The exact mechanism by which this protein decreases dynamic growth is only partially understood. We found that the addition of DdPFK to tubulin led

to the formation of amorphous aggregates (Figures 2C and 8) which included both type of proteins and mean sequestration of tubulin dimers. Then, the binding of DdPFK to tubulin prevents its normal polymerization and facilitates further interactions with free tubulin and enzyme molecules. However, the highly substoichiometric effect of DdPFK suggests that this enzyme does not inhibit microtubule assembly simply by sequestering tubulin. An inhibitory interaction of DdPFK with microtubule free ends (49, 50) is unlikely, since binding of the enzyme to these structures was not detected (Figure 7). On the other hand, association of readily visible growing microtubules with tubulin–DdPFK aggregates that could arrest further dimer addition, as demonstrated for tubulin–colchicine complexes (50, 51), was not observed. We hypothesize that tubulin–DdPFK complexes and/or free enzyme may also bind to very early forms of tubulin polymers, or nucleation sites, thus blocking microtubule formation. The process seems to be reversible, as microtubules eventually formed even in the presence of DdPFK (Figures 1B and 2D). This finding evidences that no permanent modification of tubulin took place upon binding of the inhibitor and suggests that taxol promotes slow dissociation of tubulin from the heterocomplex, thus shifting the equilibrium to normal polymerization. DdPFK has no depolymerizing effect, as observed with less-strongly stabilized microtubules grown by GTP in the presence of MAPs (Figure 4) or glutamate. Thus, it is in agreement with the lack of interaction between the enzyme and taxol-stabilized microtubules.

The tubulin C-terminal domain is not involved in the binding of DdPFK, since polymerization of subtilisin-digested tubulin was inhibited (Figure 5) as efficiently as that of the untreated protein. RmPFK has also been reported (50) not to bind this tubulin region. However, both kinases may bind to tubulin to different sites, as the action of DdPFK was not affected by RmPFK even at a higher concentration (Figure 1B). Direct interaction of RmPFK with tubulin, as indicated by our copelleting experiments (Figure 6), did not prevent microtubule assembly. Tubulin–RmPFK complexes were previously demonstrated (13) by fluorescent anisotropy and shown (18) to be able to polymerize into microtubules, additionally cross-linking them in a periodic form that could be visualized by immunoelectron microscopy. This represents a further expression of the distinct interaction exerted on tubulin by both isozymes.

The slime mold has been proposed (53) as a source of potential microtubule inhibitors on the basis that most of its cytoplasmic tubulin is in an unpolymerized state (54, 55). In fact, a strong protein inhibitor with an  $M_r$  of 13 000 which works through a catalytic modification of tubulin was isolated from *D. discoideum* (53). Interestingly, the mitotic spindle is intranuclear in this organism (55, 56), the nuclear envelope remaining practically intact during cell division and thus keeping mitosis relatively isolated from cytoplasmic inhibitors. However, a transient formation of a microtubule array during interphase has also been described in the slime mold cytoplasm (57), therefore requiring the action of tubulin regulators as well. *D. discoideum*  $\alpha$ - and  $\beta$ -tubulins are among the most divergent members of the highly conserved family of tubulins (58). We did not test the action of DdPFK on *D. discoideum* tubulin, since this protein has not been successfully purified from the slime mold because of its low

content and the occurrence of high levels of proteases and tubulin inhibitors, as already pointed out (57, 58). However, the interaction of DdPFK with bovine brain tubulin devoid of the hypervariable acidic C-termini suggests that it can bind *D. discoideum* tubulin as well. The high affinity of this cytosolic isozyme (25) for tubulin with an  $IC_{50}$  value of 32 nM is within the range of its intracellular concentration determined in *D. discoideum* cells, 62 nM (25), and therefore supports the idea that DdPFK might be of physiological relevance as a cytoplasmic inhibitor of microtubule assembly. This is a novel function for DdPFK, which in contrast to PFK from other cells does not seem to play a role in the control of glycolysis (25). Since the activity of DdPFK was not influenced by tubulin, their association into a hetero-complex does not appear to be important for the regulation of this metabolic pathway in the slime mold, but for the control of microtubule formation. Although several enzymes of glycolysis have been described to be affected upon interaction with tubulin (13–16), this is the first example to the best of our knowledge in which a glycolytic enzyme acts not only as a catalyst but also as a potent protein factor affecting tubulin dynamics.

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