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Nucleoside Diphosphate Kinase Does Not Directly Interact with Tubulin nor Microtubules

Ronald Melki*, Ioan Lascu+, Marie-France Carlier* and Michel Véron+[1]

* Laboratoire d'Enzymologie, CNRS, 91198, Gif-sur-Yvette, France

+ Unité de Biochimie Cellulaire, CNRS-URA 1129, Institut Pasteur, 75724, Paris cedex 15, France

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Nucleoside diphosphate kinase has been shown to play a role in proliferation and development. Microtubules have been evoked as a possible target of NDP kinase action; in particular it was proposed that NDP kinase could regulate the cellular pool of polymerizable GTP-tubulin by direct phosphorylation of tubulin bound GDP. We show that this reaction does not occur *in vitro* and also that NDP kinase does not bind to microtubules both in the presence and absence of MAPs. Thus, any possible physiological effect of NDP kinase on microtubule dynamics is exerted only by modulating the concentrations of free guanine nucleotides in the vicinity of microtubules. © 1992 Academic Press, Inc.

NDP kinases, are ubiquitous enzymes that catalyze the transfer of the γ-phosphate of 5'-triphosphate nucleotides to 5'-diphosphate nucleotides, and play a major role in the synthesis of nucleoside triphosphates [1].

Recent studies have focused on the possible role of NDP kinases in the regulation of development and growth control. NDP kinase was cloned from *Dictyostelium discoideum* [2] and found highly homologous to counterparts in higher eucaryotes [3], the *Drosophila awd* and human *nm23* gene products subsequently shown to be NDP kinases [4,5]: The involvement of *awd* in *Drosophila* development [6] and of *nm23* in proliferation [7,8] and possibly in metastasis [9,10,11] point out to a role of NDP kinase in cellular regulation pathways, but its exact site(s) of action to exert such a function, remain(s) unknown. There is evidence for a role of NDP kinases in the regulation of the activity of guanyl nucleotide binding proteins and it has been suggested that NDP kinase might synthesize GTP in the vicinity of G-proteins and thus

[1]To whom correspondence should be addressed.

Abbreviations used: Nucleoside Diphosphate Kinase, NDP kinase; Bovine Serum Albumin, BSA;[2-(N-morpholino)ethanesulfonic acid], MES; Ethylene glycol bis[ß-aminoethyl ether]-N,-N,N',N'-tetraacetic acid, EGTA; Microtubule Associated Proteins, MAPs; Thin Layer Chromatography, TLC.

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participate in their activation activity by a channeling mechanism. An alternative possibility has also been proposed: NDP kinases might activate GTP binding proteins by directly phosphorylating the protein bound GDP *in* situ, thus obviating the need for a nucleotide exchange factor that classically controls the amount of GTP bound protein [12] [13, 14 for reviews]. However, recent results indicated that this attractive hypothesis is unlikely [15-16],[Kahn, personal communication].

NDP kinases have also been proposed to play a role in the control of microtubule polymerization, a process involving GDP/GTP exchange. Indeed, copurification of brain NDP kinase with microtubules has been reported [17-21] as well as direct transphosphorylation of tubulin bound GDP by NDP kinase [17, 21]. The implication of NDP kinase in the regulation of microtubule assembly may be responsible for the observed condensation of chromosomes from *awd* mutant larvae, which look quite similar to colchicine-treated wild-type chromosomes [4]. *In vitro* studies have emphasized the crucial role of the relative concentrations of dimeric GTP-tubulin and GDP-tubulin in the control of microtubule dynamics [22, 23], but no protein has thus far been identified that could modulate the rate of nucleotide exchange on dimeric tubulin, as in the case of G-proteins. In this paper, we describe experiments designed to assess whether NDP kinase simply acts on the pool of free GDP and GTP, or directly interacts with tubulin and microtubules.

MATERIALS AND METHODS

Chemicals. GTP, GDP, DTT, rabbit muscle pyruvate kinase and lactate dehydrogenase were from Boehringer. MES was from Calbiochem, EGTA, ATP, dTDP, NADH, BSA and PEP from Sigma. [^{3}H]GDP was from Amersham. All other chemicals were Merck analytical grade.

Tubulin. Tubulin was purified from fresh pig brains by 3 polymerization cycles [24] followed by phosphocellulose chromatography [25]. Polymerization buffer was 50 mM MES pH6.8 containing 4 M glycerol, 0.5 mM EGTA, 6 mM $MgCl_2$ and nucleotides as indicated. Polymerization was monitored turbidimetrically at 350nm using a Uvikon (Kontron) spectrophotometer equipped with a thermostated 0.43 cm light path cuvette.

NDP kinase. *Dictyostelium* NDP kinase was purified from recombinant bacteria as described [2,3]. Human NDP Kinase A and B was purified from erythrocytes as described [5]. Both preparations were homogenous when analyzed by SDS gel.

Nucleotide exchange kinetics on tubulin. The rate of bound [^{3}H]GDP dissociation from [^{3}H]-GDP-tubulin (prepared as described in [22]) was monitored using two independent assays: a linked enzyme assay using pyruvate kinase [22, 26]and PEI-cellulose TLC separation of [^{3}H]GTP and [^{3}H]-GDP [27]; and a direct assay using filtration on nitrocellulose [28]. In the latter assay, 1 ml aliquots were filtered at time intervals following addition of 100 μM GTP to a solution of 1 μM [^{3}H]GDP-tubulin. [^{3}H] radioactivity was quantitated by liquid scintillation.

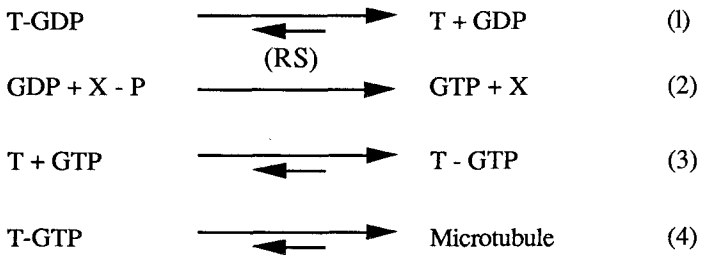
Binding of NDP kinase to microtubules. The association of NDP kinase to microtubules was measured in a sedimentation assay. Tubulin was polymerized at 37°C for 15 min in the presence of variable amounts of NDP kinase. Microtubules were sedimented at 37°C at 300,000xg for 5 min in the TL 100 Beckman tabletop ultracentrifuge. NDP kinase activity was measured in the supernatants of sedimented

samples and compared to total activity in the corresponding sample prior to sedimentation using a coupled enzyme assay: pyruvate kinase-lactate dehydrogenase in the following reaction mixture: 50 mM Tris-HCl pH 7.4, 50 mM KCl, 6 mM $MgCl_2$, 1 mM PEP, 0.1 mg/ml NADH, 0.5 mM ATP, 0.1 mM dTDP, 6.5 u/ml PK and 10 u/ml LDH.The amount of NDP kinase present in the pellets and supernatants was also estimated by SDS-PAGE [29].

Immunoblot assay of NDP kinase. Proteins separated by polyacrylamide gel electrophoresis were transferred to nitrocellulose (Schleicher & Schull). The sheets were then incubated for 1 h in 50 mM Tris pH 8.0, 25 mM NaCl, 1 mM EDTA, 0.3 % Tween 20 and 30 mg/ml skimmed milk. Anti-NDP kinase antibodies (1:250) were then added to the blocking solution . After 1 h incubation nitrocellulose sheets were washed 5 times for 10 min with the washing buffer (50 mM Tris-Cl- pH 8.0, 25 mM NaCl, 1 mM EDTA, 0.3 % Tween 20), then incubated for 1 h in the blocking solution containing a peroxidase-anti-rabbit immunoglobulin conjugate (1:250). Following 6 washes with the washing buffer, they were incubated in chromogenic substrate (130 mM Tris, pH 7.5, 400mM NaCl, 0.5 mg/ml HRP color development reagent (Bio-Rad), 0.045 % H_2O_2, for 15 minutes under dim light. The colorimetric reaction was stopped by washing filters in distilled water.

RESULTS AND DISCUSSION

It is well known that GDP-tubulin (T-GDP) is unable to polymerize into microtubules. However polymerization is readily observed in the presence of a GTP regenerating system (RS), *via* the following sequence of reactions taking place along the long arrows:



Examples of GTP regenerating systems are acetate kinase (X-P = acetyl-phosphate), pyruvate kinase (X-P = PEP) or NDP kinase (X-P = any nucleotide triphosphate). In the presence of sufficient amounts of X-P and RS, the rate of GDP dissociation from tubulin (reaction 1) is kinetically limiting in the production of T-GTP and in initiation of microtubule assembly. Therefore the time courses of polymerization of T-GDP in the presence of large amounts of either X-P + RS or free GTP, added at time zero, should be identical. It has previously been shown, however, that the lag time preceding the onset of tubulin polymerization was shorter when brain NDP kinase and ATP (or GTP) were added to GDP-tubulin at time zero, than when free GTP was added [17, 21]. The interpretation proposed was that direct phosphorylation of bound GDP by NDP kinase would occur at a faster rate than GDP dissociation from tubulin.

The time course of [^{3}H]-GDP dissociation from tubulin was monitored at 25°C with two independent methods, a direct filter assay and two linked enzyme assays using

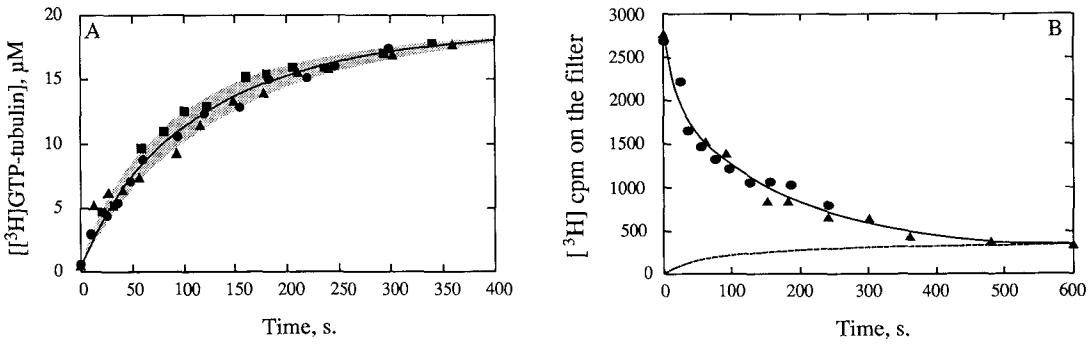


Figure 1. Kinetic measurements of GDP dissociation from tubulin.
Panel A: linked enzyme assay using pyruvate kinase and NDP kinase.
[3H]GDP-tubulin 1:1 complex at 20 µM was equilibrated at 25°C in assembly buffer containing 2 mM PEP or 2 mM ATP. At time zero, pyruvate kinase (1500 u/ml) or NDP kinase (1.5 µM) was added. The time course of [^{3}H] GTP regeneration in the presence of pyruvate kinase (●) and NDP kinase (▲ and ■ corresponding to two independent experiments) is shown. The grey zone represents the measured variation between the different kinetics of [^{3}H] GTP regeneration.
Panel B: direct filtration assay.
[^{3}H]GDP-tubulin 1:1 complex at 1 µM in assembly buffer was equilibrated at 25°C. At time zero, 100 µM GTP was added. The time course of GDP dissociation from tubulin was measured using the nitrocellulose filter assay (see Methods). Data coming from two independent experiments are shown (●, ▲) The adsorption of free [^{3}H]GDP in the time course of the experiment (dashed line) was derived from a control triplicate measurement of the amount of trapped [^{3}H]GDP upon filtration of 1 ml assembly buffer containing 1µM [^{3}H]GDP, 100 µM GTP and 1 µM GTP-tubulin.

either pyruvate kinase and PEP or NDP kinase and ATP or GTP. The results of these experiments, displayed in figure 1, show that GTP was regenerated at the same rate on tubulin by direct exchange of GTP for bound GDP, or by action of either pyruvate kinase or NDP kinase. These data therefore show no evidence for *in situ* phosphorylation of bound GDP by NDP kinase at a rate faster than GDP dissociation from tubulin. Both pyruvate kinase and NDP kinase therefore phosphorylate free GDP, as described by reactions 1-3.

An experiment analogous to those performed previously with brain NDP kinase [17, 21]was carried out using pig brain tubulin and NDP kinase from *Dictyostelium discoideum* and exactly the same result, shown in figure 2, was observed, i.e. the same lag time preceding polymerization was shorter when ATP or GTP+ NDP kinase was added to tubulin at time zero, than when free GTP, was added. Therefore *Dictyostelium* NDP kinase has the same effect as the brain enzyme. A slightly faster polymerization (but with the same lag) was observed when ATP, rather than GTP was used as a phosphate donor (not shown). Figure 2 also shows that, puzzlingly, an identical shortening of the lag preceding polymerization was observed upon addition of pyruvate kinase + 2 mM PEP, or of BSA in mass amounts comparable to either pyruvate kinase or NDP kinase. In conclusion, the data in figures 1 and 2, while consistent with each other, are clearly inconsistent with the interpretation formely proposed [17, 21] for the effect of NDP kinase in microtubule assembly. Again note that, in order for a direct

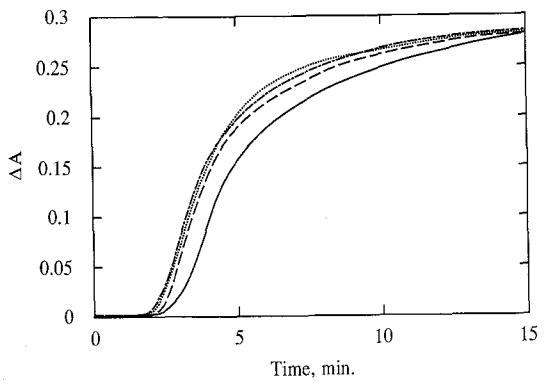


Figure 2. Tubulin assembly in the presence of NDP kinase, pyruvate kinase, bovine serum albumin and GTP.
GDP-tubulin (20 μM) in assembly buffer was incubated at 37°C for 2 min. Tubulin assembly was induced, at time zero, by addition: 250 μM GTP (——), 250 μM GTP and 0.1 μM NDP kinase (— — —), 2 mM PEP and 600 u/ml pyruvate kinase (·····), 250 μM GTP and 0.2 mg/ml BSA (— · — · —) . Turbidity at 350 nm was recorded (light path 0.5 cm).

phosphorylation of bound GDP to account for a 1 minute shortening of the lag, the regeneration of GTP-tubulin should have occured at an approximately 10 fold higher rate in the linked enzyme assay than in the direct exchange assay. The shortening of the lag time observed in the presence of pyruvate kinase, NDP kinase or BSA may be due to some non specific protein effect, e.g. on the stabilization of short oligomers, nuclei of microtubule assembly, which are present at very low concentrations ($\sim 10^{-9}$ M) at the onset of polymerization.

The issue of the possible binding of NDP kinase to microtubules was addressed next. Tubulin was polymerized at 20 μM in the presence of *Dictostelium* NDP kinase at a series of concentrations in the range 0-1.5 μM. The gel electrophoresis patterns of the pellets and supernatants of sedimented samples, displayed in figure 3, clearly show no

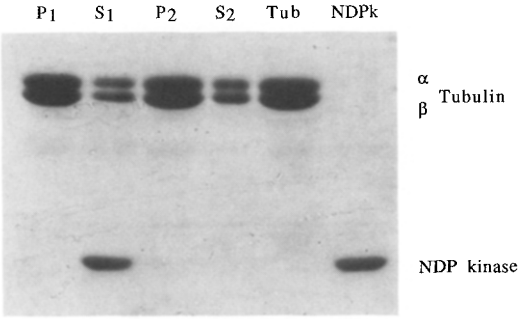


Figure 3. SDS-PAGE patterns of the pellets and supernatants of sedimented mixtures of microtubules and NDP kinase.
Tubulin (20 μM) in polymerization buffer was assembled for 15 min in the presence of NDP kinase (0 or 1.5 μM) and sedimented as described under the Materials and Methods section. SDS gel electrophoresis profiles of the pellet (P) and supernatant (S) fractions are shown.

Table 1. Binding assay of NDP kinase to microtubules

NDP kinase total concentration, nM	NDP kinase activity in the sample	
	Total	microtubule supernatant
3.25	6.5	6.1
6.5	10.3	10.2
13	20.1	19.4

Tubulin (20 μM) was polymerized in assembly buffer in the presence of Dictyostelium NDP kinase at the indicated concentration (left column). NDP kinase activity was measured using the coupled enzyme assay (see methods) in the microtubule-NDP kinase mixture (total activity) and in the supernatant of sedimented microtubules. NDP kinase activity is expressed in nmoles of NADH consumed per minute in a total volume of 1 mL at 25°C in the coupled enzyme assay.

binding of NDP kinase to microtubules. The total absence of NDP kinase in microtubule pellets, as judged by the Coomassie Blue stain, was confirmed using a more sensitive immunoblot assay (data not shown). In order to determine whether the binding of NDP kinase to cell microtubules, observed in immunofluorescence [4] might have been mediated by interaction of NDP kinase with MAPs, the same experiment was repeated with three-times cycled whole microtubule proteins. The same negative result was obtained (data not shown). NDP kinase activity was also measured in mixtures of microtubules/NDP kinase and in the supernatants of sedimented samples. As shown in Table I, all the activity was recovered in the supernatant. Finally, when binding of human erythrocyte NDP kinase (either A or B isozyme [5]) to microtubules w/o MAPs was measured using the same assay as above, identical negative results were obtained (data not shown).

In conclusion, our results do not substantiate the hypothesis of direct phosphorylation of tubulin-bound GDP by NDP kinase. It is interesting to note that this hypothesis is not supported by the structure of the enzyme which has recently been solved at high resolution [30]. Indeed, the histidine residue which is phosphorylated during catalysis is buried in the active site pocket and not likely to be available for interaction with the β-phosphate of GDP on the G-protein. In addition, our results show no evidence of direct interaction between NDP kinase and either tubulin or microtubules. This conclusion is in agreement with the findings that brain NDP kinase did not bind to a tubulin affinity column [21], that a very small proportion of brain cytoplasmic NDP kinase was found associated to a twice cycled microtubule protein preparation [20], and that NDP kinase did not copurify with microtubules or dynamin preparations from chick brain [31, 32], or calf brain [33].

Although the present data are not in support of the direct interaction of NDP kinases with the microtubule system, they do not eliminate the possibility that NDP kinase may affect microtubule dynamics by controlling the level of dimeric GTP-tubulin and GDP-tubulin *via* phosphorylation of free nucleotides. The subcellular distribution of NDP kinase isozymes may then allow a spatially controlled regulation of microtubule dynamics in the living cells. This possibility has not been tested in the present work.

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