Volume 296, number 1, 21–24 FEBS 10562 January 1992

Microtubule-associated protein autophosphorylation alters in vitro microtubule dynamic instability

Nadia Raffaelli*, Paul S. Yamauchi** and Daniel L. Purich

Department of Biochemistry and Molecular Biology, University of Florida College of Medicine, Gainesville, FL 32610-0245, USA

Received 24 September 1991; revised version received 11 November 1991

While phosphorylation of high-molecular-weight microtubule-associated proteins (MAPs) alters the assembly properties of microtubules in vitro, virtually nothing is known about the influence of MAP phosphorylation on the time-scale of microtubule polymer length redistribution. The latter has been used as an index of microtubule assembly/disassembly turnover as predicted by the dynamic instability model (Mitchison, T.M. and Kirschner, M.W. (1984) Nature 312, 237–242). We have now determined that under conditions leading to the incorporation of 8–10 mol phosphoryl groups per mol MAP-2 (and about 0.2 mol phosphoryl groups per mol MAP-1 and tau), we can reproducibly observe significant acceleration in the polymer length redistribution process in a manner consistent with greater microtubule dynamic instability. We have also found that MAP phosphorylation resulted in more extensive release of MAPs from microtubules as a function of increasing salt concentration. These results are consistent with a weakening of MAP-microtubule interactions upon phosphorylation.

Cytoskeleton; Tubulin; Assembly/disassembly

1. INTRODUCTION

Specification of microtubule involvement in cellular morphology and motility depends upon the participation of microtubule-associated proteins (MAPs), a group of cytoskeletal proteins displaying one or several of the following properties: (i) ability to co-polymerize with tubulin during microtubule assembly; (ii) action on tubulin or another MAP in enzyme-catalyzed modification reactions; or (iii) attachment to microtubules in ATP-dependent force-generating processes [1,2]. Brain tissue is especially rich in tubulin and MAPs, and a number of studies have demonstrated that the latter undergo phosphorylation by endogenous brain protein kinases [3–5]. In particular, MAP-2 and the tau proteins, both members of a group of fibrous cytomatrix proteins cross-linking microtubules in dendrites and axons, are extensively phosphorylated. Theurkauf and Vallee [6] showed that MAP-2 contains cAMP protein kinase activity and that MAP-2 is multiply phosphorylated; moreover, Tsuyama et al. [7] demonstrated that rat brain MAP-2, after isolation from focused microwave-irradiation of brain tissue in the living animal, contained about 46 covalently bound phosphoryl groups per mol MAP-2. While there is some evidence that MAP phosphorylation can affect the rate and extent of microtubule polymerization in vitro [5], relatively little is known about the role of MAP phosphorylation in neurons. Because microtubules are subjected to processes of assembly and disassembly, a great deal of interest centers on characterizing the GTP-dependent polymerization process [1]. In the Mitchison and Kirschner [8] dynamic instability model, length-changes in microtubules at steady-state are thought to arise from an overall balance of 2 processes: the first involving slow growth of the majority of microtubule polymers; and the second arising from the rapid disassembly of a smaller fraction of tubules. They proposed that microtubules exhibit this behavior as a result of the addition and release of tubulin dimers to and from the growing and depolymerizing microtubules, respectively. One possibility is that MAPs, by virtue of their ability to promote microtubule assembly and to reduce the critical tubulin concentration needed for assembly [3], could reduce the dynamic instability of microtubules. Accordingly, factors that alter the strength of MAP-microtubule interactions may play a regulatory role in the assembly/disassembly of microtubules. Under conditions that led to the incorporation of 8–10 phosphoryl groups per MAP-2 molecule, we now report that we have reproducibly observed significant acceleration in the polymer length redistribution process in a manner indicative of greater microtubule dynamic instability. We also found that MAP phosphorylation resulted in

**Present address:* Biochemistry Institute, Faculty of Medicine and Surgery, University of Ancona, via Monte d'Ago, 60100 Ancona, Italy.

***Present address:* Department of Orthopaedic Surgery, University of Rochester, Rochester, NY 14623, USA.

Correspondence address: D.L. Purich, Department of Biochemistry and Molecular Biology, University of Florida College of Medicine, Gainesville, FL 32610-0245, USA.

a higher level of release of MAPs from microtubules as a function of salt concentration, suggesting that weaker MAP-microtubule interactions result from phosphorylation.

2. MATERIALS AND METHODS

Bovine brain microtubule protein, containing tubulin and MAPs was isolated through 2 assembly/disassembly cycles [9], stored at −80°C until use, and then subjected to an additional assembly/disassembly purification cycle just prior to use. MAPs were separated from tubulin using phosphocellulose chromatography [9], and dephosphorylation of MAPs was accomplished by treatment with 1.5 U protease-free alkaline phosphate (Sigma Chemical Co.) per mg MAPs in 0.1 M 2(*N*-morpholino)ethanesulfonic acid, 1 mM magnesium sulfate (pH 6.8) buffer for 30 min. Protein kinase phosphorylation conditions were as described by Coughlin et al. [4] using an ATP/GTP-regenerating system with acetyl phosphate and acetate kinase. Microtubule assembly was measured by optical density changes at 350 nm, and microtubule length measurements were accomplished using indirect immunofluorescence microscopy methods [9,10]. Glutaraldehyde-fixed microtubules were labeled by the addition of Texas red-conjugated goat anti-mouse IgG (diluted 200 times) after treating microtubule samples with mouse anti-β-tubulin antibodies (diluted 70 times). Microtubule lengths were measured for about 400 tubules with a 1% accuracy using photomicrographs (900× magnification) and a GTCO automatic digitizer. SDS polyacrylamide gel electrophoresis with 7.5% cross-linking gels were stained with Coomassie brilliant blue [9]. Protein levels were estimated using an LKB laser densitometer.

3. RESULTS AND DISCUSSION

The objective of this study was to assess changes in the time-scale of microtubule length redistribution in the presence of unphosphorylated or phosphorylated MAPs. We chose conditions that would minimize differences in the initial polymer length distribution by assembling a single sample of microtubule protein, containing a 3-to-1 [tubulin]/[dephospho-MAPs] protein weight concentration ratio. After assembly, this sample was divided into equal aliquots: one was permitted to undergo autophosphorylation by the addition of 0.05 mM ATP and 20 μM cAMP for 30 min at 37°C in the presence of an ATP/GTP-regenerating system; the other sample was incubated under identical conditions in the absence of any added ATP. (With companion samples containing the same ATP and cAMP concentrations plus 2×10^7 dpm [γ-^{32}P]ATP, we carried out SDS gel electrophoresis after autophosphorylation, and we excised the MAP-1, MAP2 and tau protein bands for scintillation counting. Only MAP-2 exhibited significant ^{32}P incorporation, corresponding to 8–10 mol phosphoryl groups per mol MAP-2. For comparison, the level of MAP-1 and tau radiolabeling corresponded to less than 0.2 mol phosphoryl groups per mol for each of these proteins.) After autophosphorylation, we mechanically sheared the assembled microtubules containing either dephospho- or phospho-MAPs [10], and aliquots were removed and fixed immediately or at 7, 20, 40 and 60 min after shearing. These samples were

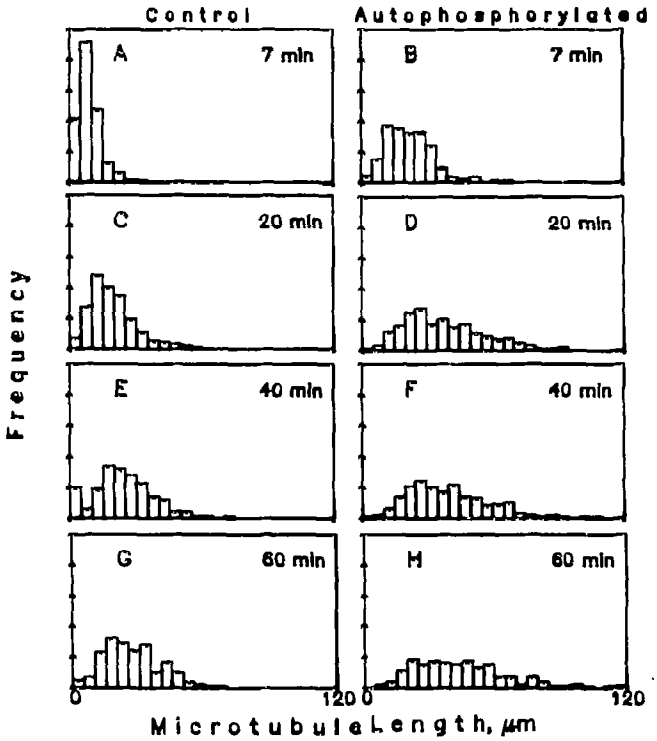

Fig. 1. Microtubule length distribution plots for assembled microtubules containing dephospho- and phospho-MAPs. Microtubule protein (2.7 mg/ml) was assembled in a pH 6.8 buffer containing 100 mM 2(*N*-morpholino)ethanesulfonic acid, 1 mM $MgCl_2$, 1 mM EGTA and 100 mM KCl for 30 min at 37°C, and then processed as described in the text. Panels A, C, E and G depict the results with dephospho-MAPs; B, D, F and H correspond to the samples containing autophosphorylated microtubule-associated proteins.

then diluted in fixation buffer and stained with anti-β-tubulin antibodies and secondary antibodies as described by Yamauchi and Purich [9]. The findings presented in Fig. 1 correspond to the polymer length distributions of microtubules in the absence and presence of cAMP protein kinase phosphorylation. The reader will note that the autophosphorylated microtubule protein underwent significantly faster length distribution over the 1-h time-course studied, as indicated by the broadening of the distribution favoring formation of longer tubules. It should also be noted that the data in Fig. 1 frames 1B,D,F,H are virtually indistinguishable from tubule length redistribution measurements made with pure tubulin [10], as confirmed by us. Therefore, MAP phosphorylation under the conditions presented here virtually abolishes any effect of MAPs on the dynamic instability of microtubules. This is shown even more directly in Fig. 2, which represents the time-evolution of the average microtubule length for the samples shown in Fig. 1. Both phosphorylated and unphosphorylated MAP-containing microtubule samples started off with nearly identical average lengths and polymer number concentrations. Phosphorylated microtubule samples, however, exhibited about a 3-fold

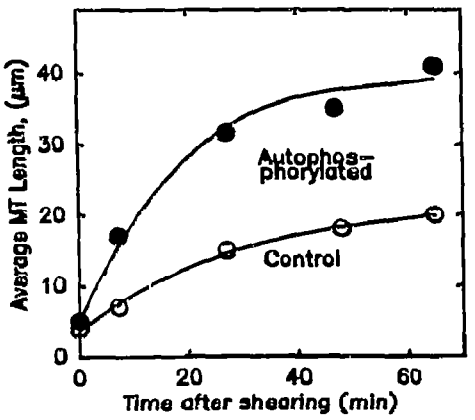

Fig. 2. Time-evolution of the average polymer length of assembled micro-tubules containing dephospho- and phospho-MAPs. See legend to Fig. 1 and the text for details.

increase in the rate of length redistribution, as indicated by the slope over the first 20 min. The phosphorylated microtubule protein samples also reached nearly twice the average microtubule length within approximately 1 h after mechanical shearing, a result that was obtained in 3 independent experiments. Together, these data demonstrate that microtubule protein phosphorylation accelerates tubule length redistribution to levels comparable to that observed by Kristofferson et al. [10] for microtubule samples assembled from tubulin in the absence of any MAPs.

When cAMP was omitted from the reaction mixture described above, the level of ATP-dependent autophosphorylation was significantly less (1–2 pmol P/mol MAP-2), and length redistribution kinetics were virtually identical to samples with dephospho-MAPs. Moreover, in companion experiments, we found that replacement of ATP by the nonhydrolyzable analogues App(CH_2)p or App(NH)p in the autophosphorylation step failed to give similar results in the length redistribution, further suggesting that the ATP effect is limited to phosphorylation and not merely ATP binding.

Acceleration of microtubule length redistribution upon autophosphorylation most probably occurs through weakening of the stability interactions of MAPs with microtubules. Evidence that MAPs and tubules interact largely by ionic forces has been adduced by many investigators [1,2]. To probe this issue further, we examined the binding strength of dephospho- and phospho-MAPs to microtubules at various salt concentrations known to alter MAP binding to microtubules. A sample of microtubule protein was first assembled, and again split into 2 identical aliquots, with only one subjected to autophosphorylation by the addition of ATP and cAMP. Next, we transferred equivalent volumes of each sample to pairs of tubes containing assembly buffer and additional NaCl (to the levels indicated), and the microtubule-bound and -unbound MAPs were separated by ultracentrifugation at 160 000 × *g* for 1 h in a Beckman Airfuge. SDS gel electrophoresis of the separated samples was followed by dye staining, and densitometry was used to estimate the amount of MAPs released from microtubules by the above salt treatment. As shown in Fig. 3, both MAP-1 and MAP-2 display a higher sensitivity to salt desorption when the microtubules are subjected to autophosphorylation conditions. Indeed, despite the fact that less than 0.2 mol P per mol MAP-1 was observed, the level of MAP-1 desorption increased by a factor of 2. The length redistribution kinetic studies were conducted at ionic strengths corresponding to the 0.05 M NaCl values in this figure, and one can readily recognize in Fig. 3 that the difference between the salt desorption properties of dephospho- and phospho- MAP-1 and MAP-2 is especially evident in that salt concentration range.

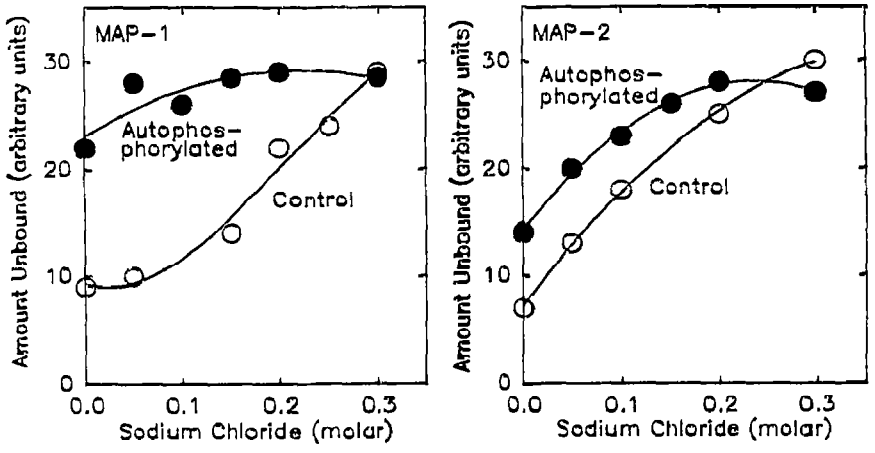

Fig. 3. Salt-desorption of dephospho- and phospho-MAPs from taxol-stabilized microtubules. Microtubule protein (9.6 mg/ml) was assembled as described in Fig. 1. One sample also contained 0.5 mM ATP and 20 μM cAMP. After cold depolymerization (at 4°C for 20 min), assembly was induced by the addition of 20 μM taxol at 37°C for 30 min. Aliquots (0.1 ml) were then transferred to microcentrifuge tubes containing 50 μl of the same buffer containing additional NaCl at the designated concentrations. The samples were then centrifuged (160 000 × *g*) for 1 h at 37°C in an Airfuge; then, the supernatant and pellet fractions were subjected to SDS gel electrophoresis, Coomassie brilliant blue dye binding, and densitometry to estimate the amount (arbitrary densitometry units) of MAP-1 and MAP-2 released in the presence of added salt.

The detailed role(s) of protein phosphorylation in controlling neuronal cytoskeletal processes remain to be elucidated, but in vitro studies promise to shed light on molecular properties of cytoskeletal components that may offer regulatory options. Ultimately, one would like to understand the factors that control the number of microtubules per cell, their rate of turnover, and their spatial distribution in living cells. This is a challenging goal that has been only partially achieved through the use of photobleaching [11,12] and biotinylated-tubulin [13] exchange experiments. In particular, Schulze and Kirschner [13] recently presented evidence for the occurrence of both stable and dynamic microtubules in African Green monkey kidney fibroblasts. They found that the average BSC-1 cell contains about 700 microtubules with approximately 500 growing at 4 μm min^{-1}, 100 shrinking at approximately 20 μm min^{-1}, and the remaining tubules exhibiting more stability with respect to exchange. Likewise, we have used [^{14}C]guanine to metabolically label a pheochromocytoma cell line (PC12), and we have analyzed the time-course of the rise in specific radioactivity of the cytosolic GTP and microtubule cytoskeletal-bound GDP; this study demonstrated that these neuronal cells display very rapid microtubule assembly/disassembly turnover, with only a 1–3 min delay in labelling of the above pools (J.M. Angelastro and D.L. Purich, unpublished findings). Interestingly, this nerve growth factor-responsive cell line contains very little MAP-2 and relatively lower amounts of tau protein than found in brain tissue, but MAP kinases are thought to influence neurite outgrowth in response to this growth factor. Nonetheless, studies of the intracellular dynamics of microtubules have not yet reached the stage where the impact of protein phosphorylation can be directly addressed, and new experimental approaches must be developed to address this issue.

REFERENCES

[1] Purich, D.L. and Kristofferson, D. (1984) Adv. Prot. Chem. 36, 133–212.
[2] Olmsted, J.B. (1986) Annu. Rev. Cell Biol. 2, 421–457.
[3] Sloboda, R.D., Dentler, W.L. and Rosenbaum, J.L. (1976) Biochemistry 15, 4497–4505.
[4] Coughlin, B.A., Dentler, W.L. and Purich, D.L. (1980) Biochem. Biophys. Res. Commun. 92, 89–94.
[5] Jameson, L. and Caplow, M. (1981) Proc. Natl. Acad. Sci. USA 78, 3413–3417.
[6] Theurkauf, W.E. and Vallee, R.B. (1983) J. Biol. Chem. 258, 7883–7886.
[7] Tsuyama, S., Terayama, Y. and Matsuyama, S. (1987) J. Biol. Chem. 262, 10886–10892.
[8] Mitchison, T. and Kirschner, M.W. (1984) Nature 312, 237–242.
[9] Yamauchi, P.S. and Purich, D.L. (1987) J. Biol. Chem. 262, 3369–3375.
[10] Kristofferson, D., Mitchison, T. and Kirschner, M. (1986) J. Cell Biol. 102, 1007–1019.
[11] Saxton, W.M., Stemple, D.L., Leslie, R.J., Salmon, E.D., Zavortink, M. and McIntosh, J.R. (1984) J. Cell Biol. 99, 2175–2186.
[12] Soltys, B.J. and Borisy, G.G. (1985) J. Cell Biol. 100, 1682–1689.
[13] Schulze, E. and Kirschner, M.W. (1987) J. Cell Biol 104, 277–288.