Vol. 184, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1992 Pages 414-418

ADPRibosylation of Chicken Red Cell Tubulin and Inhibition of Microtubule Self-Assembly In Vitro by the NAD$^+$-Dependent Avian ADPRibosyl Transferase[1]

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Received January 30, 1992

Summary: Chicken erythrocyte tubulin was found to undergo NAD$^+$-dependent ADPribosylation *in vitro* in the presence of ADPRtransferase also isolated from avian red blood cells. Unlike the low level of ADPR incorporation catalyzed by *Cholera* and *Pertussis* toxins (*i.e.*, <0.005 mol ADPR/mol tubulin), the avian system displayed a much higher stoichiometry of 0.8-1.2 mol ADPR/mol tubulin. Modification resulted in potent inhibition of microtubule self-assembly, even in the presence of bovine brain microtubule-associated proteins or with the addition of pre-assembled microtubules.

The principal microtubule cytoskeleton component tubulin is known to serve as a post-translational modification substrate for tyrosination and acetylation (1,2). This heterodimeric protein binds guanine nucleotide in two ways: GTP interacts at the nonexchangeable nucleotide site; and GTP or GDP can bind at the exchangeable site, the former promoting assembly and the latter inhibiting that process (3). As a GTP-binding protein with sequence homology with other so-called G-proteins (4), tubulin has been examined with regard to its ability to serve as an ADPR acceptor in reactions catalyzed by mono-ADPribosyltransferase activities of *Cholera* and *Pertussis* toxins (5-7). The level of modification achieved in those studies never exceeded 0.005 mol ADPR per mol brain tubulin, and such modification was without effect on microtubule assembly/disassembly.

[1]Supported in part by National Institutes of Health Research Grant GM-44823.
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0006-291X/92 $1.50

West and Moss (8) isolated NAD^+:arginine-mono-ADPR transferase from turkey erythrocytes, and availability of this transferase in high purity afforded us with the opportunity to re-examine tubulin ADPribosylation. We recently demonstrated that bovine tubulin can accept ADPR groups at a level of 1.2 mol/mol (9), but those studies employed tubulin and ribosylating enzyme obtained from two widely diverse organisms. We have reinvestigated this matter using components obtained from avian red blood cells. We now report that 0.8-1.2 mol ADPR can be incorporated per mol tubulin and that modification inhibits both nucleation and elongation steps in microtubule assembly.

Experimental Procedures

Chicken erythrocyte tubulin was prepared as described by Murphy and Wallis (10). Turkey erythrocyte ADPR transferase, isolated as outlined elsewhere (8), was the generous gift of Dr. Joel Moss. [^{14}C]NAD^+ was purchased from ICN Radiochemicals, Inc.

Incorporation of radiolabel from [^{14}C]NAD^+ into tubulin was quantitated by liquid scintillation counting of tubulin precipitated from the reaction mixture by the addition of an equal volume of 25% trichloroacetic acid (w/v) for 30 min at 4°C followed by centrifugation (10 min, 4°C, 14,000 xg). Pellets were washed twice by resuspension in 8-10% trichloroacetic acid, followed by intervening centrifugation, and then solubilized in 1% SDS. Alternatively, we used SDS polyacrylamide gel electrophoresis with 7% gels as described by Laemmli (11), and proteins were visualized with Coomassie Blue dye. After drying gels on a paper support, autoradiographs were obtained using Kodak X-OMAT film. Microtubule assembly was examined as described by MacNeal and Purich (12), and seeded assembly experiments used microtubules prepared from unmodified bovine brain tubulin.

Results

To learn whether red cell tubulin could undergo ADPribosylation in the presence of NAD^+ and avian erythrocyte ADPR transferase, we first tested for [^{14}C]ADPR incorporation using trichloroacetic acid to precipitate protein samples after a 30 min reaction period in the presence of 5 μg/mL turkey transferase. We demonstrated that ADPR incorporation under these conditions was approximately 0.6 mol per mol tubulin, based on scintillation counting for estimating the extent of covalently incorporated radioactivity and SDS electrophoresis and gel densitometry for determining the amount of tubulin. We then studied the reaction conditions necessary for ADPribosylation, and the bar graph in Fig. 1A indicates the amount of ADPR covalently attached to tubulin in the presence of several transferases, at two concentrations of the turkey erythrocyte enzyme, or with the microtubule-stabilizing drug taxol. *Cholera* and *Pertussis* toxin yielded only very low levels of ADPR incorporation into brain microtubule protein (5-7). We also confirmed that ADPribosylation occurs in an enzyme concentration-dependent manner. Taxol (10 μM) is sufficient to polymerize more than 95% of the tubulin into microtubules, and this agent also inhibited ADPR incorporation, a result indicating that unpolymerized tubulin is the ADPR acceptor for the transferase.

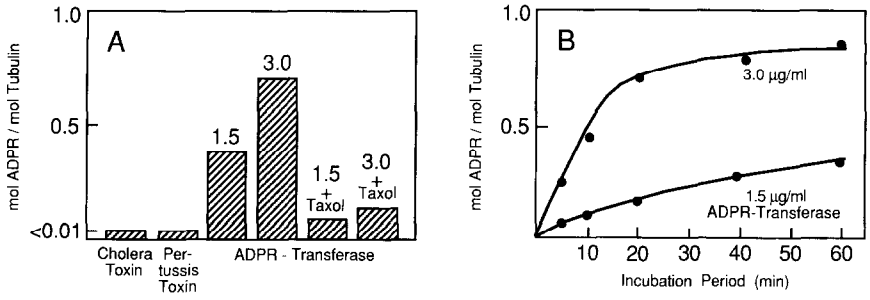

Fig. 1. ***A, Extent of tubulin ADPribosylation under the various reaction conditions.*** Purified red cell tubulin (1.5 mg/mL) was incubated for the indicated periods with turkey ADPR transferase (1.5 or 3 μg/mL), Cholera toxin (30 μg/mL), or Pertussis toxin (25 μg/mL), in addition to 0.1 mM NAD^+ (including 100,000 cpm [^{14}C]NAD^+), 1 mM $MgSO_4$, and 1 mM EGTA. Reactions were terminated after one hour by addition of 1% sodium dodecyl-sulfate and boiling for 2 min. ***B, Time-course of ADPR incorporation into chicken erythrocyte tubulin.*** Conditions were as described in Fig. 1, using the turkey red cell enzyme (1.5 and 3 μg/mL).

We next examined the time-course of ADPribosylation of chicken red cell tubulin by the avian ADPR transferase (Fig. 1B). ADPR incorporation reached a plateau value of 0.88 mol ADPR per mol tubulin dimer within 45-60 min. In four separate experiments using two different preparations of chicken red cell tubulin, we obtained stoichiometries with the chicken red cell tubulin of 0.8 to 1.2 mol per mol. In agreement with our recent findings on bovine brain tubulin (10), autoradiography of a SDS gel electropherogram revealed that both α and β tubulin poly-peptide chains were covalently modified; likewise, laser densitometry indicated there was roughly equal modification of each (data not shown).

Because tubulin polymerization is directly linked to GTP hydrolysis (12), we recognized the possibility that such ADPribosylation could alter the kinetics and/or the extent of microtubule self-assembly. The findings presented in Fig. 2 show that ADPribosylated chicken tubulin failed to assemble, whereas tubulin incubated with the transferase in the absence of NAD^+ underwent facile assembly. Microtubule-associated proteins (MAPs) are well known for their ability to promote assembly (3), and because the isolation of avian erythrocyte MAPs has not been reported, we utilized bovine brain heat-stable MAPs. We tested whether addition of bovine brain MAPs (at a ratio 0.2 mg MAPs per mg chicken tubulin) would promote polymerization. In the absence of ADPribosylation, heat-stable bovine brain MAPs stimulated tubule assembly; however, conditions leading to covalent modification at levels of 0.23 mol ADPR per mol tubulin blocked assembly, even with added brain MAPs. These observations indicate that ADPribosylated tubulin cannot undergo self-assembly. The latter process requires tubulin to participate in both nucleation and elongation reactions (3); accordingly, we used pre-assembled microtubule seeds to obviate the

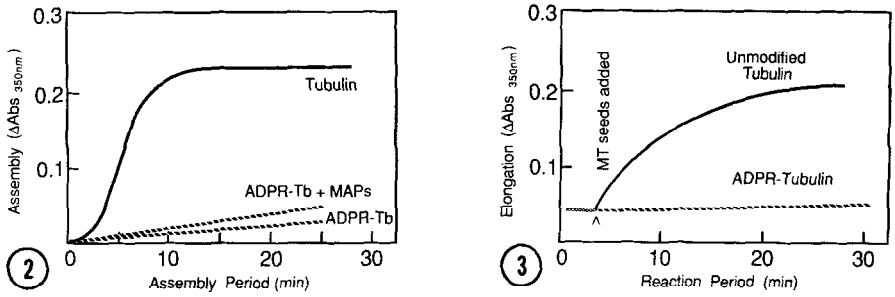

Fig. 2. ***Assembly properties of unmodified red cell tubulin (1.5 mg/mol) and ADP-ribosylated red cell tubulin (1.8 mg/mL).*** Polymerization was initiated by warming from 4° to 37°C in the presence of 1 mM GTP (and 0.35 mg/mL MAPs, where indicated) in assembly buffer. Polymerization was measured as the turbidity change at 350 nm.

Fig. 3. ***Elongation experiments with pre-formed microtubule seeds.*** Microtubules were prepared by incubating bovine brain tubulin (2.0 mg/mL) in 0.1 M piperazine-N,N'-bis [ethanesulfonic acid] (pH 6.8) containing 1 mM magnesium chloride, and 1 mM EGTA, and 2 M glycerol; after assembly, tubules were pelleted by centrifugation, resuspended in the same buffer, and sheared mechanically by rapid passage through a 24 gauge syringe needle. Elongation was carried out with 0.2 mg/mL sheared microtubule seeds and 1.5 mg/mL of unmodified or 1.55 mg/mL ADPribosylated chicken tubulin.

requirement for nucleation, and the results in Fig. 3 demonstrated that even under these seeded assembly conditions ADPR-tubulin cannot participate in elongation on the 20-30 min time-scale typical for tubulin elongation.

Discussion

Mono-ADPribosylation is catalyzed by certain bacterial toxins and several eukaryotic enzymes (13). The ability to serve as a substrate for mono-ADPribosylation *in vitro* appears to be a characteristic property of GTP-binding proteins, including G_i and G_s regulatory subunits of adenylate cyclase, the retinal regulatory protein transducin, elongation factor-2, and the proto-oncogene Ha-ras (13,14). ADPribosyltransferase activities have been identified in a variety of animal tissues including turkey erythrocytes (8) and brain (15). Recent studies suggest that reversible mono-ADPribosylation may regulate the 78 kD glucose- and heat shock-sensitive protein (GRP78) in eukaryotes (16). We now describe conditions for ADPribosylation of chicken erythrocyte tubulin using the avian erythrocyte monoADPR-transferase. These and other related findings with bovine brain tubulin (10) are in harmony with the suggestion by Sternlicht *et al.* (4) that tubulin shares structural features with other GTP-binding proteins. That each subunit can be ADPribosylated also fits well with the likelihood that each α and β chain has a guanine nucleotide site. Photolabeling experiments with 8-azido GTP resulted in modification only of the β-subunit (17), suggesting that this

polypeptide chain contains the exchangeable site. Presumably, the nonexchangeable site resides in the α subunit, and the primary sequences of both α and β subunits are indeed highly related (18). Localization of the amino acid residues containing ADPR groups may provide more information about the proximity of modified sites to the guanine nucleotide binding sites on tubulin.

The physiologic significance of these observations remains to be elucidated, and avian erythrocytes may become a useful model system for exploring intracellular ADPribosylation of tubulin. Our use of turkey red cell enzyme with chicken red cell tubulin is still subject to limitations imposed by any cross-species enzyme specificity study. Because we were particularly interested in obtaining unambiguous evidence of ADPribosylation *in vitro*, we chose to work with the best characterized components found in the same cell type (8,10). We have not yet determined whether [^{14}C]NAD$^+$ can be transported into avian erythrocytes, and metabolic labeling to form radioactive NAD$^+$ in the red cells may require radiolabeled adenine, adenosine, ribose, or orthophosphate precursors. Interestingly, avian erythrocyte tubulin is assembled into a single marginal band microtubule underlying the cell membrane, and the amount of unpolymerized tubulin may prove to be too low for effective ADPribosylation *in vivo*. In any event, the capacity of chicken erythrocyte tubulin to undergo ADPribosylation *in vitro* should stimulate further efforts to clarify interactions of avian red cell tubulin and ADPR transferase in living cells.

References

1. Terry, B. J. and Purich, D. L. (1982) *Adv. Enzymol.*(A. Meister, ed.) **53**, 113-161.
2. Scaife, R. M. and Purich, D. L. (1985) *Curr. Topics in Cell. Regul.* **27**, 107-116.
3. Purich, D. L. and Kristofferson, D. (1984) *Adv. Prot. Chem.* **36**, 133-207.
4. Sternlicht, H., Yaffe, M. B. and Farr, G. W. (1987) *FEBS Lett.* **214**, 226-235.
5. Kaslow, H. R., Groppi, V. E., Abood, M. E. and Bourne, H. R. (1981) *J. Cell Biol.* **91**, 410-413.
6. Amir-Zaltsman, Y., Ezra, E., Scherson, T., Zutra, A., Littauer, U. Z. and Salomon, Y. (1982) *EMBO J.* **1**, 181-186.
7. Hawkins, D. J. and Browning, E. T. (1982) *Biochemistry* **21**, 4474-4479.
8. West, R. E., Jr. and Moss, J. (1986) *Biochemistry* **25**, 8057-8062.
9. Scaife, R. M., Wilson, L. and Purich, D. L. (1992) *Biochemistry* **31**, 310-316.
10. Murphy, D. B. and Wallis, K. T. (1985) *J. Biol. Chem.* **258**, 8357-8364.
11. Laemmli, U. K. (1970) *Nature* **227**, 680-685.
12. MacNeal, R. L. and Purich, D. L. (1978) *J. Biol. Chem.* **253**, 4683-4687.
13. Ueda, K. and Hayaishi, O. (1985) *Ann. Rev. Biochem.* **54**, 73-100.
14. Tsai, S., Adamik, R., Moss, J., Vaughan, M., Manne, V. and Kung, H. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8310-8314.
15. De Wolff, M. J. S., Vittii, P., Ambesi-Impiombato, F. S. and Kohn, L. D. (1981) *J. Biol. Chem.* **256**, 12287-12296.
16. Carlsson, L. and Lazarides, E. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4664-4668.
17. Geahlin, R. L. and Haley, B. E. (1979) *J. Biol. Chem.* **254**, 11982-11987.
18. Cleveland, D. W., Lopata, M. A., MacDonald, R. J., Cowan, N. J., Rutter, W. J. and Kirschner, M. W. (1980) *Cell* **20**, 95-105.