ADP-Ribosylation of microtubule proteins as catalyzed by cholera toxin

Y. Amir-Zaltsman1, E. Ezra1, T. Scherson2, A. Zitra2, U. Z. Littauer2, and Y. Salomon1

Departments of 1Hormone Research and 2Neurobiology, The Weizmann Institute of Science, Rehovot 76100, Israel

Communicated by U.Z.Littauer
Received on 28 December 1981

Incubation of purified rat brain tubulin with cholera toxin and radiolabeled [32P] or [8-3H]-NAD results in the labeling of both α and β subunits as revealed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Treatment of these protein bands with snake venom phosphodiesterase resulted in quantitative release of labeled 5'AMP, respectively labeled with the corresponding isotope. Two-dimensional separation by isoelectric focusing and SDS-PAGE of labeled and native tubulin revealed that labeling occurs at least in four different isotubulins. The isoelectric point of the labeled isotubulins was slightly lower than that of native purified tubulin. This shift in mobility is probably due to additional negative charges involved with the incorporation of ADP-ribosyl residues into the tubulin subunits. SDS-PAGE of peptides derived from [32P]ADP-ribosylated α and β tubulin subunits by Staphylococcus aureus protease cleavage showed a peptide pattern identical with that of native tubulin. Microtubule-associated proteins (MAP1 and MAP2) of high molecular weight also were shown to undergo ADP-ribosylation. Incubation of permeated rat neuroblastoma cells in the presence of [32P]NAD and cholera toxin results in the labeling of only a few cell proteins of which tubulin is one of the major substrates.

Key words: tubulin/MAPS/cholera toxin/ADP-ribosylation

Introduction

ADP-ribosylation has recently emerged as a potentially important mechanism for controlling the activity of several proteins (Hayaishi and Veda, 1977; Hinz et al., 1978; Pappenheimer, 1977). Some bacterial toxins have been shown to catalyze NAD-dependent ADP-ribosylation of key regulatory proteins. ADP-ribosylation of elongation factor 2 diphtheria toxin (Pappenheimer, 1977) leads to inhibition of protein synthesis. Cholera toxin catalyzes ADP-ribosylation of the GTP-binding protein of the hormone-sensitive adenylate cyclase system, thereby leading to more persistent cyclase stimulation (Cassel and Pfueffer, 1978; Johnson et al., 1978; Gill and Meren, 1978). The common denominator of these two acceptor proteins is that they both specifically bind and cleave GTP and that ADP-ribosylation inhibits their activity. It was, therefore, of interest to test whether tubulin, which apparently is one of the most abundant GTP-binding proteins in eukaryotic cells can serve as acceptor for ADP-ribosylation catalyzed by cholera toxin.

In this study we demonstrate that rat brain tubulin, as well as high molecular weight (mol. wt.) microtubule-associated proteins (MAP1 and MAP2), serve as substrates for cholera toxin NAD-dependent ADP-ribosylation.

Results

Rat brain microtubule proteins were obtained by two assembly-disassembly cycles. The fraction obtained contains both tubulin and MAPs. Incubation of this fraction with [32P]NAD and cholera toxin and subsequent polyacrylamide gel electrophoresis under denaturing conditions resulted in the labeling of both the α and β tubulin subunits as well as MAP1 and MAP2 (Figure 1). Further purification of the microtubule proteins by phosphocellulose chromatography separated the tubulin from the MAPs. Incubation of the phosphocellulose-purified tubulin fraction with cholera toxin and labeled NAD causes considerable labeling of the tubulin subunits (Figure 1, lane 6). It was also noted that the β subunit was labeled more heavily than the α subunit. The position of the radiolabeled α and β subunits correlated well with the position of the Coomassie brilliant blue stained bands of purified tubulin (lane 8). In addition some radioactivity was incorporated into high mol. wt. protein bands, which had a mobility identical to that of marker rat brain MAP1 and MAP2. It should be noted that the high mol. wt. MAPs are present at very low concentrations in the purified tubulin fraction and were not detectable by Coomassie brilliant blue staining yet they appear to be labeled by this reaction. In the absence of cholera toxin no labeling of any of these proteins was observed (Figure 1, lane 7).

To examine whether additional MAPs are labeled in this reaction, we incubated the MAP fraction, purified by phosphocellulose column chromatography, with [32P]NAD and cholera toxin. This fraction contains the tau factors, the high mol. wt. polypeptides (MAP1 and MAP2) as well as other proteins (Weingarten, 1975). Figure 1 (lane 2) shows considerable labeling of MAP1 and MAP2, while tau factors 1–4, were only weakly labeled, thus indicating the selective nature of the cholera toxin-mediated reaction. In the absence of cholera toxin, no labeling was observed (Figure 1, lanes 3 and 5, respectively). In a control experiment (Figure 1, lane 1) cholera toxin was incubated with [32P]NAD in the absence of any other acceptor proteins. Under these conditions, i.e., 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), labeled cholera toxin runs out of the slab gel. Consequently one could conclude that the labeled bands seen in lanes 2, 4, and 6 represent brain proteins.

To permit labeling at high specific radioactivity, experiments were carried out at micromolar concentrations of radiolabeled NAD. Since the K_m of cholera toxin for NAD is high (3–4 mM; Mekalanos, 1979) the fraction of labeled tubulin obtained was minute, and did not exceed 0.1%. It was therefore imperative to identify the labeled proteins by independent methods. We first subjected the presumptive labeled tubulin bands to isoelectric focusing followed by SDS-PAGE (Figure 2). Protein staining of the purified tubulin following the two-dimensional electrophoresis showed four spots (Figure 2A). Autoradiography of this slab gel (Figure 2B) revealed a similar picture with respect to the separation of the α and β tubulin subunits. Superposition of the autoradiogram (Figure 2B) on the Coomassie brilliant
blue stained gel (Figure 2A) showed that the mobility in the second dimension of the various isotubulins on SDS-PAGE was identical. In contrast, the various spots on the autoradiogram were slightly shifted in the acid direction with an average ΔpI of 0.18 for both the α and β subunits.

Cholera toxin is also labeled with [32P]ADP-ribosylation. The labeled toxin separates well from the tubulin subunits on two dimensional gel electrophoresis as seen in lane 2, Figure 2B. The spot at the center of Figure 2A (indicated by an arrow) originates in the cholera toxin preparation but is not labeled by incubation with radiolabeled NAD.

Another approach to verify the identity of the labeled tubulin bands was to examine their peptide distribution on SDS-PAGE following limited digestion with *Staphylococcus aureus* protease. As shown in Figure 3, proteolytic digestion of α (lanes 2, 3) and β tubulin (lanes 5, 6) with *S. aureus* protease resulted in different peptide distribution for the two subunits on SDS-PAGE (15–20%). Staining with Coomassie brilliant blue (lanes 2, 5) and autoradiography (lanes 3, 6) indicated that the major cleavage peptides of α and β tubulin incorporated label following incubation of purified tubulin with [32P]NAD and cholera toxin. Excised bands of α and β tubulin which were not digested with protease were loaded in lanes 4 and 7 respectively.

We next examined the nature of the tubulin modification.

![Image of SDS-PAGE gel](image)

**Fig. 1.** SDS-PAGE of tubulin and MAPs incubated with [32P]NAD and cholera toxin. The reaction mixtures contained [32P]NAD 16.3 Ci/mmol and the protein to be labeled with or without cholera toxin as indicated. 10 μg of labeled protein fraction were loaded in each lane, and the resulting autoradiographic pattern is shown. Lane 1, cholera toxin alone with [32P]NAD; lanes 2, 3, MAPs after separation from tubulin by phosphocellulose chromatography incubated with or without cholera toxin; lanes 4, 5, assembled tubulin (second cycle) incubated with or without cholera toxin; lanes 6, 7, phosphocellulose-purified tubulin incubated with or without cholera toxin; lane 8 is a Coomassie brilliant blue staining of lane 5.

![Image of two-dimensional gel](image)

**Fig. 2.** Two-dimensional gel electrophoresis of purified tubulin labeled with [32P]NAD. Labeling of purified tubulin was performed as described in Materials and methods using cholera toxin and [32P]NAD 45 Ci/mmol. Labeled purified tubulin (13 μg protein) was subjected to isoelectric focusing. The resulting isoelectric focusing gels were separated (second dimension) by SDS-PAGE (7.5–15% polyacrylamide gradient, 20 cm long). Protein staining of the gel (part A) and autoradiogram (part B) are shown. Markers separated on SDS-PAGE only are: lane 1 (A,B) unlabeled tubulin mixed with cholera toxin which has been previously incubated with [32P]NAD under standard labeling conditions but with no tubulin; lane 2 (A,B) contains labeled tubulin. The dashed markings on parts A and B indicate the location of the corresponding isotubulin spots as found on the autoradiogram (part B) and on the protein stained gel (part A) respectively. The arrow (part A) indicates an unlabeled peptide originating from the cholera toxin preparation.

It was assumed that if ADP-ribosylation is involved, then a transfer of the adenine moiety of NAD to tubulin should be observed. We therefore tested the action of cholera toxin on purified tubulin using [8-3H]NAD (Figure 4) and we were able to show that tubulin is indeed labeled in this reaction and that the position of the labeled protein corresponded upon gel electrophoresis to that of unlabeled tubulin (α and β tubulin bands were clearly seen after staining with Coomassie
Fig. 3. S. aureus protease cleavage peptides of [3H]ADP-ribosylated purified tubulin. Tubulin (phosphocellulose fraction) was incubated with labeled [3H]NAD 35 Ci/mmol or unlabeled NAD and cholera toxin as described in Materials and methods. The ADP-ribosylated protein was subjected to 8% SDS-PAGE: 5 μg protein/slot for the radiolabeled and 40 μg protein/slot for the unlabeled purified tubulin. The stained gels were dried and the corresponding α and β bands were excised, prepared, proteolytically digested by S. aureus protease, and subjected to gel electrophoresis as described in Materials and methods. The resulting peptide maps of α (lanes 2–4) and β (lanes 5–7) subunits of purified tubulin are shown.

brilliant blue but this pattern diffused following further treatment with salicylate. It should be noted that the [3H]NAD is labeled at the α position adjacent to the 5'-carbon of the adenosine moiety. It therefore appears that the radioactively labeled derivatized tubulin contains at least the adenosine and the α phosphate of NAD.

In other experiments (Table I), we were able to show that [3H] or [32P]5'-AMP are released from 3H- or 32P-labeled tubulin by treatment with snake venom phosphodiesterase. Purified 3H- and 32P-labeled tubulin was prepared by incubation with cholera toxin and [8-3H]NAD or [32P]NAD, respectively. The labeled protein preparations were subjected to SDS-PAGE on 8% polyacrylamide and the radioactive bands were then excised. The protein was extracted and precipitated with 10% trichloroacetic acid. The resulting pellets of the labeled tubulin preparations were incubated with snake venom phosphodiesterase for 4.5 h. Reaction products were identified by t.l.c. on polyethyleneimine cellulose. Table I shows that quantitative release of [32P] or [3H]5'-AMP from the respective labeled tubulin bands depended on the presence of phosphodiesterase. In the absence of the enzyme the radioactivity remained bound to the protein and did not migrate from the origin. 5'-AMP seems to be the sole product released under these conditions as no radioactivity was found anywhere else on the t.l.c. plate. It was therefore concluded that the labeling of tubulin by [32P] or [8-3H]NAD following incubation with cholera toxin is likely to result from an ADP-ribosylation reaction.

Table I. Distribution of radioactive products released from ADP-ribosylated tubulin after treatment with snake venom phosphodiesterase

<table>
<thead>
<tr>
<th>NAD labeling</th>
<th>Snake venom phosphodiesterase</th>
<th>Origin (c.p.m., %)</th>
<th>5'-AMP</th>
<th>Total c.p.m. loaded</th>
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<tr>
<td>[3H]NAD</td>
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<tr>
<td>-</td>
<td></td>
<td>99</td>
<td>&lt;1</td>
<td>370</td>
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<td>+</td>
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<td>&lt;1</td>
<td>99</td>
<td>2911</td>
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<tr>
<td>[8-3H]NAD</td>
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<td></td>
<td>85</td>
<td>9</td>
<td>494</td>
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<td>+</td>
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<td>&lt;1</td>
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4 Spots corresponding with NAD as well as the intervening spaces between spots were also extracted and counted but contained no significant radioactivity.

Purified tubulin (100 μg) was labeled with [3H]NAD 35 Ci/mmol or [8-3H]NAD 41 Ci/mmol and cholera toxin, and electrophoresed on 8% SDS-PAGE (10 μg/lane) as described in Materials and methods. Bands from three lanes were pooled for extraction and digestion by phosphodiesterase as described in Materials and methods. Reaction products from incubations with or without phosphodiesterase were separated by t.l.c. on polyethyleneimine cellulose. Spots corresponding to 5'-AMP were extracted and counted as described in Materials and methods.

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reaction mixture of the jor proteins blastoma tubulin of cells collected, [32P]NAD cellular gross (lane 2). lane 1, 2, 3, 4, 5. SDS-PAGE stained labeled with [32P]NAD and cholera toxin. The reaction was carried out as described in the legend to Figure 5 lane 5. The labeled proteins were subjected to isoelectric focusing. The resulting isoelectric focusing gels were separated (second dimension) by SDS-PAGE (8–12% polyacrylamide gradient, 10 cm long). The arrow indicates the position of tubulin migration.

1) revealed many more proteins as compared with the toxin-mediated ADP-ribosylated products (lane 5). The identity of the ADP-ribosylated tubulin was further established by two-dimensional gel electrophoresis (Figure 6) and limited protease digestion (not shown).

**Discussion**

It was demonstrated in this study that incubation of purified rat brain tubulin with radiolabeled NAD and cholera toxin leads to the labeling of α and β tubulin subunits (Figures 1, 4). High mol. wt. microtubule-associated proteins MAP1 and MAP2 are also labeled by this procedure (Figure 1). The finding that radiolabeled 5′-AMP may be released from such modified tubulin preparations by snake venom phosphodiesterase (Table 1) supports the view that protein labeling results from ADP-ribosylation catalyzed by cholera toxin. This reaction seems to involve at least four tubulin forms (Figure 2), as judged by autoradiography. It should be born in mind that each of these spots (Figure 2), represents a mixture of several isotubulins (Gozes et al., 1979) which are not well-resolved by this procedure. The labeled bands seem to represent modified proteins and not poly(ADP-ribose) since treatment with S. aureus protease resulted in their degradation. By electrophoretic mobility on one- and two-dimensional gel electrophoresis and by peptide mapping we identified the labeled proteins as α and β tubulin.

It was not surprising to find that the extra negative charges added to the polypeptide chains by the ADP-ribose moiety resulted in an acid shift in mobility of the modified tubulin subunits (Figure 2). The rather uniform acid shift in pI (∆pI = 0.18) of the four tubulin forms indicates that a similar degree of modification of these proteins has probably taken place. The α and β tubulin subunits, as well as MAP1 and MAP2, may thus be considered as potential targets for cholera toxin action in vivo.

It was shown that several proteins can serve as substrates for the ADP-ribosylation reaction (Watkins et al., 1980). We, therefore, chose a permeated neuroblastoma cell system to...
examine the specificity of this reaction. Under these conditions only a few of the cell proteins were labeled and one of the major substrates of this reaction was tubulin (Figure 5). A small proportion of the tubulin subunits (Zisaee and Lit-
tauer, 1979; Gozes and Littauer, 1979) have been reported to be associated with cell membranes. It is, therefore, not unlikely that these proteins may have been labeled in the course of cholera toxin dependent ADP-ribosylation of cell membranes in vitro. Indeed, studies performed with cholera toxin in relation to the GTP-binding protein associated with adenylate cyclase have reported the presence of membrane-bound 52 – 53 K labeled protein bands (Johnson, 1978; Hud- son and Johnson, 1980). In the light of the present findings the relationship of these proteins to microtubules may be tested. Interestingly cholera toxin catalyzed ADP-ribosylation of the membrane-bound GTP-binding protein of pigeon erythrocytes (Enomoto and Gill, 1980) or of purified 45 and 52 K subunits of this protein from rabbit liver (Northrop et al., 1980) requires the presence of yet another macro-
molecular factor. In contrast, the exogenous addition of such a factor for ADP-ribosylation of purified rat brain tubulin by cholera toxin seems not to be essential. The same is also true for purified rat brain actin consisting of β and γ subunits which we found in this study to be readily labeled by NAD in the presence of cholera toxin (data not shown).

It is interesting to note that endogenous ADP-ribosyltrans-
ferase activity is present in animal cells (Moss and Vaughan, 1978). It is therefore possible that tubulin and MAPs may be ADP-ribosylated by such enzymes in vivo. The physiological implications of ADP-ribosylation of microtubule proteins may be viewed as potential post-translational modifications that regulate the structure of the microtubular pro-
teins and their assembly (Littauer et al., 1980). These also include the control of interaction of microtubule proteins with other cytoskeletal networks, membranes, and elements of chromatin.

Materials and methods

The labeling was performed as described previously (Amir-Zaltzman et al., 1980). The labeling reaction in a final volume of 100 μl contained 50 mM potassium phosphate pH 7.2, 0.5 mM ATP, 0.1 mM GTP, 20 mM thymidine, 20 mM arginine, 5 mM ADP-ribose, 10 μM [32P]NAD or [8-3H]NAD, 100 – 200 μg acceptor proteins, and 22 μg cholera toxin. Incubation was for 20 min at 30°C. The reaction was terminated by addition of ice cold 80% acetone and the material was stored at –20°C.

Electrophoresis and peptide mapping

Prior to electrophoresis, the acetone-precipitated proteins were centrifuged at 20,000 g for 15 min and the pellets were taken up in 3% SDS containing 5% mercaptoethanol and incubated for 1 h at 60°C. SDS-PAGE was performed on slab gels (1.6 mm x 13 cm x 18 cm) according to Laemmli, 1970 with 7.5% - 15% or 15% - 20% polyacrylamide gradients, or with constant 8% polyacrylamide. Separation was accomplished on thin chromatographed (100 – 120 V) for 14 – 16 h. Gels were stained with Coomassie brilliant blue, dried, and exposed to X-ray films (Agfa Gevaert RP.2). Gels containing H-labeled proteins were treated with sodium salicylate prior to autoradiography (Chamberlin, 1979).

Protease digestion of excised tubulin bands from 8% gels was performed according to Cleveland et al., 1978, with slight modifications as previously described (Gozes and Littauer, 1978; Gozes et al., 1979) using 15% – 20% polyacrylamide gradient and 25 ng S. aureus V8 protease/Slot. Isoelectric-
focusing-electrophoresis of protein samples was performed as previously described (Gozes and Littauer, 1978).

Phosphodiesterase treatment of ADP-ribosylated proteins

The identification of radioactive products released from “ADP-ribosylated” tubulin was carried out by digestion with snake venom phosphodiesterase according to Waters et al., 1980, with the following modifications: labeled tubulin bands were excised from non-fixed non-stained 8% gels, cut into small pieces, and soaked for 12 hr in 1.5 ml 10 mM Tris-aceate pH 7.0 at room temperature with light shaking. Bovine serum albumin (100 μg) was added to each tube before precipitation with ice cold 5% trichloroacetic acid. The pellet was extracted twice with dry ethyl ether. Washed precipitates were suspended in reaction buffer and subjected to snake venom phosphodiesterase as described by Waters et al., 1980. Samples of the reaction mixture were loaded on to thin layer polyethyleneimine/chromatographed in the presence of 0.01 μmol of unlabelled carrier NAD and 5-AMP with 0.25 M LiCl at room temperature. Spots were identified by u.v. light. Extraction and counting of radioactive nucleotides were performed as described earlier (Salomon and Rodbell, 1975).

ADP-ribosylation of permeated neuroblastoma cells

Rat neuroblastoma B104 cell line was kindly provided by D. Schubert of the Salk Institute, La Jolla, CA. Stock cultures were maintained in Dulbecco-Vogt modification of Eagle’s medium (DMEM) supplemented with 8% fetal calf serum in a humidified atmosphere of 5% CO2/95% air at 37°C and sub-
cultured every 4 – 6 days with 0.25% pancreatin in DMEM. Cells were grown to the confluent state, washed three times with phosphate buffered saline (PBS) at room temperature, incubated in a solution containing 0.25 mM EDTA, 0.125 mM EGTA, 30 mM Tris and 0.25 mM sucrose, pH 7.8 for 10 min at 37°C. The detached cells were collected by centrifugation for 5 min at 500 g. The cells were washed with PBS, resuspended in cold 150 mM sucrose, 80 mM KCl, 35 mM Hepes (7.4), 5 mM potassium phosphate (7.4) (solution B) at 1 x 10^6 cells/ml. The cells were chilled on ice for 5 min, 1/10 volume of 1 mg/ml lysoscellin in solution B was added and the suspension incubated for 1 min at 4°C (Miller, 1978). The suspension was then warmed to 37°C. Aliquots of 0.1 ml of the permeated cells were incubated for 45 min at 30°C in a reaction mixture (final volume 0.3 ml) containing: 10 μM [32P]NAD (1-5 x 10^6 c.p.m.), 50 mM potassium phosphate buffer, and, when indicated, 48 μg activated cholera toxin. The suspension was then centrifuged for 5 min at 500 g in the cold, washed once with 0.1 ml of solution B, and the pellet dissolved in a solution containing 9.5 M urea, 2% NP-40, and 3% β-mercaptoethanol.

Acknowledgements

We thank Dr. H.R. Lindner for helpful discussions and Mrs. M. Kopelowitz for excellent secretarial assistance. This work was supported by grants to H.R.L., by the Ford Foundation and Population Council, Inc., NY and the Rockefeller Foundation, to S.S. by the U.S.-Israel Binational Science Founda-
tion (BSF), Jerusalem, and to U.Z.L. by the Muscular Dystrophy Associa-
tion, Y.S. is the incumbent of the Charles W. and Tillie Lobin Career Development Chair.

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