Synthesis of Azidotubulin: A Photoaffinity Label for Tubulin-Binding Proteins†

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ABSTRACT: A photoaffinity label for the identification of tubulin-binding proteins was synthesized from phosphocellulose-purified bovine brain tubulin and (*N*-hydroxysuccinimidyl)-4-azidosalicylic acid. The azidotubulin derivative retained the ability to undergo temperature-dependent microtubule assembly and disassembly. When incubated with purified τ protein, the azidotubulin and τ formed cross-linked complexes upon photoactivation. When ^{125}I-labeled azidotubulin was used to photoaffinity label tubulin-binding proteins within the kinetochore of isolated mammalian chromosomes, a 130-kDa band was identified on autoradiographs of SDS–polyacrylamide gels of the ^{125}I-labeled azidotubulin/chromosome preparations. The 130-kDa complex was isolated by antitubulin affinity chromatography and analyzed by immunoblotting using both antitubulin and kinetochore-specific sera obtained from human patients with the autoimmune disease scleroderma CREST. The immunoblots demonstrated that the 130-kDa band that was observed on autoradiographs was a complex of a subunit of the tubulin dimer and an 80-kDa CREST-specific kinetochore protein. The binding of azidotubulin to the 80-kDa kinetochore protein was significantly decreased when chromosomes were treated with a mixture of 9 parts underivatized tubulin to 1 part azidotubulin prior to photolysis. The formation of the 130-kDa azidotubulin/kinetochore protein complex was not inhibited by pretreating the chromosomes with CREST serum prior to incubation with azidotubulin. Azidotubulin should be a useful probe for the identification and characterization of tubulin-binding proteins.

Microtubules are cytoskeletal components that participate in many cellular processes including ciliary and flagellar motility, organelle transport, cell shape changes, and mitosis. With the exception of the specialized microtubules found in cilia and flagella, most microtubules in cells are dynamic polymers that rapidly exchange subunits with a soluble tubulin pool. In addition, these labile microtubules can quickly disassemble and be reassembled by the cell as it either responds to extracellular signals or prepares for the process of cell division (Kirschner & Mitchison, 1986).

The ability of microtubules to assemble and disassemble facilitates their rapid purification from cell homogenates in vitro. During purification, a number of proteins coassemble with the α- and β-tubulin dimers. Proteins that copurify with tubulin by cycles of assembly and disassembly are termed microtubule-associated proteins (MAPs),[1] and different MAPs have been isolated from a variety of cell and tissue types and studied in detail (Cleveland et al., 1977; Herzog & Weber, 1978; Bulinsky & Borisy, 1980; Parysek et al., 1984; Huber et al., 1985; Bloom et al., 1985; Goldstein et al., 1986; Vallee & Collins, 1986; Aizawa et al., 1987). The ability to identify MAPs via copurification with microtubules from tissue homogenates depends on the MAPs being soluble. However, in certain instances microtubule-binding proteins may be insoluble and, therefore, would not be associated with microtubules during tubule assembly. In this paper, a photoactivatable azidotubulin (N_3-Tb) derivative is described that should be useful for the identification of MAP-like molecules that cannot be identified by coassembly with tubulin into microtubules.

Photoaffinity labeling has been effectively used to identify the receptors and binding sites for a number of proteins. Specifically, lectins (Ji, 1977; Ji & Ji, 1982), insulin (Yip et al., 1978; Jacobs et al., 1979), glucagon (Johnson et al., 1981), calmodulin (Andreasen et al., 1981), fibronectin (Perkins et al., 1979), and thrombin (Carney et al., 1979) have been efficiently derivatized with photoactivatable compounds and subsequently used as probes to label their respective binding proteins. In the present study we outline the synthesis of both N_3-Tb and ^{125}I-labeled N_3-Tb and demonstrate that these compounds can be used to identify both soluble and insoluble microtubule-binding proteins. Specifically, the N_3-Tb derivative was used to produce covalently cross-linked complexes with both purified τ protein in solution and an insoluble 80-kDa protein from the mammalian kinetochore.

In an earlier paper, tubulin was cross-linked to kinetochores by using Lomant's reagent (Lomant & Fairbanks, 1976), a reversible cross-linking compound. From those experiments, a tubulin-binding complex in the kinetochore was identified consisting of proteins of molecular weights 24000, 80000, and 110000 (Balczon & Brinkley, 1987). Because of the nature of the cross-linker employed in those experiments, it could not be stated equivocally which protein in the complex was the tubulin-binding component of the kinetochore. The N_3-Tb derivative was used to further investigate the nature of the interaction of tubulin with the kinetochore in vitro, and it was found that N_3-Tb could be photochemically cross-linked to the 80-kDa kinetochore protein, indicating that the 80-kDa protein is either the microtubule-binding component of the kinetochore in the in vitro assay system used or directly contiguous to the microtubule-binding site.

Azidotubulin should be a useful probe for studying the

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[1] Abbreviations: N_3-Tb, azidotubulin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); NHS-ASA, (*N*-hydroxysuccinimidyl)-4-azidosalicyclic acid; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PEM buffer, 80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.9; MAP, microtubule-associated protein; CHO, Chinese hamster ovary; CREST, Calcinosis, Raynaud's phenomenon, Esophageal dysmotility, Sclerodactyly, and Telengectasia.

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interaction of microtubules with various cellular components both in vitro and in vivo.

Materials and Methods

Materials. ^{125}I was purchased from Amersham. (*N*-Hydroxysuccinimidyl)-4-azidosalicylic acid (NHS-ASA) was purchased from Pierce. Peroxidase-labeled antimouse and peroxidase-labeled antihuman antibodies were purchased from Boehringer-Mannheim.

Protein Purification. Twice-cycled microtubules were obtained from bovine brains by use of the methods reported by Shelanski et al. (1973). Tubulin was purified from the twice-cycled microtubule protein by phosphocellulose chromatography (Weingarten et al., 1975). Protein-containing fractions were pooled, aliquoted, and frozen at −80 °C. τ was purified from twice-cycled microtubule protein by chromatography on Bio-Gel A 1.5 M (Kim et al., 1986).

Protein concentrations were determined by use of the procedures described by Bradford (1976).

Cell Culture and Chromosome Isolation. Chinese hamster ovary (CHO) cells were grown in McCoy's 5A containing 10% controlled process serum replacement (CPSR) 4 (Sigma). Cells were mitotically arrested by incubation in the presence of 10 μg/mL vinblastine sulfate for 12–14 h. Mitotic cells were collected and homogenized, and the chromosomes were isolated on a sucrose gradient (Mitchison & Kirschner, 1985). The isolated chromosomes were collected and frozen at −80 °C until use.

Synthesis of Azidotubulin (N_3-Tb). Phosphocellulose-purified tubulin was assembled in 80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.9 (PEM buffer), and 0.5 mM GTP containing 25% glycerol. After incubation at 37 °C for 40 min, a 10× molar excess of NHS-ASA (in DMSO) was added (the remainder of this procedure was performed under dark room conditions). After 10 min, the reaction was quenched by the addition of lysine to a final concentration of 10 mg/mL. The assembled microtubules were pelleted at 100000*g* for 30 min at 30 °C. The microtubules were disassembled by resuspending them in PEM buffer containing 0.1 mM GTP and left at 4 °C for 40 min. The tubulin was then desalted on a Bio-Gel P-6DG column to remove any unincorporated NHS-ASA. Peak protein containing fractions were pooled and glycerol was added to a final concentration of 25%, and GTP was added to 0.5 mM. The tubulin was incubated for 60 min at 37 °C to assemble microtubules, and the microtubules were subsequently pelleted at 100000*g* for 30 min. The pelleted microtubules were resuspended in a small volume of PEM containing 0.1 mM GTP and left at 4 °C for 30 min. The solution was centrifuged at 100000*g* for 30 min at 4 °C, and the supernatant was collected, brought to 0.5 mM with GTP, and frozen in aliquots at −80 °C. By use of this procedure, approximately 30–35% of the original starting tubulin is recovered at the final step.

For some experiments, the tubulin was derivatized with ^{125}I-labeled NHS-ASA. The iodination of NHS-ASA was performed by using chloramine T in either acetone or dimethyl sulfoxide as previously described (Ji & Ji, 1982; Ji et al., 1985). The ^{125}I-labeled NHS-ASA was purified by chromatography on thin-layer silica gel plates in benzene/chloroform/ethyl acetate/acetic acid (1:1:1:0.1 v/v) (Ji & Ji, 1982). The silica gel plate was dried, and the radioiodinated product was identified by autoradiography. The radioactive region was collected, and the ^{125}I-labeled NHS-ASA was extracted from the gel with 95% ethanol. Tubulin was then derivatized with the ^{125}I-labeled NHS-ASA according to the procedure outlined above.

Determination of the Molar Incorporation of NHS-ASA into Tubulin during the Synthesis of N_3-Tb. To determine the amount of NHS-ASA incorporated per mole of tubulin, N_3-Tb was synthesized according to a modification of the previously described protocol. Microtubules were assembled by incubating phosphocellulose-purified tubulin for 40 min at 37 °C in PEM buffer containing 25% glycerol and 0.5 mM GTP. A 10× molar excess of NHS-ASA was dissolved in DMSO, and a tracer amount of ^{125}I-labeled NHS-ASA (100 000 cmp) was added to the solubilized NHS-ASA. The NHS-ASA/^{125}I-labeled NHS-ASA mixture was added to the assembled microtubules. After 10 min, the reaction was quenched with lysine and the microtubules were pelleted as described above. The supernatant (super 1) was removed and saved, and the microtubule pellet was disassembled by resuspension in cold PEM buffer containing 0.1 mM GTP. After 40 min at 4 °C, the derivatized tubulin was desalted into PEM on a Sephadex G-25 column. Both the protein-containing peak and the small peak of unincorporated ^{125}I-labeled NHS-ASA were collected. In addition, the supernatant (super 1) from the microtubule assembly step in this procedure was desalted into PEM on a Sephadex G-25 column. Both the protein peak and the unincorporated ^{125}I-labeled NHS-ASA fraction were collected. The ^{125}I-labeled N_3-Tb fractions from both columns and the unincorporated ^{125}I-labeled NHS-ASA fractions from each desalting column were then counted by using an LKB 1272 Clinigamma counter. By use of this procedure, it was determined that between 70 and 75% of the ^{125}I-labeled NHS-ASA was incorporated into the N_3-Tb (data not shown). It should be mentioned that the amount of NHS-ASA incorporated per mole of tubulin decreased significantly as the age of the NHS-ASA reagent increased.

Photoaffinity Labeling. The conditions for the identification of tubulin-binding proteins using N_3-Tb were as follows. To determine whether the α- and β-subunits of N_3-Tb could be cross-linked to one another or whether neighboring tubulin subunits in microtubule polymers would be cross-linked to each other, N_3-Tb (5.0–6.0 mg/mL) was incubated at 37 °C for 12–15 min in PEM buffer containing 0.5 mM GTP. The samples were then photolyzed for 1–2 min at a distance of 5 cm by using a Model UVG-11 Mineralight lamp (UVP, Inc.). The samples were then mixed with an equal volume of 2× electrophoresis sample buffer and analyzed by SDS–PAGE (Laemmli, 1970). To determine whether N_3-Tb could be cross-linked to known tubulin-binding proteins, N_3-Tb was mixed with column-purified τ protein and subsequently incubated and photolyzed as described above. Cross-linked complexes were then identified by immunoblotting (Towbin et al., 1979) using monoclonal antibodies against the τ polypeptides (see below).

Photochemical cross-linking of N_3-Tb to kinetochore proteins was performed under the following conditions. Purified CHO chromosomes were incubated with either a mixture of N_3-Tb and ^{125}I-labeled N_3-Tb or N_3-Tb only at 37 °C for 5 min. The chromosomes were rinsed three times with PEM buffer, photolyzed as described above, run on SDS gels, and autoradiographed.

To characterize the product that was formed by the photolysis of N_3-Tb to kinetochore proteins, the N_3-Tb/kinetochore protein complex was purified by antitubulin chromatography (Balczon & Brinkley, 1987), dialyzed against distilled water, and lyophilized. The lyophilized protein was run on SDS gels and analyzed by immunoblotting using antitubulin and sera from patients with the CREST variation of the autoimmune disease scleroderma.

Antibodies. Antitubulin was produced in sheep and affinity-purified according to previously published methods (Brinkley et al., 1980). Monoclonal antibody against the τ polypeptides was a gift from Dr. L. I. Binder (The University of Alabama at Birmingham).

All scleroderma CREST sera (CREST, Calcinosis, Raynaud's phenomenon, Esophageal dysmotility, Sclerodactyly, and Telengectasia) were obtained from the Comprehensive Arthritis Center at The University of Alabama at Birmingham. Before use, the sera were tested for antikinetochore staining properties by indirect immunofluorescence and by immunoblotting against proteins of isolated chromosomes. For most procedures, whole CREST serum was used. However, for some experiments it was necessary to affinity purify antibodies that were specific for the individual kinetochore proteins. The purification of monospecific antikinetochore antibodies was performed according to previously published methods (Olmsted, 1981; Earnshaw & Rothfield, 1985). Briefly, the proteins from isolated CHO chromosomes were run on 12.5% SDS gels and subsequently transferred to nitrocellulose. The nitrocellulose was then incubated for 2 h at room temperature with CREST serum (1:500) in 3% powdered milk. The blot was rinsed three times with 3% powered milk, and a thin strip was cut from one end of the blot. The thin nitrocellulose strip was then incubated in peroxidase-labeled antihuman antibody in 3% powdered milk for 90 min (the remainder of the blot was stored at 4 °C in PBS) at room temperature. The strip was rinsed three times with PBS and developed with 4-chloro-1-naphthol. The thin nitrocellulose test strip and the remainder of the blot were then aligned, and antibody-binding regions of the blot were cut out and transferred to individual test tubes. The blots were then rinsed as previously described (Earnshaw & Rothfield, 1985), and the monospecific kinetochore antibodies were eluted with either 50 mM sodium citrate, pH 2.5, or 3 M potassium thiocyanate and 0.5 M NH_4OH (Earnshaw & Rothfield, 1985). The purified antibodies were rapidly desalted into PBS on Bio-Gel P-6DG columns and then lyophilized.

PAGE and Immunoblotting. SDS–PAGE was performed according to the methods published by Laemmli (1970). Transfer of proteins to nitrocellulose was performed according to the methods of Towbin et al. (1979). The nitrocellulose blots were then probed with either CREST antiserum (1:500), antitubulin (10 μg/mL), or anti-τ (1:1000) for 2 h. The blots were then treated with the appropriate peroxidase-labeled second antibody (1:500) for 90 min, rinsed, and developed with 4-chloro-1-naphthol. All antibodies were diluted in 3% powdered milk in PBS.

Results

An azidotubulin derivative was synthesized from phosphocellulose-purified 6S tubulin and NHS-ASA by use of the scheme outlined under Materials and Methods. Approximately 30–35% of the original starting tubulin was recovered as N_3-Tb at the final step of this procedure, and the N_3-Tb was assembly competent. According to the procedure that was described, a ratio of 10 mol of NHS-ASA was used for every mole of tubulin in the synthesis of N_3-Tb. When this ratio was used, approximately 7 mol of the azido group was incorporated per mole of tubulin (data not shown). N_3-Tb has also been synthesized by using a 3:1 molar ratio of NHS-ASA tubulin, and the N_3-Tb formed under these conditions retained the assembly and cross-linking characteristics of N_3-Tb that was synthesized by using the 10:1 ratio of NHS-ASA tubulin. However, all of the experimental results described in this paper were obtained by using N_3-Tb that was synthesized exactly as described under Materials and Methods.

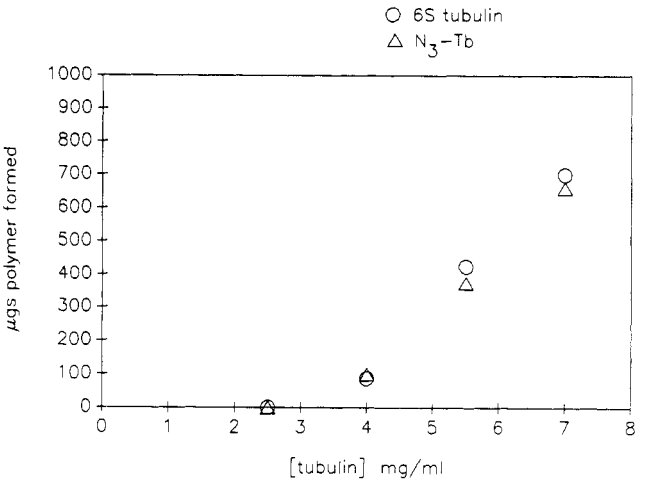

FIGURE 1: Amount of microtubule polymer formed when N_3-Tb was incubated under assembly conditions was compared to amount of polymer formed when an identical amount of nonderivatized tubulin was incubated under the same conditions. Solutions of 200 μL of either N_3-Tb (Δ) or phosphocellulose-purified tubulin (O) at either 2.5, 4.0, 5.5, or 7.0 mg/mL in PEM buffer containing 0.5 mM GTP were warmed to 37 °C for 30 min. The samples were then centrifuged at 100000*g* in a type 75 rotor for 30 min. The supernatant was removed, and the bottom of the tube was rinsed twice with warm PEM buffer. The pellets were resuspended in 400 μL of PEM containing 0.1 mM GTP by passing the material through a 26-gauge needle 8–10 times. The suspended microtubules were then left at 4 °C for 60 min, and the amount of tubulin in each sample was determined by using the procedures described by Bradford (1976).

The ability of the N_3-Tb derivative to assemble into microtubules was assayed by determining the amount of microtubule polymer formed when different concentrations of N_3-Tb were incubated under microtubule assembly conditions. As Figure 1 shows, the amount of microtubule polymer formed was virtually identical when equal amounts of either phosphocellulose-purified tubulin or N_3-Tb were warmed to 37 °C for 30 min. The microtubules that formed from N_3-Tb were morphologically identical with microtubules assembled from nonderivatized tubulin when observed by whole mount electron microscopy (not shown).

Following the synthesis of N_3-Tb, the derivatized protein was characterized both by examining its ability to interact with other tubulin molecules and by testing whether the N_3-Tb could be cross-linked to τ protein, a known microtubule-binding protein. As demonstrated in Figure 2, N_3-Tb was not effectively cross-linked to other N_3-Tb monomers. When N_3-Tb was incubated under microtubule assembly conditions, photolyzed, and then run on SDS gels, higher molecular weight tubulin complexes were not observed. When this experiment was repeated with ^{125}I-labeled N_3-Tb, over 98% of the radioactive counts were in the α- and β-tubulin bands with less than 2% of the counts being in higher molecular weight regions of the gel (data not shown). Furthermore, when this experiment was performed under SDS–PAGE conditions that allowed for complete separation of the tubulin subunits, it was determined that virtually identical amounts of ^{125}I-labeled NHS-ASA were incorporated into both the α- and β-subunits (data not shown). These results demonstrated that neither the α- and β-subunits of N_3-Tb nor neighboring N_3-Tb monomers were efficiently cross-linked to one another and that both the α- and β-subunits of tubulin incorporated NHS-ASA during the synthesis of N_3-Tb.

To further characterize the N_3-Tb, the derivative was incubated with column-purified bovine brain τ to determine whether N_3-Tb could be cross-linked to known MAPs. Fol-

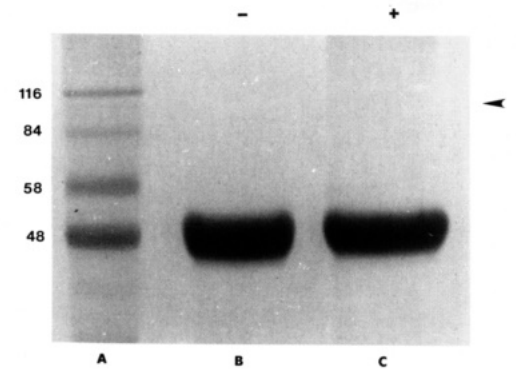

FIGURE 2: N_3-Tb (5.5 μg/mL) was incubated at 37 °C for 8 min and then divided into two samples. One aliquot was suspended directly in sample buffer, while the other aliquot was UV-irradiated before being prepared for electrophoresis. The two samples were then run on an 8.5% polyacrylamide gel and the gel was subsequently stained with Coomassie blue. (Lane A) Molecular weight standards. (Lane B) Control sample that was not UV-irradiated ("–" UV) prior to electrophoresis. (Lane C) UV-photolyzed sample ("+" UV). The arrowhead to the right of lane C shows that little, if any, N_3-Tb cross-linked to other N_3-Tb molecules by UV irradiation.

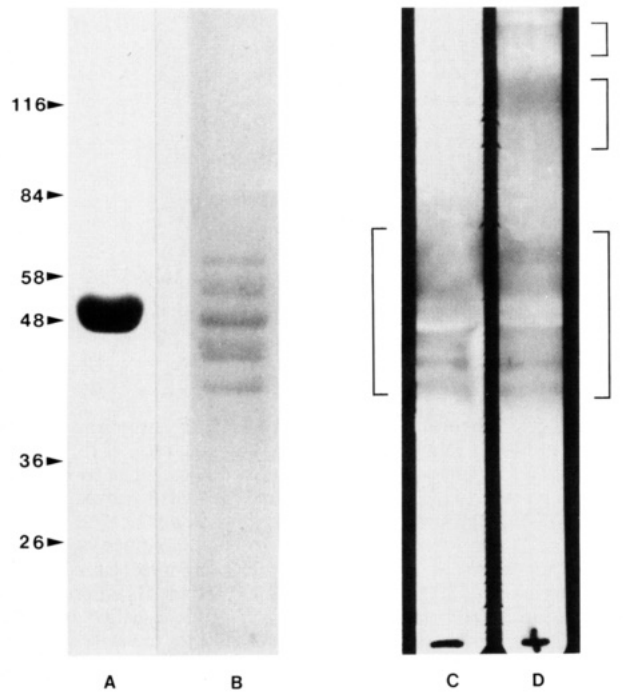

FIGURE 3: N_3-Tb and column-purified bovine brain τ protein were incubated together under microtubule assembly conditions as described under Materials and Methods. Half of the sample was UV-irradiated (+), while the other aliquot was suspended directly in sample buffer without UV photoactivation (–). The protein samples were run on SDS gels and transferred to nitrocellulose, and the blots were subsequently probed with monoclonal anti-τ antibody. (Lane A) Coomassie-stained gel of the phosphocellulose-purified tubulin used in this experiment. (Lane B) Coomassie-stained gel of the column-purified τ used in this experiment. (Lane C) Control sample of N_3-Tb and τ that was not UV-irradiated prior to electrophoresis and immunoblotting. The brackets to the left of lane C show the polypeptides recognized by the τ antibody. (Lane D) N_3-Tb/τ sample that was photoactivated prior to electrophoresis and immunoblotting. The brackets to the right of lane D show the cross-linked comlexes that were formed between N_3-Tb and τ. Molecular masses are given in kilodaltons.

lowing UV irradiation, the samples were run on SDS gels, transferred to nitrocellulose, and then probed with a monoclonal antibody against the τ polypeptides. As demonstrated in Figure 3, two different N_3-Tb/τ complexes were formed. The lower molecular weight complex, between 95 000 and

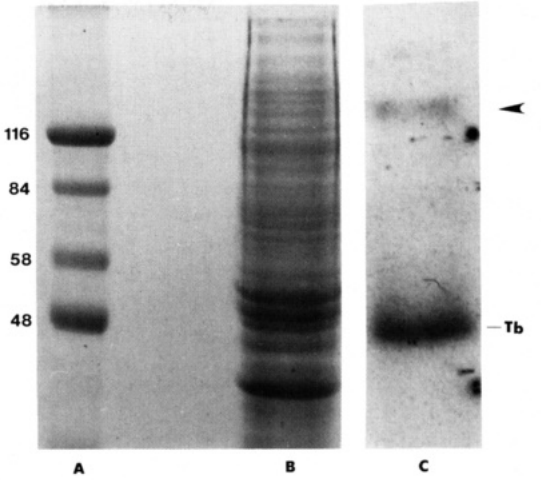

FIGURE 4: ^{125}I-labeled N_3-Tb and purified CHO chromosomes were incubated, rinsed, and photoactivated as described under Materials and Methods. The sample was then electrophoresed, and the gel was dried and autoradiographed. (Lane A) Molecular weight standards. (Lane B) Coomassie-stained gel of the ^{125}I-labeled N_3-Tb/CHO chromosome preparation. (Lane C) Autoradiograph of lane B. In addition to the ^{125}I-labeled N_3-Tb band (Tb), a band at 130 kDa was resolved (arrowhead).

115 000 corresponded in molecular weight to a complex that would be formed between a τ polypeptide and either an α- or β-tubulin subunit. The higher molecular weight complex, between 140 000 and 180 000, was in the molecular weight range of a complex that would be composed of either an α- or β-tubulin subunit and two τ polypeptides or two tubulin subunits and one τ polypeptide. The two high molecular weight N_3-Tb/τ complexes were not observed in control samples of N_3-Tb and τ that were not UV-irradiated. These results demonstrated that N_3-Tb could be cross-linked to tubulin-binding proteins and suggested that N_3-Tb would be a useful probe for investigating the interactions that occur between microtubules and MAP-like molecules.

To determine whether N_3-Tb could be used for identifying insoluble microtubule-binding proteins, ^{125}I-labeled N_3-Tb was incubated with isolated CHO chromosomes and then photolyzed. By use of the incubation conditions described under Materials and Methods, tubulin was bound specifically to the proteins of the kinetochore region of the chromosomes (Mitchison & Kirschner, 1985; Balczon & Brinkley, 1987). When the photolyzed ^{125}I-labeled N_3-Tb/chromosome preparations were observed following autoradiography of polyacrylamide gels, two radioactive bands were observed (Figure 4). . The lower molecular weight band corresponded to ^{125}I-labeled N_3-Tb, while the higher molecular weight band migrated with an apparent molecular mass of 130 kDa; this corresponds to the mass of a covalent complex formed between either an α- or β-tubulin subunit plus a protein or group of proteins of 80 kDa.

The composition of the 130-kDa protein complex was investigated by immunoblotting using antitubulin and kinetochore-specific sera obtained from human patients with the autoimmune disease scleroderma CREST. For these experiments, the 130-kDa protein complex was purified by antitubulin affinity chromatography and concentrated by lyophilization. The lyophilized protein complex was then suspended in sample buffer, run on polyacrylamide gels, and transferred to nitrocellulose, and the blots were probed with either antitubulin or CREST serum. As Figure 5 shows, the 130-kDa band was recognized by both antitubulin and antikinetochore antibodies by immunoblotting. Furthermore, the CREST-reactive N_3-Tb/kinetochore protein complex was not identified

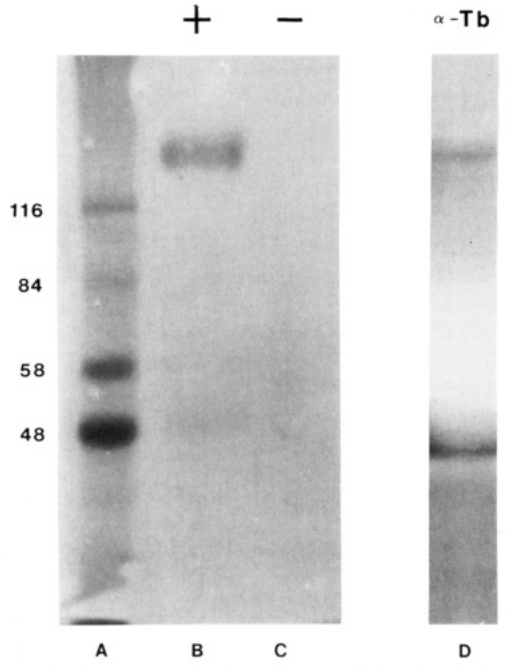

FIGURE 5: To characterize the 130-kDa band identified in Figure 4, an N_3-Tb/CHO chromosome preparation was solubilized and passed over an antitubulin affinity column. The affinity-purified proteins were lyophilized, run on an SDS gel, and characterized by immunoblotting using antitubulin and antikinetochore serum from a patient with scleroderma CREST. (Lane A) Molecular weight standards. (Lanes B and C) Affinity-purified proteins were transferred to nitrocellulose and probed with CREST serum. (Lane B) N_3-Tb/chromosome preparation was UV-irradiated (+) prior to solubilization and affinity purification. (Lane C) Control sample in which the N_3-Tb/CHO chromosome preparation was not UV-irradiated (−) prior to solubilization and affinity purification. (Lane D) Sample that was treated exactly as the sample in lane B, but probed with antitubulin (α-Tb). The 130-kDa complex reacts both with CREST serum and with antitubulin antibody. A CREST-reactive band was not identified in the control sample that was not UV-photolyzed.

in control samples that were treated in the same manner, but which were not UV-photolyzed prior to antitubulin affinity chromatography. The results of these experiments suggest that N_3-Tb should be a useful probe for investigating the interaction of microtubules with microtubule-binding proteins that are not readily soluble and, as a result, cannot be identified by copurification with microtubules from cellular homogenates.

To further characterize the 130-kDa N_3-Tb/kinetochore protein complex, the individual antikinetochore antibodies were affinity-purified from nitrocellulose blots according to previously published methods (Olmsted, 1981; Earnshaw & Rothfield, 1985), and each was subsequently blotted against the 130-kDa complex. A family of kinetochore proteins ranging from 14 to 140 kDa has been identified on immunoblots of mammalian chromosomes by using scleroderma CREST sera (Cox et al., 1983; Earnshaw et al., 1984; Guldner et al., 1984; Earnshaw & Rothfield, 1985; Valdivia & Brinkley, 1985; Palmer et al., 1987; Kingwell & Rattner, 1987). The actual CREST serum used in these experiments recognized proteins of 18, 22, 52, and 80 kDa on immunoblots of isolated CHO chromosomes (Figure 6A). Monospecific antibody against each of these proteins was produced by specifically eluting the individual antibodies from nitrocellulose as described, and the purity of each antibody was subsequently verified by immunoblotting against CHO chromosomes. The monospecific antibodies were then blotted against the 130-kDa complex. As shown in Figure 6B,C, monospecific anti-80-kDa antibody reacted with the 130kDa N_3-Tb/kinetochore protein complex. Purified antibodies against the 18-, 22-, and 52-kDa

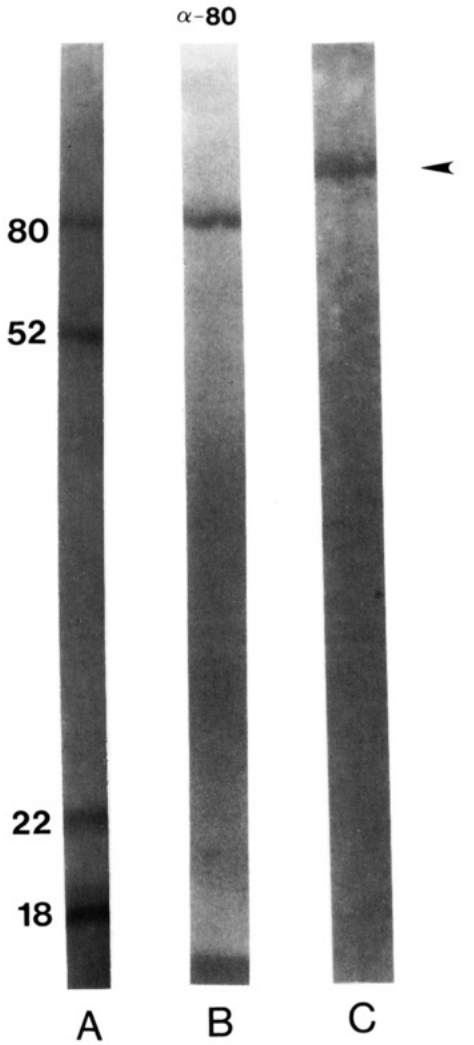

FIGURE 6: To determine which of the CREST antigens was cross-linked to N_3-Tb, monospecific antibody against each of the CREST antigens was affinity-purified from nitrocellulose blots by using the procedures described under Materials and Methods. The monospecific CREST antibodies were subsequently blotted against the 130-kDa complex. (Lane A) Immunoblot of CHO chromosomes using serum from scleroderma CREST patient S.H. This serum recognized proteins of M_r of 18000, 22000, 52000, and 80000. (Lane B) Before blotting against the 130-kDa complex, each of the affinity-purified antibodies was immunoblotted against CHO chromosomes to verify that each antibody was monospecific. Affinity-purified anti-80-kDa antibody (α-80) recognized only the 80-kDa protein. The affinity-purified antibodies against the 18-, 22-, and 52-kDa proteins also reacted specifically with their appropriate antigens (not shown). (Lane C) Each of the affinity-purified CREST antibodies was blotted against the 130-kDa complex. Monospecific anti-80-kDa antibody recognized the 130-kDa complex. The affinity-purified antibodies against the 18-, 22-, and 52-kDa polypeptides did not recognize the 130-kDa N_3-Tb/kinetochore protein complex (not shown).

polypeptides did not react with the 130-kDa complex, demonstrating that these proteins were not components of the 130-kDa N_3-Tb/kinetochore protein complex (not shown). The results of these experiments suggest that the 80-kDa CREST antigen is either the microtubule-binding component of the kinetochore in the in vitro system used or directly contiguous to the microtubule-binding region.

In some samples, the 130-kDa complex formed between the N_3-Tb and the 80-kDa kinetochore protein actually appeared as two or more closely migrating bands on immunoblots (see Figure 5). This was most probably due to UV-induced photobreakdown of the tubulin subunit (data not shown), although UV-induced photobreakdown of the 80-kDa kinetochore

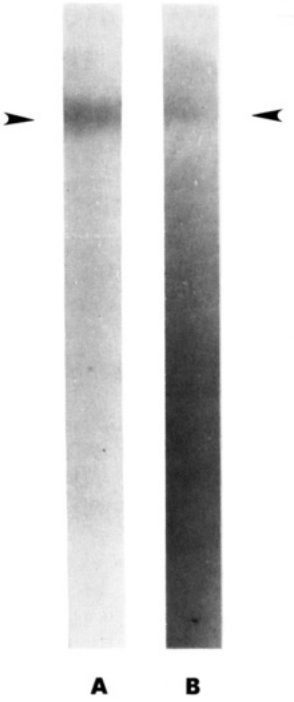

FIGURE 7: Binding of N_3-Tb to the 80-kDa protein could be competitively inhibited by unlabeled tubulin. Isolated CHO chromosomes were incubated with either N_3-Tb or N_3-Tb and unlabeled tubulin at a ratio of 9 parts tubulin to 1 part N_3-Tb. Each sample was UV-irradiated, solubilized, and subjected to antitubulin affinity chromatography. The relative amount of 130-kDa complex formed in each sample was determined by immunoblot analysis using scleroderma CREST serum. (Lane A) 130-kDa complex isolated after incubation with N_3-Tb only. (Lane B) 130-kDa complex isolated after incubation with tubulin and N_3-Tb at a ratio of 9 parts tubulin to 1 part N_3-Tb. The amount of 130-kDa complex formed was greatly reduced in sample B.

protein has not been completely eliminated.

The interaction of N_3-Tb with the 80-kDa CREST antigen could be competitively inhibited by unlabeled 6S tubulin. Isolated CHO chromosomes were incubated with a mixture of 9 parts tubulin to 1 part N_3-Tb and then UV-irradiated. The 130-kDa complex that formed during this treatment was purified by antitubulin affinity chromatography and analyzed by immunoblotting using CREST serum. The amount of 130-kDa complex formed during the co-incubation of CHO chromosomes with tubulin and N_3-Tb was greatly reduced when compared to a control sample that consisted of an equal number of chromosomes that were incubated with N_3-Tb only (Figure 7). No 130-kDa complex could be identified on blots when chromosomes were incubated with both tubulin and N_3-Tb at a ratio of 250 parts 6S tubulin to 1 part N_3-Tb (data not shown).

The cross-linking of N_3-Tb to the 80-kDa kinetochore protein could not be inhibited by CREST serum. Purified CHO chromosomes were pretreated for 30 min at 4 °C with CREST serum, rinsed, and then resuspended in N_3-Tb and incubated under microtubule assembly conditions. The preparation was photolyzed, and the proteins were solubilized and subjected to antitubulin affinity chromatography. The eluted proteins were lyophilized, run on SDS gels, and subsequently immunoblotted with antikinetochore CREST serum. The pretreatment of chromosomes with CREST serum did not inhibit the formation of the 130-kDa complex, and preparations of chromosomes that had been preincubated with CREST antibodies formed approximately equivalent amounts of 130-kDa complex as control chromosome samples that were not treated with the scleroderma serum (data not shown). These results suggest that the epitopes recognized by the CREST antibodies used in these experiments are not the regions of the 80-kDa protein that interact with N_3-Tb.

Discussion

Azido groups have been efficiently coupled to proteins, nucleotides, and sugars and then subsequently used to identify either receptors or binding proteins for the derivatized ligands. In this paper, we describe an assembly-competent N_3-Tb derivative that was synthesized from phosphocellulose-purified tubulin and NHS-ASA. N_3-Tb was characterized both by its ability to interact with bovine brain τ and an 80-kDa protein from the mammalian kinetochore and by its inability to be photochemically cross-linked to other N_3-Tb molecules. The failure of N_3-Tb molecules to be cross-linked to one another is obviously an artifact, as tubulin monomers are certainly nearest neighbors in assembled microtubules. However, this artifact can be readily explained. By their very nature, cross-linking compounds will only form covalent bonds between neighboring proteins if the amino acid side chains that the cross-linking reagents interact with are in the proper molecular orientation to allow for cross-linking to occur. Therefore, the inability of N_3-Tb to be photochemically coupled to other N_3-Tb molecules suggests that the amino acids that are important in the interaction of tubulin monomers during the assembly of microtubules are not the same amino acids that NHS-ASA couples to during the synthesis of N_3-Tb. As a result, N_3-Tb monomers, as well as α- and β-subunits of the same N_3-Tb molecule, are not effectively cross-linked to other N_3-Tb molecules.

The inability of the N_3-Tb derivative synthesized by using NHS-ASA to photochemically cross-link to other N_3-Tb molecules does not preclude the possibility of producing an N_3-Tb derivative that will have the ability to cross-link to other N_3-Tb subunits. The *N*-hydroxysuccinimide esters, such as NHS-ASA, react specifically with amine functional groups. There are several photoactivatable cross-linkers commercially available that react with functional groups other than primary and secondary amines. These cross-linking reagents might be useful for synthesizing an N_3-Tb that will have the ability to cross-link to other N_3-Tb monomers. Such an N_3-Tb derivative might be useful for studying the interactions that occur between tubulin molecules during microtubule assembly.

N_3-Tb was able to form cross-linked complexes with τ, a known microtubule-binding protein. As Figure 3 demonstrated, N_3-Tb and τ formed two different sets of complexes upon UV irradiation. The lower molecular weight complex was in the molecular weight range of a covalently bonded complex that would have been formed between either an α- or β-tubulin subunit and a τ polypeptide. The larger complex was in the molecular weight range of a complex that would have formed between either a tubulin subunit and two τ polypeptides or two tubulin subunits and one τ molecule. If the latter of these two possibilities proves to be the case, it seems unlikely that the two tubulin subunits would have been derived from the same tubulin monomer since we have demonstrated that the α- and β-subunits of tubulin do not cross-link to one another. A more likely explanation would be that the τ polypeptide was cross-linked to two tubulin subunits from different tubulin monomers. This suggests that τ has more than one tubulin-binding site per molecule. Experiments are presently under way using monoclonal antibodies that are specific for either the α- and β-subunits of tubulin that will allow both for the identification of which tubulin subunit binds to τ and for the complete characterization of the higher mo-

lecular weight N_3-Tb/τ complex.

When N_3-Tb was photochemically coupled to CHO chromosomes in vitro, a 130-kDa complex was formed. Upon further analysis, it was demonstrated that the 130-kDa complex was composed of an α- or β-tubulin subunit and an 80-kDa kinetochore protein. In an earlier paper, we had shown that a high molecular weight complex was formed when tubulin was cross-linked to tubulin by using Lomant's reagent (Balczon & Brinkley, 1987). In addition to tubulin, proteins of M_r of 24000, 80000, and 110000 were identified in that complex. Because of the nature of the cross-linker used in the previous study, it was not possible to state which of the proteins was the tubulin-binding component of the kinetochore. To further investigate the binding of tubulin to kinetochore proteins in vitro, the N_3-Tb derivative was photochemically coupled to CHO chromosomes, and a 130-kDa N_3-Tb/kinetochore protein complex was formed (Figure 4). Immunoblot analysis demonstrated that the 130-kDa complex was composed of a tubulin subunit and an 80-kDa kinetochore protein (Figures 5 and 6). These results strongly suggest that the 80-kDa kinetochore protein is the microtubule-binding component of the mammalian kinetochore in the in vitro system used, although the possibility that the 80-kDa protein is directly adjacent to the microtubule-binding site while some other kinetochore protein actually attaches to microtubules cannot be completely eliminated from these experiments.

In summary, an N_3-Tb derivative was synthesized and photochemically coupled to τ and an 80-kDa kinetochore protein. N_3-Tb should be very useful for studying the interaction of tubulin with various microtubule-binding proteins.

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