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Direct photoaffinity labeling of tubulin with colchicine

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ABSTRACT **Ultraviolet irradiation of the [^{3}H]colchicine–tubulin complex leads to direct photolabeling of tubulin with low but practicable efficiency. The bulk (70% to >90%) of the labeling occurs on β-tubulin and appears early after irradiation, whereas α-tubulin is labeled later. The labeling ratio of β-tubulin to α-tubulin (β/α ratio) is reduced by prolonged incubation, prolonged irradiation, urea, high ionic strength, the use of aged tubulin, dilution of tubulin, or large concentrations of colchicine or podophyllotoxin. Glycerol increases the β/α ratio. Limited data with [^{3}H]podophyllotoxin show that it covalently bound with a similar β/α distribution. Vinblastine, on the other hand, exhibits preferential attachment to α-tubulin. The possibilities that colchicine binds at the interface between α-tubulin and β-tubulin, that the drug spans this interface, and that both subunits may contribute to the binding site are suggested.**

Despite the fact that colchicine has been used as an antimicrotubule agent for many years, there is no unanimity regarding the location of the high-affinity binding site for the drug in the tubulin dimer, which is formed by the noncovalent association of the similar but not identical α and β monomers. Several studies have assigned the site to the α-subunit, but uncertainties exist regarding the specificity of the reactions used. Thus, *N*-bromoacetyldesacetylcolchicine showed nonspecific alkylation (1), photoaffinity labels used long spacer arms (2, 3), and studies with limited proteolysis could have been affected by rearrangements during proteolysis in the damaged protein (4). Colchicine binding to a site on β-tubulin has been proposed on the basis of indirect experiments dealing with the reactivity of cysteine residues in β-tubulin (5) and by findings that most tubulin mutations that confer colchicine resistance occur in β-tubulin genes (6–8).

The excitation maximum of colchicine occurs at a higher wavelength than that of the tryptophan residues of tubulin; hence, direct photolabeling of tubulin with colchicine, without irradiating the protein, appeared to be feasible. However, stoichiometric covalent binding would not be expected for such a reaction because the efficiency of direct photolabeling tends to be <25% (9) because of the short colchicine fluorescence lifetime (of 1.14 ns) (10) with little intersystem crossing to the triplet state or long lifetimes (11), and because of the powerfully competing photoisomerization reaction to form lumicolchicines from excited-state colchicine, which causes dissociation of the ligand (12–14). Nevertheless, such a reaction might be less subject to the specificity problems noted above and thus increases the probability that colchicine will cross-link to the "correct" site. The following study explores the conditions for the direct photolabeling reaction, the localization of the covalently bound colchicine, and the factors influencing the distribution of the drug on tubulin. A portion of this material has been presented (15).

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MATERIALS AND METHODS

Tubulin was prepared from rat brain by cycling and phosphocellulose chromatography (16) or with 1 M sodium glutamate (17) and was >98% pure. Several preparations (courtesy of Dan L. Sackett of this laboratory) were used. All preparations were drop frozen and stored in liquid nitrogen. [*ring-C-methoxy*-^{3}H]Colchicine was from Amersham (4.2 Ci/mmol; 1 Ci = 37 GBq) or New England Nuclear (25.7 Ci/mmol) and was diluted in ethanol, divided into siliconized Eppendorf tubes, and dried under vacuum. The tubulin–colchicine complex was formed by incubation at 37°C for 30–60 min in the dark and in the absence of added GTP.

After incubation the tubulin–colchicine complex was irradiated at 4°C with a high-pressure mercury lamp as described (18), at a lamp output of 88 ± 2 W. Samples were irradiated under 2 cm of a 20% $CuSO_4 \cdot 5H_2O$ solution (19). This provided <1% transmission below 305 nm, 50% transmission at 322 nm, and 97% transmission at 353 nm, the absorption maximum of colchicine. In the geometry of the system, this provided ≈35 mW/cm^2 to the sample (measured with a Newport 815 power meter calibrated to 404 nm and by using a 3-OD unit filter), or about 2.4 mW to the surface of the 20-μl sample. In addition, the $CuSO_4$ solution was an excellent red and infrared filter above 570 nm, thus reducing evaporative losses from the sample. A Corning 54 filter gave higher covalent isotope yields, but the transmitted light overlapped the protein spectrum and was not used. In general, better radioactive yields were obtained at high tubulin-to-colchicine ratios, presumably because of an improved rate of covalent binding with respect to lumicolchicine formation.

Irradiated samples were boiled for 2 min with an SDS/mercaptoethanol loading solution and electrophoresed on SDS/8% polyacrylamide gels at 20 mA until the dye was expelled plus 15 min to enhance monomer separation (20). After staining and destaining, half the gel was washed in water, photographed, impregnated with 1 M sodium salicylate enhancer for 5–15 hr, dried, and exposed for radioautography. The other, duplicate half of the gel was washed with water for ≈30 min, and ≈7-mm bands were then cut from each lane, dissolved in 300 μl of 30% H_2O_2 at 55°C for 3–5 hr, mixed with 10 ml of scintillation fluid (Packard UltimaGold), allowed to stand at 4°C for 12 hr to reduce chemiluminescence, and assayed for radioactivity for 10 min at a 2.0- to 18.6-keV (1 eV = 1.602×10^{-19} J) window. Under these conditions, the radioactive yield in the monomer bands was 2–5%.

Direct photolabeling with [^{3}H]podophyllotoxin was carried out as above except that, because of the excitation maximum near 290 nm, a 2-cm pathlength quartz cell containing 0.3% bovine serum albumin in 0.9% NaCl was used as filter, which still permitted red-edge excitation of the ligand. We thank Dan L. Sackett for this suggestion. The [^{3}H]podophyllotoxin

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was generously provided by M. Flavin of the National Heart and Lung Institute. It was checked for purity as before (21) and repurified by TLC on flexible silica plates.

[^{3}H]Vinblastine (Amersham) was preincubated as above and irradiated under a 2-cm quartz cell containing 0.0123 M potassium hydrogen phthalate as filter. Subsequent treatment was as for colchicine.

RESULTS

Binding of colchicine to tubulin at 37°C was a necessary precondition for covalent photolabeling of tubulin. As shown in Fig. 1 *C* and *D*, with increasing incubation times (and a fixed 5-min irradiation time), there was a gradual increase in covalent label, and this was largely confined to β-tubulin until preincubation times exceeded 15 min, at which point significant labeling of α-tubulin was also seen. Without incubation, the amount of label was invariably small but not zero; this may be due, in part, to short periods of room-light exposure during handling. The radioautographs (Fig. 1*C Right*) provide clear evidence of the late appearance of labeling in the α monomer, whereas the protein stain (Fig. 1*C Left*) shows little variation with α- or β-tubulin with time of incubation. Most subsequent experiments were carried out with 30- to 60-min incubations at 37°C in the dark. Results using tubulin from bovine brain were similar, as were results using microtubule protein, but these data are not detailed here. No covalent binding of colchicine occurred with human transferrin, carbonic anhydrase, rabbit muscle lactic dehydrogenase, lysozyme, or pancreatic ribonuclease A. Various preparations of bovine serum albumin from fraction V to recrystallized protein incorporated from 5.4% to 11% as much ^{3}H as did β-tubulin incubated and irradiated under identical conditions. This is not surprising in view of the known low-affinity binding that has been reported (22).

When the time of irradiation was studied at a fixed preincubation period (45 min, 37°C), the number of total colchicine dpm that was covalently bound to tubulin increased progressively with a more rapid initial phase lasting several minutes (Fig. 1*B*). In other experiments, even 15-sec irradiation times caused highly significant increases over unirradiated samples, which generally exhibited only 2–3 times the background dpm (data not shown). Similar results could be obtained by precipitation of the tubulin with 10% cold trichloracetic acid, but removal of unbound colchicine was less satisfactory than with electrophoresis. The radioautographs in Fig. 1*A Right* again reveal that the labeled colchicine first appeared in β-tubulin alone and gradually appeared in α-tubulin after 2–5 min of irradiation at 88-W lamp power output. Progressive formation of a faint covalent dimer band (labeled D) can be seen in both Coomassie-stained gels of Fig. 1 (Fig. 1 *A* and *C Right*). The reproducibility for replicate electrophoresis, band cutting, and counting procedures had standard errors of 4.2% and 18.2% for β and α bands, respectively ($n = 6$). The labeling ratio of β-tubulin to α-tubulin (β/α ratio) had a standard error of 6.2%. Because different numbers of dpm were applied in some experiments, the β/α ratio was used to reduce errors resulting from such experimental variations. Thus, in the above figure, during the early incubation or irradiation periods, the β/α ratios ranged from 3 to 10 and decreased at later times as the amount of ^{3}H in the α-tubulin monomer increased. Higher values could be attained if the free colchicine was first removed from the bound colchicine in a Sephadex G-10 spin column and then irradiated. Under these conditions, β/α ratios as high as 13–14

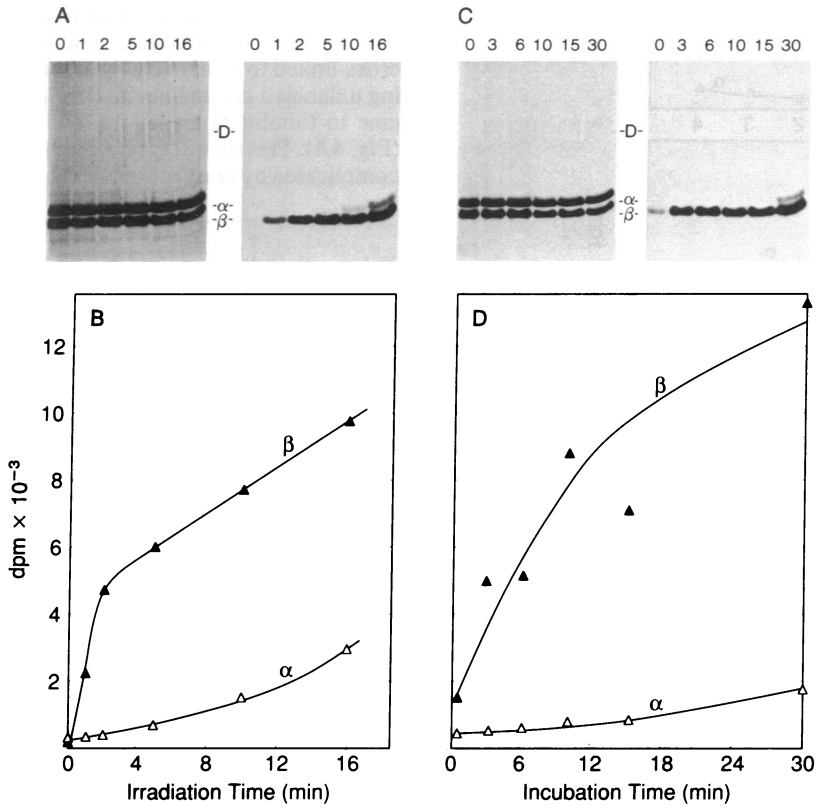

FIG. 1. Effect of incubation and irradiation times on photolabeling of tubulin with [^{3}H]colchicine. (*A* and *B*) Thirty-two micromolar tubulin was incubated with 2.4 μM colchicine for 45 min and irradiated at 87 W for various times. (*A Left*) Coomassie-stained gel. (*A Right*) Radioautograph of the gel in *A Left*. (*C* and *D*) Twenty-five micromolar rat brain tubulin and 2.1 μM colchicine were incubated at 37°C for various times and were then irradiated at 88 W for 5 min. (*C Left*) Coomassie-stained gel. (*C Right*) Radioautograph of the gel in *C Left*. D, covalent tubulin dimer.

could be obtained; however, it was not necessary to use this separation routinely.

The well-known lability of tubulin suggested that the gradual covalent labeling of α-tubulin with colchicine and net loss of colchicine from β-tubulin coincided with minimal changes in tubulin. (Whether this implies that isomerization to lumicolchicine and consequent debinding of the label competes more successfully with covalent bond formation in certain conditions and not others remains to be determined.) We, therefore, expected to see an increase in the β/α ratio when glycerol (a well-known tubulin stabilizer) was the solvent, and this turned out to be the case as shown in Fig. 2*A*. In some experiments, β/α ratios in excess of 20 could be achieved with glycerol. It is clear that the dpm in α-tubulin remained constant and that the increase in the β/α ratio is due almost entirely to increased labeling of β-tubulin. Taxol, a microtubule stabilizer and assembly promoter, did not enhance the β/α ratio in concentrations of 3–300 μM and appears to act differently (data not shown). On the other hand, destabilizing agents, such as urea or high ionic strength, caused marked reductions in the β/α ratio, sometimes to values <1.0 (Fig. 2 *B* and *C*, respectively). With urea, β-tubulin labeling showed a gradual decline with increasing concentration, approaching β/α values of 1.0 or less (Fig. 2*B*). The initial rise was not seen in all experiments. Labeling of α-tubulin increased up to ≈2.5 M urea and then fell off. By contrast, the fall in the β/α ratio after incubation with NaCl was due largely to a rise in the labeling of α-tubulin (Fig. 2*C*). It seems reasonable to conclude that conformational manipulations of tubulin influence the distribution of covalently bound colchicine between the β and α subunits.

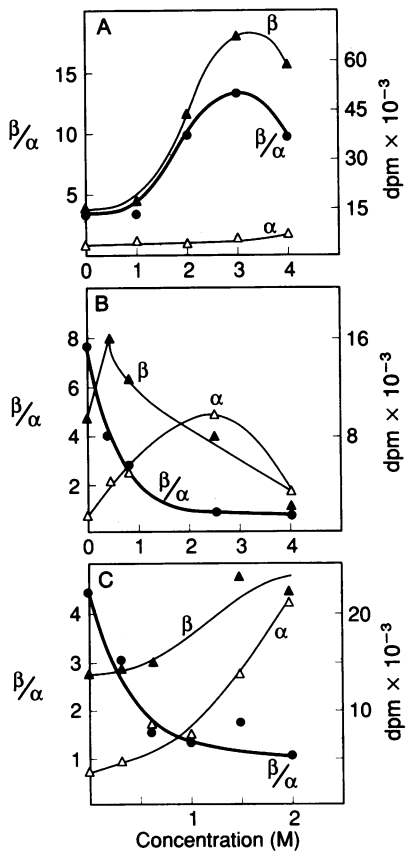

FIG. 2. Changes in the [^{3}H]colchicine photolabeling distribution between α- and β-tubulin produced by agents that alter protein conformation. All samples contained 49 μM rat brain tubulin and were irradiated at 92 W for 5 min. (*A*) One to 4 M glycerol was present during a 60-min incubation with 5.0 μM [^{3}H]colchicine. (*B*) Zero to 4 M fresh urea was present during a 45-min incubation with 4.4 μM [^{3}H]colchicine. (*C*) Three-tenths to 2.0 M NaCl was present during a 45-min incubation with 4.0 μM [^{3}H]colchicine. ●, β/α ratio; ▲, dpm in β-tubulin; △, dpm in α-tubulin.

Additional evidence that α-tubulin labeling may result from structural modifications in the dimer comes from the observations that older preparations of tubulin showed lower β/α ratios than fresh preparations, despite storage in liquid nitrogen. Aging might thus cause structural changes in tubulin leading to "exposure" of the α-subunit binding domain. To test this hypothesis, rat brain tubulin was "aged" for up to 6 hr at 25°C. Samples were then incubated at 37°C for 30 min with [^{3}H]colchicine, irradiated, and electrophoresed as before. It is apparent from Fig. 3 that the β/α ratio of covalent label decreased by more than half over the 6-hr aging period; this occurs largely through loss of β-tubulin label.

Sackett *et al.* (23) have recently presented evidence that the fluorescent dye Nile red has its high-affinity binding site at or near a hydrophobic contact surface between the α and β monomers. Colchicine did not interfere with Nile red binding. Because the change in covalent [^{3}H]colchicine distribution upon irradiation, urea treatment, etc. suggests that the bound colchicine may have access to both subunits, we considered the possibility that colchicine might bind to another portion of that hydrophobic subunit interface. Because the Nile red site could be abolished by separation of the α and β subunits by dilution of the dimer (K_d < 1 μM) (23), we attempted to influence the β/α ratio of covalent labeling by dilution of tubulin. Because of the low efficiency of cross-linking, we were limited in the degree of dilution that permitted analysis. It was, nevertheless, possible to show a decreasing β/α ratio upon dilution of tubulin, with a β/α ratio of 7.8 at 10 μM rat brain tubulin to a ratio of 1.4 at 0.3 μM tubulin. Thus, preferential and total labeling of β-tubulin is lost upon dilution. This suggests that colchicine may span both subunits.

A number of attempts were made to saturate the photolabeling process with excess colchicine. The number of dpm cross-linked to tubulin was markedly decreased with increasing unlabeled colchicine. As the initial molar ratio of colchicine to tubulin exceeded 10, the β/α ratio approached 1.0 (Fig. 4*A*). Precise interpretation of these results is, however, complicated by two factors: (*i*) the internal filter effect and (*ii*)

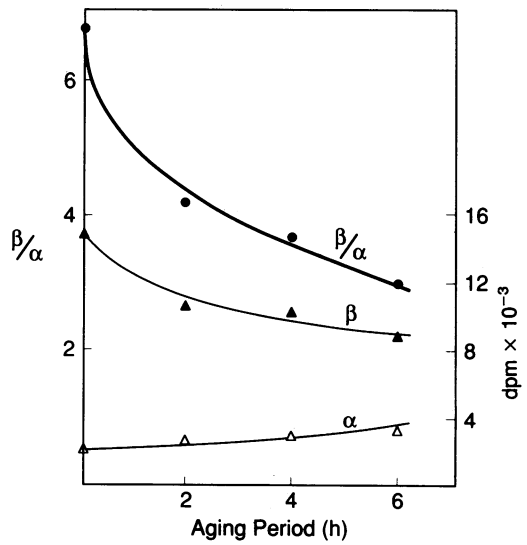

FIG. 3. Effect of aging of tubulin on photolabeling with [^{3}H]colchicine. Rat brain tubulin was kept at 25°C. Samples were withdrawn at 0–6 hr and incubated at 37°C for 30 min with 4.0 μM [^{3}H]colchicine. Samples were cooled and irradiated at 88-W lamp power for 5 min, separated on SDS/8% polyacrylamide gels, and counted as described in *Materials and Methods*. ●, β/α ratio; ▲, dpm in β-tubulin; △, dpm in α-tubulin.

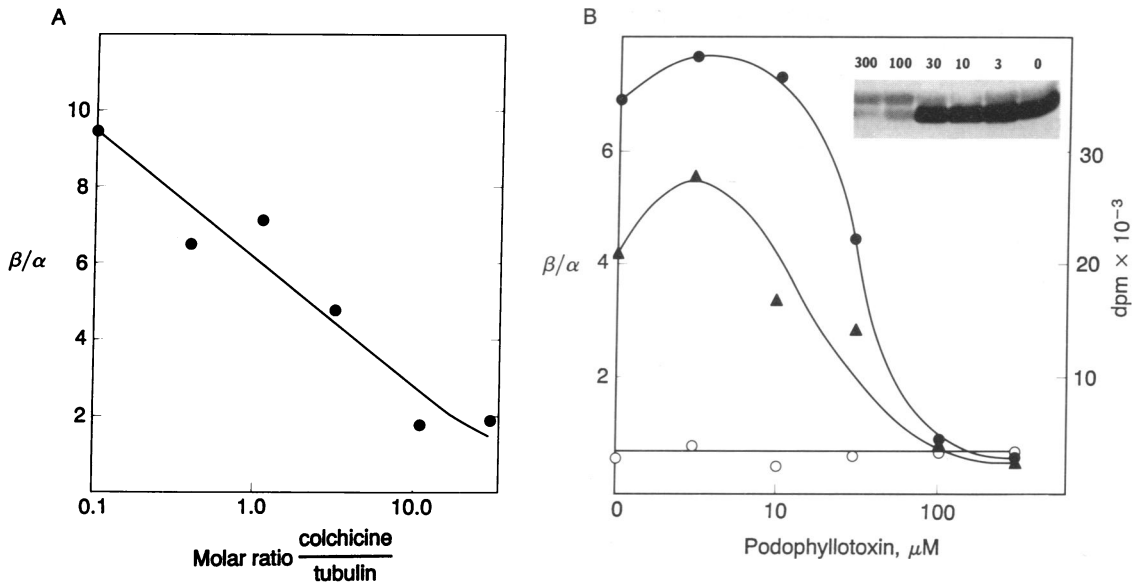

FIG. 4. Competition of [^{3}H]colchicine binding and photolabeling by colchicine and podophyllotoxin. (*A*) Forty-nine micromolar pure rat brain tubulin was incubated for 1 hr at 37°C with 4.9 μM [^{3}H]colchicine and increasing concentrations of unlabeled colchicine to achieve molar ratios of 0.1 to 30. Samples were irradiated at 92 W. (*B*) Forty-nine micromolar tubulin was incubated with 4.9 μM [^{3}H]colchicine for 1 hr at 37°C in the presence of increasing concentrations of podophyllotoxin. Irradiation was for 5 min at 92 W. ▲, dpm in β-tubulin; ○, dpm in α-tubulin; ●, β/α ratio. (*Inset*) Radioautogram from which data were obtained.

photoisomerization to lumicolchicines, which may be different for β- and α-tubulin-bound colchicine and which would rapidly lower the molar ratio to well below zero time. To circumvent this, we used podophyllotoxin, which binds, in part, to the same site (21) but does not absorb at the excitation wavelengths used here. As can be seen in Fig. 4*B*, inhibition of binding with podophyllotoxin decreased the yield of covalently bound colchicine, with 50% inhibition occurring at 20 μM. More importantly, there was a marked diminution of photolabeling of the β subunit with little change or an increase in α-subunit labeling since the binding of colchicine and podophyllotoxin is competitive (21). The sudden decrease of the β/α ratio as podophyllotoxin concentrations exceeded 30 μM is clearly demonstrated in the radioautogram (Fig. 4 *Inset*). Attempts to explore the β-tubulin specificity by the use of single-ring (A-ring) analogues, such as 3,4,5-trimethoxy congeners of benzoic acid, benzaldehyde, phenylacetic acid, or phenylpropionic acid, were unsuccessful at concentrations as high as 3 mM. In addition, we investigated several nontropolonic C-ring analogues [on the basis of the high activity of combretastatin (24)] such as eugenol and isoeugenol but were not able to achieve preferential inhibition of the α- or β-subunit labeling of tubulin with these compounds.

As an alternative approach we carried out direct photolabeling with [^{3}H]podophyllotoxin. It was anticipated that the results might be less specific than for colchicine because the absorption spectra of tubulin (λ_{max} = 279 nm) and podophyllotoxin (λ_{max} = 290 nm) show considerable overlap, and it would not be possible to shield the protein completely. Nevertheless, as shown in Fig. 5, there was a time (of irradation)-dependent increase in covalent labeling, and the β/α ratio was 2–3 (data not shown). This is consistent with the marked reduction in the β/α ratio illustrated in Fig. 4 and strengthens the case that the cross-linking of [^{3}H]colchicine occurs at its specific binding site. By contrast, [^{3}H]vinblastine, which binds to an independent and different site on tubulin, showed a distinct preference for cross-linking to the α subunit with α/β of ≈3 and contrasts markedly with colchicine cross-linking as shown in Fig. 5*B*.

DISCUSSION

The original impetus for this study was the possibility that exclusive labeling of α-tubulin by photosensitive colchicine derivatives (2) might have resulted from the use of large spacer groups. Subsequent use of shorter spacers yielded β-tubulin labeling as well (3); this was, however, ascribed to binding of colchicine to a second, lower affinity binding site. In the present study we found, by contrast, that β-tubulin labeling is strongly favored and that when the molar ratio of colchicine to tubulin was increased from 0.1 to 30, the β/α labeling ratio *decreased*. This renders unlikely any explanations based on filling a low-affinity site of β-tubulin, although it would be consistent with such a site on α-tubulin.

The possibility that an activated form of one of the lumicolchicines is an intermediate in covalent linkage formation should be considered. However, the photochemistry of colchicine is extremely complex (25), and no mechanism can be offered at present.

The present data are consistent with three models. (*i*) The drug binds only to β-tubulin. The site on α-tubulin is close and

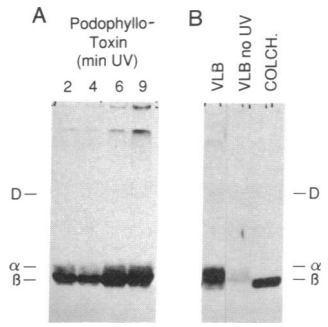

FIG. 5. (*A*) Forty-nine micromolar rat brain tubulin was incubated for 10 min with 290 μM [^{3}H]podophyllotoxin and irradiated for 2–9 min at 92 W under a 2-cm filter of bovine serum albumin (0.5 mg/ml) in 0.15 M NaCl. (*B*) Thirty micromolar rat brain tubulin was incubated at 37°C for 30 min with 64 μM [^{3}H]vinblastine (VLB) and irradiated for 7 min at 92 W under 2 cm of 0.0123 M potassium hydrogen phthalate. Colchicine (COLCH.) was treated as in Fig. 4. D, covalent tubulin dimer.

when bulky substituents are used, or, under mild denaturing conditions, α-tubulin becomes labeled. (*ii*) A certain degree of lower affinity labeling occurs on the same α-monomer of the same dimer or on an α-monomer of an isomeric tubulin dimer that prefers labeling on this monomer. (*iii*) Colchicine spans the α/β dimer interface and the distribution of colchicine label between the α- and β-tubulin monomers is influenced by bulky substituents on the B ring as well as by structural alterations in the dimer. We favor the third mechanism in which each monomer contributes an actual or potential binding domain to the site. The reasons for this are as follows. (*i*) Covalent labeling is predominantly on β-tubulin, especially at short time intervals of ultraviolet irradiation, and the diminution of the β-tubulin preference under a variety of conditions that may "alter" the structure of the tubulin dimer (26)—such as aging of the protein, long incubation or irradiation times, treatment with urea, or increased ionic strength. In a number of experiments, loss of β-tubulin labeling appeared as gains in α-tubulin labeling, although this was rarely stoichiometric (e.g., Figs. 2 and 3). Hence, there must be losses of [^{3}H]colchicine from the β-tubulin binding domain following these manipulations that occur in addition to any transfer of the label from β-tubulin to the α-tubulin binding domain. (*ii*) Colchicine photosensitizes tubulin to covalent dimerization by ultraviolet light. This can be seen in Fig. 1 (band labeled D) but can be enhanced by changes in the irradiation conditions (to be detailed elsewhere). (*iii*) Colchicine causes an increase in dimer association (2- to 3-fold decrease in K_d) (27). It is of considerable interest that podophyllotoxin, which shares only the A-ring binding domain with colchicine (21), does not cause such a change in the dimer association despite equivalent ligand affinity (27). Presumably, the lignan moiety of podophyllotoxin does not span the α–β interface effectively. (*iv*) Finally, the findings that colchicine binds primarily to β-tubulin and that occupancy of this site leads to reduced accessibility of the carboxyl terminus of α-tubulin to a specific peptide antibody (ref. 28; J. L. Morgan, personal communication) can be rationalized if the binding site is located at the monomer/monomer interface.

Several authors have previously postulated that the colchicine binding site on the tubulin dimer receives contributions from both the α and β subunits (5, 24, 29). Although no direct data supporting this proposal were supplied in those studies, the results presented here, as well as the promotion of covalent α-β cross-links by colchicine (unpublished results), provide presumptive evidence for such a proposal. They also demonstrate the greater contribution of the β-tubulin domain to the native binding site and emphasize the greater lability of the binding domains on β-tubulin than the α-tubulin domain. Similar conclusions have been reached for the α-γ and α-δ subunit contacts of the nicotinic receptor by direct photoaffinity labeling with *d*-tubocurarine (30).

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