Molecular Mechanism of Colchicine Action: Induced Local Unfolding of β-Tubulin

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ABSTRACT: Colchicine, the classic antimitotic poison, disrupts cell division by preventing proper assembly of microtubules in the mitotic spindle. Colchicine is known to act by binding to tubulin, the heterodimeric subunit of microtubules. How this binding to tubulin changes the structure of the protein and results in polymerization poisoning has not been characterized. The structural locus of spectroscopically detected conformational changes induced by colchicine is unknown. We report here that colchicine induces the unfolding of a small region in the carboxyl-terminal region of β-tubulin, around Arg-390. This unfolding is detected by proteolysis with trypsin and chymotrypsin. Chymotrypsin cleaves this region after Phe-389, and trypsin cleaves after Lys-392. The unfolded region appears to be the carboxyl end of an amphipathic helix in the absence of colchicine, and we propose that this unfolding prevents contacts necessary for assembly. Our results suggest that β-tubulin is exposed on the growing end of the microtubule, which provides a mechanism for coupling GTP hydrolysis to polymerization.

Colchicine is one of the oldest drugs still in use. It was known in herbal form since well before the time of Dioscorides (Eigsti & Dustin, 1955). Purified colchicine is still in use today, to produce polyploids and amphidiploids in plant breeding, to induce mitotic arrest in animal cell cytogenetics, and to treat gout and familial Mediterranean fever (Garcia-Gonzales & Weisman, 1992; Malkinson, 1982; Moreland & Ball, 1991; Zemer et al., 1993). In toxic doses, colchicine causes metaphase arrest due to disruption of the normal functioning of the mitotic spindle. Spindle function is disrupted due to interference with microtubule polymerization. This interference is mediated by binding of colchicine to the microtubule subunit protein tubulin. It was in fact tracing this binding with radioactive colchicine that led to the original isolation of tubulin (Borisy & Taylor, 1967; Wilson & Friedkin, 1967). Under most conditions, the tubulin–colchicine complex will not polymerize, but can nonetheless add to the growing end of a microtubule, poisoning its further growth. This explains why colchicine toxicity is observed at concentrations of colchicine much lower than the concentration of tubulin (Margolis & Wilson, 1977; Taylor, 1965). Yet how colchicine binding changes the structure of tubulin is not known.

The tubulin molecule is a heterodimer, composed of two similar but nonidentical subunits, α- and β-tubulin, each about 50 000 Da in mass. Chymotrypsin cleaves β-tubulin at Tyr-281, producing two fragments which contain roughly the amino-terminal two-thirds, termed βN (sequence mass 31 kDa), and the carboxyl-terminal one-third, termed βC (see Figure 1). Similarly, trypsin cleaves native tubulin at Arg-339 (α), cutting α-tubulin into two fragments: roughly the amino-terminal two-thirds (αN, sequence mass 37.6 kDa) and the carboxyl-terminal one-third, αC (Sackett & Wolff, 1986). Both αN and βN contain a protease-sensitive site dividing two compact regions (delaVina et al., 1988), but a two-domain model is used here for simplicity. Cross-linking and protease studies with dimer vs microtubule indicate that αN and βC are in contact in the isolated dimer, while βN and αC touch in the interdimer contact formed in the polymer (Kirchner & Mandelkow, 1985). One colchicine molecule binds per tubulin dimer, primarily to β, though near to α and possibly at the α–β contact (Floyd et al., 1989; Lacey, 1988; Lin et al., 1989; Wolff et al., 1991). Colchicine binding prevents the formation of an intra-β-chain cross-link between reactive sulfhydryls, indicating a shift in tubulin conformation (Luduena & Roach, 1991). Spectroscopic evidence also indicates that conformational changes occur in tubulin after binding colchicine (Andreu & Timasheff, 1982). Because the nature and structural locus of these changes are unknown, the structural basis of colchicine's action has remained elusive.

We report here that colchicine induces a local unfolding of β-tubulin near the carboxyl end of the protein. The unfolded region can be cleaved by trypsin or chymotrypsin.

MATERIALS AND METHODS

Materials. Trypsin (TPCK-treated) and chymotrypsin were obtained from Worthington. Phenylmethanesulfonyl fluoride and leupeptin were obtained from Sigma Chemical Co., St. Louis, MO. For electrophoresis, the detergent used was sodium lauryl sulfate from Sigma Chemical Co. (Best et al., 1981; Sackett & Wolff, 1986).

Tubulin. Microtubule protein was purified from rat brain by cycles of temperature-dependent polymerization and depolymerization. Tubulin was purified from this by selective polymerization in sodium piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)/dimethyl sulfoxide (Pipes/DMSO) followed by glutamate polymerization (Hamel & Lin, 1984; Himes et al., 1977; Sackett et al., 1991). Purified tubulin was stored, drop-frozen at 25 g/L in Mes assembly buffer (MAB),[1] in liquid nitrogen until use.

Proteolytic Digestion. For digestion, tubulin was diluted to 10 μM (1 g/L) in MAB and incubated for 45 min at 30

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[1] Abbreviations: SDS, sodium dodecyl sulfate; PVDF, poly(vinylidine difluoride); MAB, Mes assembly buffer [0.1 M 2-(*N*-morpholino)-ethanesulfonic acid, 0.1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, and 0.1 mM $MgCl_2$].

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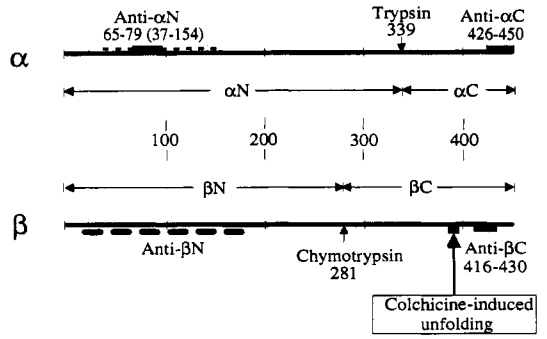

FIGURE 1: Schematic diagram of α- and β-tubulin. Residue numbers are indicated between α and β. The site of trypsin cleavage of α is indicated above the α line, and the extent of the resulting αN and αC fragments is indicated below. Similarly, the chymotrypsin cleavage site on β is shown on β as are the resulting βN and βC fragments. The sequence regions which contain the epitopes of the antibodies specific to αN, αC, βN, and βC are indicated. Further details and references are given under Materials and Methods. The site of the colchicine-induced unfolding described here is indicated on β.

°C in the presence or absence of added drug as indicated. For studies of added nucleotide, tubulin was saturated with GTP or GDP following depletion of Mg^{2+} as described (Correia et al., 1987; Sackett & Lippoldt, 1991). Samples were then incubated for 45 min at 30 °C with 1 mM GTP or GDP or 0.1 mM colchicine or no addition as indicated. Trypsin or chymotrypsin was added to 10 mg/L, and the sample was incubated on ice for 30 min, except as indicated in the figure legends. Enzyme action was halted by addition of 0.2 mM phenylmethanesulfonyl fluoride (chymotrypsin) or 0.01 mM leupeptin (trypsin). Samples were separated by SDS gel electrophoresis (Sackett & Wolff, 1986).

Immunoblots. Tubulin was incubated with and without colchicine, digested with chymotrypsin and trypsin, and separated on SDS gels as described above. The samples were then transferred to Immobilon PVDF membranes (Millipore Corp., Bedford, MA) by semi-dry electrophoresis. Blots from replicate gels were stained with Coomassie Blue for total protein, or probed with monoclonal antibodies directed to the amino- or carboxyl-terminal domains of α- or β-tubulin. The monoclonal antibodies used were DM1A and DM1B from Amersham (Arlington Heights, IL), which were used to label αC and βC, respectively. The sequence regions that contain the epitopes for these antibodies are as follows: DM1A, α426–450; DM1B, β416–430 (Breitling & Little, 1986; Ponstingl et al., 1990). TU-01 and TU-06 from STC Diagnostics (Bethlehem, PA) were used to label αN and βN, respectively (Draber et al., 1989). The sequence region that contains the epitope for TU-01 is α37–154, principally α65–79 (Grimm et al., 1987; Ponstingl et al., 1990). The epitope for TU-06 has not been localized, but only binds to βN, and therefore must be between residues β1 and β281 (Draber et al., 1989). Antibody epitope positions are summarized in Figure 1. Bound antibody was detected with alkaline phosphatase linked second antibody (Sigma Chemical Co.) and alkaline phosphatase detection kit from Bio-Rad.

Mass Spectrometry. For mass spectrometry, the fragments were electroeluted from bands excised from SDS gel separations of reactions similar to those described above. The eluted fragments were precipitated by acetone precipitation. Five volumes of −20 °C acetone were added to the samples which were then held at −20 °C for 5 h and centrifuged for 15 min at 30000*g*. The supernatant was removed, and the sample was washed by adding 80% acetone/water at −20 °C, vortexing, and recentrifuging. The supernatant was discarded, and the sample was dissolved in 2% triethylamine in water, run on a Vydac C-4 HPLC column, and eluted with a gradient of acetonitrile/trifluoroacetic acid. The peak fractions of the eluted fragment were combined and loaded directly into a Finnigan TSQ700 triple-quadrupole mass spectrometer, with a sheath of 2-methoxyethanol. Data were collected from m/z = 400–2000. Analysis was performed at the Harvard Microchemistry Laboratory (Cambridge, MA). The masses and uncertainty were evaluated from the centers of 8–10 peaks from the m/z spectrum. Deduced masses were corrected for adduct formation during electrophoresis. Adducts formed during electrophoresis and purification were determined using the chymotryptic fragment βN (sequence mass 31 001 Da). When purified from the digest by HPLC alone, βN yielded a mass of 30 996 ± 8 Da. When purified using SDS gels and HPLC, the mass obtained was 31 220 ± 46 Da. The 220-Da mass excess may be due to residual SDS or to reaction of unpolymerized acrylamide with tubulin sulfhydryls. On the basis of the mass excess for βN, the predicted mass excess for a fragment cleaved at residue 390 is 305 Da, whether based on cysteine content (7 versus 5 in βN) or on mass available for SDS binding (43 versus 32 kDaa for βN). The resulting masses are (mean ± sd) the following: colchicine–chymotrypsin = 43 408 ± 94 Da; colchicine–trypsin = 43 778 ± 44 Da.

RESULTS

Native tubulin is cleaved by trypsin and chymotrypsin, each producing two major characteristic fragments (shown diagrammatically in Figure 1). Chymotrypsin cleaves β-tubulin, producing βN and βC (Figure 2A, lane 1). Trypsin cleaves α-tubulin, producing αN and αC (Figure 2A, lane 3). In the presence of bound colchicine, the cleavage patterns change. Chymotryptic cleavage produces a new band whereas tryptic cleavage produces a much enhanced yield of a minor band (Figure 2A, lanes 2 and 4, respectively; the new band is indicated by asterisks). In both cases, the new fragment is larger than either major fragment produced in the absence of colchicine, and thus appears on the gel between β-tubulin and either αN or βN. The tryptic fragment appears slightly larger than the chymotryptic fragment. None of the new chymotryptic fragment is observed in the absence of colchicine, whereas a small amount of the tryptic fragment is observed in the control, and is much enhanced by colchicine.

The binding of colchicine is stereochemically specific. Isocolchicine differs from colchicine only by a reversal in position of an adjacent carbonyl oxygen and a methoxy group, yet this change results in nearly a 1000-fold loss of polymerization inhibitory potency (Fitzgerald, 1976). Therefore, isocolchicine provides a useful reagent to test the specificity of an effect attributed to colchicine binding. Figure 2B presents time courses of chymotrypsin digestion of tubulin in the presence of no addition, colchicine, or isocolchicine. Clearly, at all times of digestion, production of the fragment is only induced by colchicine and not by isocolchicine.

Colchicine induces GTPase activity in tubulin, and GDP–tubulin is polymerization-incompetent (Andreu & Timasheff, 1981; David-Pfeuty et al., 1979; Lin & Hamel, 1981). Therefore the structural changes observed in Figure 2A,B might be due to colchicine-induced GDP–tubulin rather than colchicine–tubulin *per se*. Figure 2C demonstrates that the new fragment was not produced from GDP–tubulin. Preincubation of tubulin with saturating amounts of GTP or GDP prior to treatment with chymotrypsin failed to produce the colchicine-dependent fragment. The ability of colchicine to

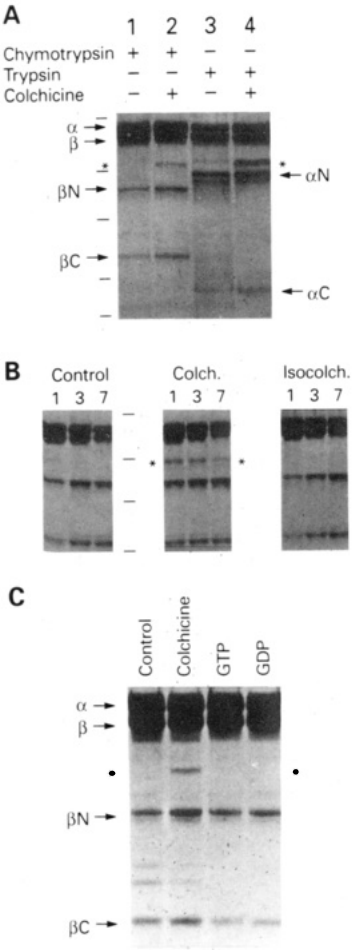

FIGURE 2: Proteolysis of tubulin reveals new (chymotrypsin) or enhanced (trypsin) cleavage due to colchicine binding. (A) Chymotrypsin cleaves β-tubulin at Tyr-281, yielding amino and carboxyl fragments βN and βC, respectively (lane 1). In the presence of bound colchicine, however, chymotrypsin yields a new band intermediate between β and βN (lane 2; the new band position is marked by an asterisk at the sides of the gel). Trypsin cleaves α-tubulin at Arg-339, yielding αN and αC, as well as several minor bands (lane 3). If colchidine is bound to tubulin before addition of trypsin, a minor band between α and αN is much enhanced (lane 4). Digestion conditions are described under Materials and Methods. All gels in panels A, B, and C are Coomassie Blue stained. (B) The specificity of the colchicine effect is demonstrated by the time course of chymotryptic digestion following preincubation of tubulin with colchicine or isocolchicine (0.1 mM). Digestion was performed at 30 °C, the same temperature at which the preincubation with or without drug addition was performed. Minutes of digestion are shown above the lanes. At no point during the time course does the colchicine-induced band (labeled with an asterisk) appear in the isocolchicine-treated sample. (C) The colchicine-induced cleavage is not due to the status of the guanine nucleotide since neither GTP- nor GDP-saturated tubulin yielded the band. Tubulin was saturated with GTP or GDP as described under Materials and Methods. The unlabeled tics indicate the position of reference proteins. From top to bottom, they are (mass in kilodaltons) bovine serum albumin (66), ovalbumin (45), carbonic anhydrase (31.5), soybean trypsin inhibitor (21.5), and lysozyme (14.4). All gels in Figure 2 were stained with Coomassie Blue.

induce production of this fragment was not affected by saturation of tubulin with either GTP or GDP (data not shown).

Immunoblot assays (Figure 3) were performed to determine from where in the tubulin heterodimer the colchicine fragments originated. The monoclonal antibodies used are specific to one of the four regions: αN, αC, βN, or βC, and epitope locations are shown in Figure 1. Colchicine-induced tryptic and chymotryptic fragments (approximate gel position is

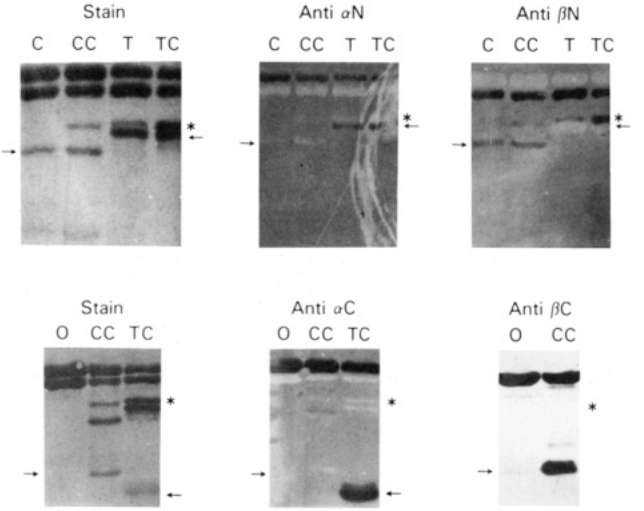

FIGURE 3: Immunoblot analysis of the colchicine-induced cleavage product limits the cleavage site to the carboxyl-terminal region of β. Lanes are labeled by treatment: 0 = no enzyme, C = chymotrypsin, CC = chymotrypsin + colchicine, T = trypsin, TC = trypsin + colchicine. The detection method is indicated: stain for total proteins or antibodies to stain specific regions. In each of the panels in the upper row, the positions of αN and βN are indicated by the arrows on the right and left sides, respectively. The positions of the colchicine-induced bands are indicated by asterisks on the right sides of the panels. The center panel demonstrates that the colchidine-induced bands do not react with anti-αN. This antibody only reacts with the expected αN bands in lanes T and TC. The right panel demonstrates that the colchicine-induced bands produced by both chymotrypsin and trypsin react with anti-βN, as does the trypsin band produced at much lower yield in the absence of colchicine (lane T). As expected, the antibody also reacts with βN in lane C and CC but not αN in lanes T and TC. The lower row, center panel, demonstrates that the colchicine bands do not react with an anti-αC antibody which does react as expected with the αC band (lane TC). The right panel shows that the colchicine bands also failed to react with an antibody to βC, with an epitope between residues 416 and 430 (Breitling & Little, 1986). The positions of αC and βC are indicated in the lower row by arrows on the right and left sides, respectively. The asterisks on the right sides of the panels indicate the positions of the colchicine-induced bands.

indicated by an asterisk in Figure 3) both reacted with a monoclonal antibody whose epitope is in βN. Neither fragment reacted with monoclonal antibodies to αN or αC. Furthermore, neither fragment reacted with an anti-βC antibody whose epitope is between residues 416 and 430 (Breitling & Little, 1986). Therefore, the bands contain only fragments from β-tubulin, and those fragments contain the amino-terminal region but have lost part of the carboxyl-terminal region of β-tubulin.

Both trypsin and chymotrypsin cleave β-tubulin in the same region (βC) following colchicine binding. In Figures 2A and 3, it can be seen that more colchicine-specific fragment is produced by trypsin than by chymotrypsin (compare Figure 2A, lanes 4 and 2, and Figure 3, lanes TC and CC). This is at least partly due to the fact that trypsin does not otherwise cleave β-tubulin whereas chymotrypsin cleaves β-tubulin at Tyr-281 in the absence and presence of colchicine. Thus, the colchicine-induced tryptic fragment can accumulate, while the chymotryptic fragment will disappear following cleavage at Tyr-281. This may be seen in Figure 2B. Note that at 7 min of digestion (at 30 °C), the colchidine-induced band has decreased in intensity relative to the 3-min point.

Knowing that the cleavage site is in the carboxyl-terminal region of β-tubulin allows the position to be determined from the sequence of β-tubulin and an accurate mass. We determined the mass of the tryptic and chymotryptic fragments in two ways: by averaging multiple SDS gel results and by

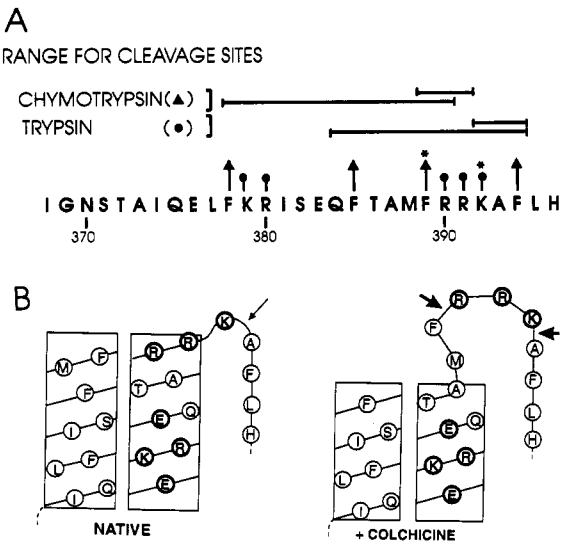

FIGURE 4: Site of cleavage was determiend by comparing the mass of the colchicine-induced fragment with the known sequence of rat β-tubulin (Ginzburg et al., 1985). (A) The fragment mass was evaluated by SDS gel electrophoresis and by electrospray mass spectrometry. For the gel analysis, multiple reactions were run on multiple gels, and the fragment was compared to nearby standards including β-tubulin (mass = 50 kDa), αN (mass = 37.6 kDa), βN (mass = 31 kDa). The results are (mean ± sd) as follows: colchicine–chymotrypsin = 42.7 ± 0.7 kDa (n = 6); colchicine–trypsin = 43.3 ● 0.7 kDa (n = 8). For mass spectrometry, fragments were eluted from SDS gels and processed as described under Materials and Methods. The resulting masses are (mean ± sd) the following: colchicine–chymotrypsin = 43 408 ± 94 Da; colchicine–trypsin = 43 778 ± 44 Da. The larger bars indicate the sequence positions which correspond to masses evaluated from gel electrophoresis (mean ● one standard deviation). The smaller bars indicate the sequence positions corresponding to the masses (± one standard deviation) determined by electrospray mass spectrometry. Potential chymotrypsin cleavage sites are indicated above the sequence by filled triangles and trypsin sites by filled circles. The trypsin site (K-392) and chymotrypsin site (F-389) consistent with the data are indicated by asterisks. (B) The same sequence region is shown on an α-helical lattice (3.6 residues/turn), opened out. In the native form, the amphipathic character of the helix is indicated by the clustering of the charged residues (in boldface) on one side. The position of weak tryptic cleavage is shown by the arrow. The presence of bound colchicine appears to unfold the end of the helix, exposing the chymotrypsin site at F-389 and enhancing cleavage at the trypsin site at K-392. These sites are indicated by arrows. The extent of unfolding may be somewhat more extensive than shown.

electrospray mass spectrometry. The ranges for the position of cleavage corresponding to the masses determined by the two methods overlap and are summarized in Figure 4A. The chymotrypsin site that is exposed by colchicine is at Phe-389, and the trypsin site that is enhanced is most likely Lys-392. Figure 4B shows this sequence displayed on an α-helical lattice, opened out to demonstrate its amphipathic character. The right side of the native helix as shown contains the charged residues (in boldface), and the other side contains the nonpolar groups. The polar side of the helix has a hydrophilic (positive)/hydrophobic (negative) index average (Hopp & Woods, 1981) of +1.92 per residue (for comparison, Arg = +3.0), and the nonpolar side average is −1.52 per residue (Val = −1.5). In the native structure (Figure 4B, left), the site of weak tryptic cleavage is indicated by the arrow. The +colchicine structure (Figure 4B, right) shows the unfolding which increases cleavage at the trypsin site and exposes a chymotrypsin site that was unexposed in the native form. Thus, colchicine unfolds a loop centered around Arg-390 which can be cut by trypsin at Lys-392 and chymotrypsin at Phe-389. This region contains the highest density of basic groups in tubulin: from residues 379–392, there are 5 basic/14 total residues. Like the homologous region of α-tubulin, this region has strong α-helical potential with the basic residues clustered on one side (as demonstrated in Figure 4B), and thus may interact with the highly acidic extreme carboxyl end of this or an adjacent molecule (Bhattacharyya et al., 1985; Szasz et al., 1986).

How extensive is this unfolded region? In Figure 2, the colchicine-induced bands do not appear to be multiple, not even closely spaced doublets. There are no potential trypsin cleavage sites carboxyl-terminal to Lys-392, but there are sites at positions 379 and 380, and there are potential chymotrypsin sites in both directions, including at Phe-378 and Phe-385. No evidence for cleavage at any of these positions has been found. In addition, cleavage by subtilisin is not affected by colchicine binding (not shown). Because subtilisin cleaves β-tubulin principally at Gln-433 (Redeker et all, 1992; Sackett et al., 1985), this indicates that the acidic extreme carboxyl-terminal region of the protein is unaltered by colchicine binding. Clearly, the unfolded region must be large enough to allow the cleavage site to reach the active site of the enzyme. The crystal structure of trypsin complexed with bovine pancreatic trypsin inhibitor indicates that a loop of about eight residues centered on the cleavage site is sufficient to allow access to the active site of the protease (Marquart et al., 1983). Taken together, these data suggest that the region unfolded by colchicine may be small, possibly confined to the region ±5 residues of Arg-390.

DISCUSSION

The results presented here identify structural changes induced in tubulin by colchicine binding. Colchicine induces a change in the conformation of tubulin sufficient to alter the pattern of proteolysis by trypsin and chymotrypsin. Colchicine binding to tubulin is a slow process which inovlves changes in the conformations of both colchicine and tubulin and results in a tight complex with modified polymerization properties (Garland, 1978). A great deal has been learned about the binding process and about the interaction of the tubulin–colchicine complex with the polymer [reviewed in Hamel (1990) and Hastie (1991)]. Yet specific structural changes induced in tubulin by colchicine have not been described. That structural changes occur seems clear from changes in spectral properties (Andreu & Timasheff, 1982), from immunological reactivities (Morgan & Spooner, 1983), and of course from polymerization inhibition. Changes in sulfhydryl reactivities indicate that structural changes occur in β and affect Cys-239 and Cys-354 [reviewed in Luduena and Roach (1991)]. In order to better understand, localize, and define colchicine-induced changes in tubulin, we used proteolysis as a probe for structural change and found specific colchicine-induced changes in trypsin and chymotrypsin cleavages.

The colchicine-induced changes in proteolysis observed are confined to β-tubulin for both enzymes and are localized near the carboxyl end. A previous report (Serrano et al., 1984) noted a colchicine-induced change in proteolysis, but attributed it to a change in α-tubulin because it was produced by trypsin. We here demonstrate by reaction with subunit- and domain-specific monoclonal antibodies that both colchicine-induced trypsin and chymotrypsin cleavages occur on β-tubulin and show that they both occur near residue 390. We do not suggest that our results prove that no colchicine-induced change(s) occur(s) in α-tubulin or that the changes we observed are the only ones that occur in β-tubulin. We do report that the structural changes detected by proteolysis localize to the carboxyl-terminal region of β-tubulin.

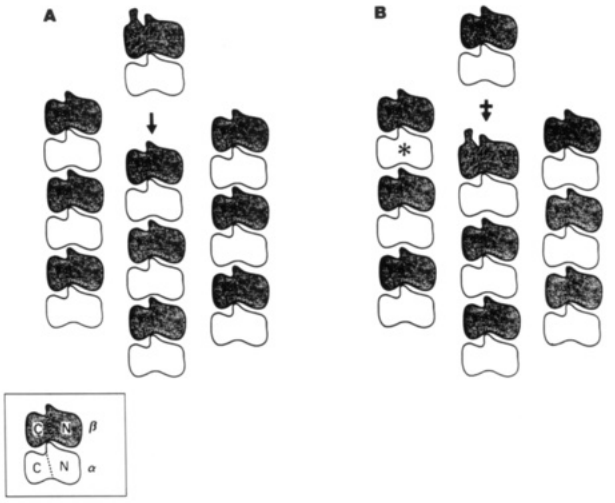

FIGURE 5: Model of colchicine poisoning of microtubule assembly. A portion of the microtubule lattice is shown in schematic form. Each of the three protofilaments shown is composed of $\alpha\beta$ dimers. As shown in the inset, the α-subunits are white, and the β-subunits are shaded. In both, the amino-terminal domain is larger than the carboxyl-terminal domain, and the shapes shown are somewhat arbitrary. The contact between αN and βC in the isolated dimer (inset) is known from cross-linking data, as is the interdimer contact between βN and αC, observed between dimers in the polymer (Kirchner & Maldelkow, 1985). In panel A, a tubulin–colchicine complex is depicted about to add to the growing polymer. The βC domain of the tubulin–colchicine complex is shown to contain a loop not present in the normal dimer. This represents the area around Arg-390 that is unfolded by colchicine and cleaved by trypsin and chymotrypsin. The contact area between this dimer and the polymer involves the unaltered α-subunit of the colchicine dimer and the surfaces of the β-subunit exposed at the end of the polymer. Since all of these surfaces are normal, the dimer can add despite the altered β-subunit conformation. Once added, however (panel B), the altered dimer impedes the addition of a further dimer longitudinally due to the unfolded loop region of βC, here shown on the distal surface of the polymer. Alternatively or in addition, the altered βC region might protrude to the side, and interfere with the addition of a dimer lateral to it, i.e., the position marked with an asterisk. Blocking addition to either position or both would impede extension of one or both protofilaments and poison the helical growth of the microtubule.

The sequence amino-terminal to the colchicine-enhanced tryptic cleavage site at Lys-392 shows high helical potential, and the amphipathic nature of the helix provides an attractive structural explanation for the altered proteolysis. The chymotryptic site at Phe-389 is not accessible in the native structure, since it is on the nonpolar side of the helix, presumably oriented away from the aqueous solvent. The tryptic site at Lys-392 is poorly accessible, being at the end of the helix. Partial unfolding of this end of the helix by colchicine increases the accessibility of Lys-392 to trypsin and exposes Phe-389 to cleavage by chymotrypsin.

These results allow us to propose a mechanism that explains a number of known features of colchicine action (Figure 5). It is known that the tubulin–colchicine complex can bind to the growing end of the microtubule, suggesting that the binding surface of the colchicine dimer is relatively unmodified (Skoufias & Wilson, 1992; Sternlicht & Ringel, 1979). Since the effects of colchicine observed here are confined to the β-subunit, it is likely that the (unmodified) α-subunit of the dimer being added makes contact with a β-subunit exposed at the growing end (Figure 5). This would allow the tubulin–colchicine complex to bind to a normal addition site (Figure 5A). However, once bound, the tubulin–colchicine complex could impede a subsequent dimer from adding to it, due to the unfolded loop in βC which would now be exposed (Figure 5B). The tubulin dimer makes lateral as well as longitudinal associations in the microtubule, and it is possible that the βC loop could impede addition laterally, i.e., interfere with the addition of the dimer marked with the asterisk in Figure 5B. Blockage of either position would interrupt the (local) helical growth of the microtubule. Complete arrest of microtubule growth may be achieved by only a few tubulin–colchicine complexes per microtubule end (Bayley and Martin, 1991; Margolis et al., 1980), or may require many to achieve complete arrest of growth (Farrel & Wilson, 1980; Sternlicht & Ringel, 1979). In any case, inhibitory concentrations of tubulin–colchicine complex are lower than the concentration of free tubulin. The primary locus of change caused by colchicine is the conformational change induced by colchicine in tubulin, here localized to the carboxyl-terminal region of β-tubulin. This model provides a mechanism for colchicine poisoning, and explains how it can do so substoichiometrically while still allowing tubulin–colchicine to add to the growing end.

This model is also relevant to GTP-cap proposed as a mechanism for the "dynamic instability" of microtubules (Bayley & Martin, 1991; Mitchison & Kirschner, 1984; O'Brien et al., 1987; Stewart et al., 1990). Since the model in Figure 5 indicates that the $\alpha\beta$ dimers are oriented in the microtubule lattice with β exposed at the growing end, coupling of GTP hydrolysis to polymerization is simple, as has been suggested (Bayley & Martin, 1991; Mandelkow & Mandelkow, 1989; O'Brien et al., 1987; Stewart et al., 1990). The α-subunit of the GTP–tubulin dimer which adds must interact with the β-subunit of the dimer previously incorporated into the microtubule. It is known that β contains the GTP whose hydrolysis is somehow coupled with polymerization (Nath et al., 1985). The exposure of β-tubulin at the growing end of the microtubule suggests a simple explanation for a single-layer GTP-cap, as mentioned above: the terminal layer of tubulin dimers will remain GTP-containing until another dimer's α-subunit adds to the polymer, interacting with the GTP-containing β-subunit of the terminal dimer, and inducing hydrolysis. Whatever the detailed mechanism of GTP hydrolysis may be, the colchicine-induced structural changes reported here suggest that this mechanism and its relation to microtubule polarity will involve exposure of β-tubulin at the growing end of the microtubule.

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REFERENCES

Andreu, J. M., & Timasheff, S. N. (1981) *Arch. Biochem. Biophys. 211*, 151–157.
Andreu, J. M., & Timasheff, S. N. (1982) *Biochemistry 21*, 6465–6476.
Bayley, P. M., & Martin, S. R. (1991) *Biochem. Soc. Trans. 19*, 1023–1028.
Best, D., Warr, P. J., & Gull, K. (1981) *Anal. Biochem. 114*, 281–284.
Bhattacharyya, B., Sackett, D. L., & Wolff, J. (1985) *J. Biol. Chem. 260*, 10208–10216.
Borisy, G. G., & Taylor, E. W. (1967) *J. Cell Biol. 34*, 525–533.
Breitling, F., & Little, M. (1986) *J. Mol. Biol. 189*, 367–370.
Correia, J. J., Baty, L. T., & Williams, R. C., Jr. (1987) *J. Biol. Chem. 262*, 17278–17284.
David-Pfeuty, T., Simon, C., & Pantaloni, D. (1979) *J. Biol. Chem. 254*, 11696–11702.
delaVina, S., Andreu, D., Medrano, F. J., Nieto, J. M., & Andreu, J. M. (1988) *Biochemistry 27*, 5352–5365.

Draber, P., Draberova, E., Linhartova, I., & Viklicky, V. (1989) *J. Cell Sci. 92*, 519–528.
Eigsti, O. J., & Dustin, P., Jr. (1955) *Colchicine—in Agriculture, Medicine, Biology and Chemistry*, Iowa State College Press, Ames, IA.
Farrel, K. W., & Wilson, L. (1980) *Biochemistry 19*, 3047–3054.
Fitzgerald, T. J. (1976) *Biochem. Pharmacol. 25*, 1383–1387.
Floyd, L. F., Barnes, L. D., & Williams, R. F. (1989) *Biochemistry 28*, 8515–8525.
Garcia-Gonzales, A., & Weisman, M. H. (1992) *Semin. Arthritis Rheum. 22*, 139–150.
Garland, D. L. (1978) *Biochemistry 17*, 4266–4272.
Ginzburg, I., Teichman, A., Dodemont, H. J., Behar, L., & Littauer, U. Z. (1985) *EMBO J. 4*, 3667–3673.
Grimm, M., Breitling, F., & Little, M. (1987) *Biochim. Biophys. Acta 914*, 83–88.
Hamel, E. (1990) in *Microtubule Proteins* (Avila, J., Ed.) pp 89–192, CRC Press, Boca Raton, FL.
Hastie, S. B. (1991) *Pharmacol. Ther. 51*, 377–401.
Himes, R. H., Burton, P. R., & Gaito, J. M. (1977) *J. Biol. Chem. 252*, 6222–6228.
Hopp, T. P., & Woods, K. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3824–3828.
Kirchner, K., & Mandelkow, E.-M. (1985) *EMBO J. 4*, 2397–2402.
Lacey, E. (1988) *Int. J. Parasitol. 18*, 885–936.
Lin, C. M., & Hamel, E. (1981) *J. Biol. Chem. 256*, 9242–9245.
Lin, C. M., Ho, H. H., Petit, G. R., & Hamel, E. (1989) *Biochemistry 28*, 6984–6991.
Luduena, R. F., & Roach, M. C. (1991) *Pharmacol. Ther. 49*, 133–152.
Malkinson, F. D. (1982) *Arch. Dermatol. 118*, 453–457.
Mandelkow, E., & Mandelkow, E.-M. (1989) in *Cell Movement* (Warner, F. D., & McIntosh, R. J., Eds.) Vol. 2, 23–45, Alan Liss, New York.
Margolis, R. L., & Wilson, L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3466–3470.
Margolis, R. L., Rauch, C. T., & Wilson, L. (1980) *Biochemistry 19*, 5550–5557.
Marquart, M., Walter, J., Deisenhofer, J., Bode, W., & Huber, R. (1983) *Acta Crystallogr. B39*, 480–490.
Mitchison, T., & Kirschner, M. W. (1984) *Nature 312*, 237–242.
Moreland, L. W., & Ball, G. V. (1991) *Arthritis Rheum. 34*, 782–786.
Morgan, J. L., & Spooner, B. S. (1983) *J. Biol. Chem. 258*, 13127–13133.
Nath, J. P., Eagle, G. R., & Himes, R. H. (1985) *Biochemistry 24*, 1555–1560.
O'Brien, E. T., Voter, W. A., & Erickson, H. P. (1987) *Biochemistry 26*, 4148–4156.
Ponstingl, H., Little, M., Kempf, T., Krauhs, E., & Ade, W. (1990) in *Laboratory Methodology in Biochemistry: Amino Acid Analysis and Protein Sequencing* (Fini, C., Floridi, A., Finelli, U. N., & Wittman-Liebold, S., Eds.) pp 151–171, CRC Press, Boca Raton, FL.
Redeker, V., Melki, R., Prome, D., LeCaer, J.-P., & Rossier, J. (1992) *FEBS Lett. 313*, 185–192.
Sackett, D. L., & Wolff, J. (1986) *J. Biol. Chem. 261*, 9070–9076.
Sackett, D. L., & Lippoldt, R. E. (1991) *Biochemistry 30*, 3511–3517.
Sackett, D. L., Bhattacharyya, G., & Wolff, J. (1985) *J. Biol. Chem. 260*, 43–45.
Sackett, D. L., Knipling, L., & Wolff, J. (1991) *Prot. Express. Purif. 2*, 390–393.
Serrano, L., Avila, J., & Maccioni, R. B. (1984) *J. Biol. Chem. 259*, 6607–6611.
Skoufias, D. A., & Wilson, L. (1992) *Biochemistry 31*, 738–746.
Sternlicht, H., & Ringel, I. (1979) *J. Biol. Chem. 254*, 10540–10550.
Stewart, R. J., Farrell, K. W., & Wilson, L. (1990) *Biochemistry 29*, 6489–6498.
Szasz, J., Yaffe, M. B., Elzinga, M., Blank, G. S., & Sternlicht, H. (1986) *Biochemistry 25*, 4572–4582.
Taylor, E. W. (1965) *J. Cell Biol. 25*, 145–160.
Wolff, J., Knipling, L., Cahnman, H. J., & Palumbo, G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2820–2824.
Wilson, L., & Friedkin, M. (1967) *Biochemistry 6*, 3126–3135.
Zemer, D., Livneh, A., Pras, M., & Sohar, E. (1993) *Am. J. Med. Genet. 45*, 340–344.