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Research Article

Griseofulvin: Interaction With Normal and Subtilisin-Treated Tubulin

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ABSTRACT Griseofulvin, an antifungal drug, possesses antimitotic activity by inhibiting microtubule assembly. The interaction of griseofulvin with tubulin is unique in that it increases the sulfhydryl titer of tubulin without affecting the hydrophobic areas. Because the C-terminal regions of both α- and β-tubulin influence the conformational and the drug-binding properties of tubulin, we decided to study the interaction of griseofulvin with αβ tubulin and tubulin treated with subtilisin to selectively remove the C-terminal regions from both the α- and β-subunits ($\alpha_s\beta_s$). We found that the apparent K_d of griseofulvin for $\alpha_s\beta_s$ tubulin (83 μM) was virtually unaltered compared to its K_d for αβ tubulin (91 μM) as determined by the tryptophan fluorescence quenching assay. The increment of the sulfhydryl titer (0.47 mol/mol) by griseofulvin was not affected by removing the C-termini from the α- and β-subunits. Moreover, the lack of effect of griseofulvin on the hydrophobic areas was not changed after the cleavage of the C-termini of the α- and β-subunits. These data therefore strongly suggest that griseofulvin binds at a certain region of tubulin distant from the C-terminal domains of the α- and β-subunits and that the C-terminal domains of both subunits do not have any conformational influence over the binding site of griseofulvin. Determining the binding site of griseofulvin will help in understanding its role in regulating microtubule assembly and dynamics. Drug Dev. Res. 53:44–49, 2001.

Key words: tubulin; griseofulvin; C-termini; subtilisin

INTRODUCTION

Microtubules, the ubiquitous cellular organelles present in almost all eukaryotic cells, serve several important cellular functions including cell shape maintenance, organelle transport, and cell division [Hyams and Lloyd, 1993]. Tubulin, the major structural component of microtubules, is a heterodimeric protein consisting of α- and β-subunits [Luduena et al., 1977]. The detailed mechanism of the interaction of drugs such as vinblastine, colchicine, and others are well studied [Bhattacharyya and Wolff, 1976; Luduena and Roach, 1981, 1991; Andreu and Timasheff, 1982a, 1982b]. Unfortunately, little attention has been focused on the mechanism of interaction of tubulin with griseofulvin, a drug produced by the mold Penicillium. Griseofulvin possesses antimitotic activity and inhibits the formation of microtubules in vivo and in vitro [Weber et al., 1976], although it has been reported that the action of griseofulvin does not involve the disruption of microtubules [Grisham et al., 1973]. There has been a great deal of controversy regarding the interaction of griseofulvin with tubulin. Some of the reports in the literature suggest that griseofulvin interacts either with tubulin directly [Sloboda et al., 1982; Wehland et al., 1977] or with one or more of the microtubule-associated proteins [Roobol et al., 1977]. Therefore, we reexamined the interaction

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of griseofulvin with tubulin in detail [Chaudhuri and Luduena, 1996]. We found that indeed, griseofulvin binds directly to tubulin with an apparent K_d of 79 μM, and its interaction with tubulin is quite different compared to the interaction of other drugs with tubulin. Moreover, it binds to a certain region on tubulin where other drugs such as colchicine do not bind, although colchicine can influence the binding of griseofulvin [Chaudhuri and Luduena, 1996].

Tubulin is labile in conformation, and it has a tendency to alter its functional conformation in a time-dependent process known as "decay" in which tubulin loses its ability to interact with ligands and to form microtubules. The cleavage of the C-terminal ends of the α- and β-subunits by subtilisin markedly inhibits the decay process [Sarkar et al., 1995; Chaudhuri and Luduena, 1997]. This suggests that decay appears to originate from the C-termini of both the α- and β-subunits. Moreover, the cleavage of the C-termini modulates the binding of the ligands to tubulin [Mukhopadhyay et al., 1990; Chaudhuri et al., 1998]. This observation raises the possibility that the C-terminal domains may interact with a certain part of the tubulin molecule in such a way that the binding of griseofulvin to tubulin may be affected. Therefore, we used subtilisin to cleave the C-terminal regions from both the α- and β-subunits ($\alpha_s\beta_s$) and studied the effect of the cleavage on the interaction with griseofulvin. We found that the apparent K_d of griseofulvin for $\alpha_s\beta_s$ tubulin (83 μM) was not significantly different than the K_d of griseofulvin to αβ tubulin (91 μM). The increment of the sulfhydryl titer of $\alpha_s\beta_s$ tubulin upon binding to griseofulvin (0.42 mole /mole) was also unaffected by griseofulvin. Moreover, the lack of effect of griseofulvin on the exposure of hydrophobic areas remained unchanged. Therefore, these data strongly suggest that griseofulvin does not bind to the C-terminal regions of tubulin and that the binding site lies in a region of tubulin which is not part of the domain whose conformation is controlled by the C-termini. Since griseofulvin inhibits microtubule assembly, our results raise the possibility that the griseofulvin-binding domain could be another critical region for modulating microtubule assembly and dynamics.

MATERIALS AND METHODS

Materials

Griseofulvin and subtilisin were purchased from Sigma (St. Louis, MO). BisANS (bis-5,5′-[8-(*N*-phenyl)-aminonapthalene-1-sulfonic acid]) was from Molecular Probes (Junction City, OR). Iodo[^{14}C]acetamide was from American Radiolabeled Chemicals Inc. (St. Louis, MO). Sources of all other materials were as previously described [Luduena et al., 1982]. Griseofulvin was dissolved in dimethylsulfoxide immediately before use.

Tubulin Preparation

Microtubules were purified from bovine cerebra by a cycle of assembly and disassembly, and the tubulin was purified therefrom by phosphocellulose chromatography [Fellous et al., 1977]. Experiments were performed in the following buffer: 100 mM Mes (2-(*N*-morpholino)-ethane-sulfonic acid., pH 6.4, 0.5 mM $MgCl_2$, 1 mM EGTA ([ethylenebis(oxyethyl-enenitrilo)]tetraacetic acid).

Sulfhydryl Group Modifications

In most experiments, tubulin with or without drugs was reacted with iodo[^{14}C]acetamide at 37°C for 2 h. After the reactions, each tubulin sample was precipitated with an equal volume of 10% trichloroacetic acid; the precipitates were collected by filtration, and the radioactivity of the filters was determined [Luduena and Roach, 1981].

Fluorescence

For studying the binding of griseofulvin to tubulin fluorometrically, aliquots of tubulin (1 μM) were incubated with different concentrations of the drugs (0–100 μM) at 37°C for 1 h. The tryptophan fluorescence of tubulin in the presence or absence of griseofulvin was measured at 335 nm in the Hitachi F-2000 spectrophotometer (Hitachi Instruments, Inc., San Jose, CA). The excitation wavelength was 296 nm. The fluorescence of each sample was corrected for inner-filter effects. The difference in fluorescence between the samples with or without drug at different drug concentrations was analyzed in a nonlinear curve-fitting program, MINSQ version 3.2 (Scientific Software, Salt Lake City, UT), using a one-site binding equation as follows:

$$F = F_m * D/(K_d + D),$$

where F is the fluorescence value at any drug concentration, F_m is the maximum fluorescence, D is the drug concentration, and K_d is the apparent dissociation constant for the drug–tubulin complex.

Digestion of Tubulin by Subtilisin

Digestion of tubulin by subtilisin was performed at 30°C for 30 min. The ratio of subtilisin to tubulin was 1:100 (wt/wt). The reaction was terminated after 30 min by the addition of 1% (wt/vol) PMSF (phenyl methyl sulfonyl fluoride) to a final concentration of 1% (vol/vol). Digestion at 30°C for 30 min cleaved the C-terminal ends of both α- and β-subunits, giving rise to the product $\alpha_s\beta_s$ tubulin [Bhattacharyya et al., 1985]. The extent of digestion of α- and β-subunits by subtilisin was determined by subjecting the αβ and $\alpha_s\beta_s$ tubulins to polyacrylamide gel electrophoresis, then staining with Coomassie blue and scanning the area of each subunit using the public

domain NIH Image program 1.58 (Anonymous FTP from zippy.nimh.nih.gov) on a Macintosh IIci computer. The image was captured using a GBC CCD 500 B camera (Testrite Instrument Co., Newark, NJ).

Other Methods

Protein concentrations were determined by a modified form of the procedure of Lowry et al. [Lowry et al., 1951; Schacterle and Pollack, 1973], using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Because the C-terminal domains of both α- and β-tubulins are flexible and modulate microtubule assembly and the binding of ligands to tubulin, we prepared $\alpha_s\beta_s$ tubulin by cleaving selectively the C-termini of both α- and β-tubulin with subtilisin (Fig. 1) to study the interaction of griseofulvin with αβ and $\alpha_s\beta_s$ tubulin. Of our population of tubulin dimers, 38% had their α chains cleaved and 86% had their β chains cleaved. We could not cleave the C-terminal ends of tubulin completely, especially from α-tubulin, under our digestion conditions. The reasons may be because of the different exposure of the C-terminal ends of the different α-isoforms to the outer environment and the extensive posttranslational modification occurring mainly at the C-terminal ends of α-tubulin. Because subtilisin cleaves at different sites in

Fig. 1. Electrophoretic analysis of αβ and $\alpha_s\beta_s$ tubulin. Digestion of αβ tubulin by subtilisin [enzyme:protein = 1:100 (wt/wt)] was performed at 30°C for 30 min. The reactions were stopped by being made 1% (vol/vol) in 1% PMSF (wt/vol). The samples were then subjected to electrophoresis on a 6% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate [Laemmli, 1970]; the gel was stained with Coomassie blue. **Lane 1:** αβ Tubulin (15 μg); **Lane 2:** $\alpha_s\beta_s$ Tubulin (15 μg)

TABLE 1. Effect of Griseofulvin on the Alkylation of αβ and $\alpha_s\beta_s$ Tubulin[a]

Reaction	^{14}C Incorporation (mol ^{14}C/mol tubulin)
αβ Tubulin + DMSO	3.66 ± 0.02
αβ Tubulin + grisefulvin	4.13 ± 0.03
$\alpha_s\beta_s$ Tubulin + DMSO	1.93 ± 0.05
$\alpha_s\beta_s$ Tubulin + griseofulvin	2.35 ± 0.04

[a]Aliquots (500 μl) of αβ and $\alpha_s\beta_s$ tubulin (5 μM) were incubated at 37°C in the presence and absence of 100 μM griseofulvin for 1 h. As a control, instead of drug, an equal volume of dimethylsulfoxide (DMSO) was added. The final concentration of DMSO was 1%. After 1 h, 36.65 μl of iodo[^{14}C]acetamide (1.2891 dpm/pmol) was added to each sample to a final iodoacetamide concentration of 500 μM. The incubation at 37°C was continued for another 1 h. Incorporation of ^{14}C was measured as described in Materials and Methods. Each reaction was done in triplicate. Standard deviations are shown.

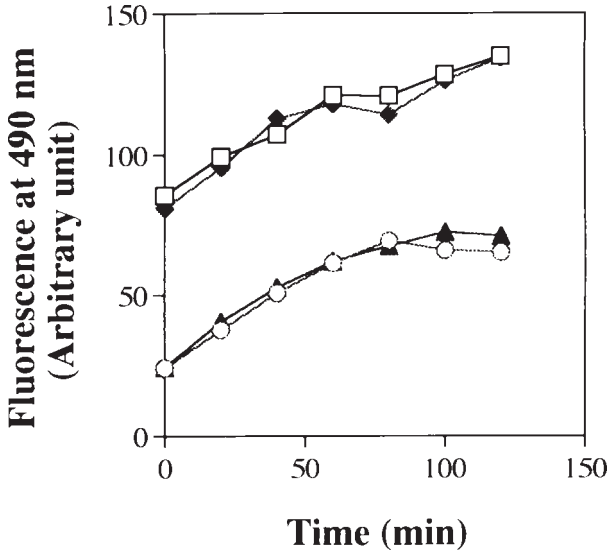

Fig. 2. Effect of griseofulvin on BisANS binding to αβ and $\alpha_s\beta_s$ tubulin. Aliquots of αβ and $\alpha_s\beta_s$ tubulin (0.2 mg/ml) were incubated in the absence or presence of 100 μM griseofulvin at 37°C. To the control sample, an equal volume of dimethylsulfoxide (DMSO) was added. At the indicated times, aliquots (1 ml) were withdrawn and mixed quickly with BisANS to give a final concentration of 10 μM. The aliquots were placed in a fluorescence cuvette and the fluorescence was measured. Excitation and emission were at 385 and 490 nm, respectively. The fluorescence values were corrected for the volume and for the fluorescence that would have been obtained if tubulin had not been present. Open squares: αβ tubulin + DMSO; filled diamonds: αβ tubulin + griseofulvin; open circles: $\alpha_s\beta_s$ tubulin + DMSO; filled triangles: $\alpha_s\beta_s$ tubulin + griseofulvin.

the C-terminal ends [Lobert et al., 1993], we were not sure exactly what regions were cleaved by subtilisin. However, subtilisin must cleave these segments from α- and β-subunits: 417 and onward from α-tubulin and 407 and onward from β-tubulin [Maccioni et al., 1986; Serrano et al., 1986; de la Vina et al., 1988]. Because tryptophan and the sulfhydryl groups are sensitive markers for determining tubulin conformation and drug binding [Luduena and Roach, 1991], we used these two probes to understand the binding of griseofulvin to tubulin. With the cleavage of the C-terminal ends, the sulfhydryl titer of tubulin was dropped by 1.73 mol/mol of tubulin (Table 1). This is interesting, because the regions of α- and β-tubulin that were cleaved by subtilisin (417 and onward in α- and 407 and onward in β) by subtilisin do not contain any cysteine residues.

Because the C-terminal regions regulate the conformation of tubulin [Sarkar et al., 1995; Chaudhuri and Luduena, 1997], it is possible that the digestion of the C-terminal ends affected the conformation of tubulin in such a way that some of the sulfhydryl groups were buried and were not accessible to the alkylating agent. As a result, the sulfhydryl titer was reduced by 1.73 mol/mol (Table 1). We previously showed [Chaudhuri and Luduena, 1996] that griseofulvin markedly increased the sulfhydryl titer of tubulin, suggesting that some of the cysteine residues that were not initially accessible to the alkylating agent were exposed on interaction with griseofulvin. Here, we also found that the sulfhydryl titer of αβ tubulin was increased by 0.47 mol/mol. We have previously observed that the sulfhydryl titer of tubulin and the titer affected by drugs varied from one preparation

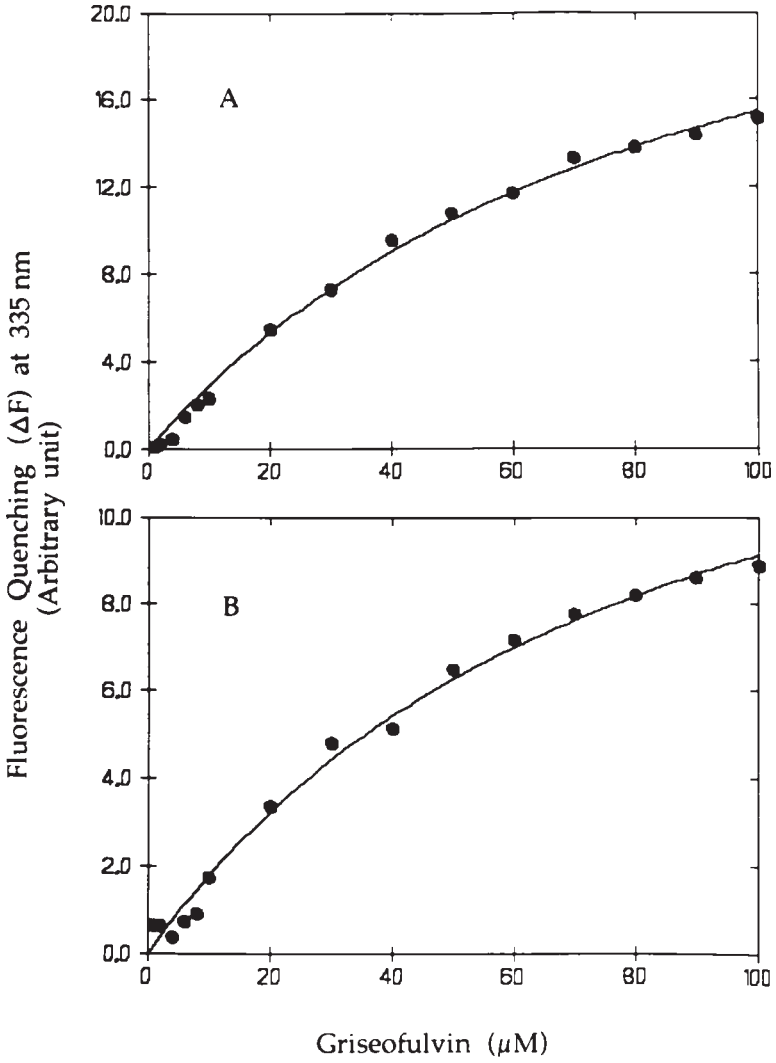

Fig. 3. Analysis of the binding of griseofulvin to αβand $\alpha_s\beta_s$ tubulin. Aliquots of αβ **(A)** and $\alpha_s\beta_s$ **(B)** tubulin (1 μM) were incubated with a series of concentrations of griseofulvin (0–100 μM) at 37°C for 1 h and then were excited at 296 nm. Emission at 335 nm was measured. The fluorescence was corrected for the inner-filter effect [Lakowicz, 1983]. The difference in fluorescence (ΔF) caused by griseofulvin was calculated and the data were fitted to a one-site model by using the nonlinear curve-fitting software program MINSQ as described in Materials and Methods.

to another. However, the important point is that griseofulvin increased the sulfhydryl titer of tubulin. In the case of $\alpha_s\beta_s$ tubulin, griseofulvin increased the sulfhydryl titer by 0.42 mol/mol, which was similar to the titer increased observed with αβ tubulin (Table 1). These data strongly suggest that the binding site of griseofulvin is not located at the vicinity of the C-terminal regions of α- and β-subunits. Moreover, the conformational change induced by the C-terminal cleavage did not alter the effect of griseofulvin. This is interesting, because the cleavage of the C-terminal regions affected the binding of drugs to tubulin [Mukhopadhyay et al., 1990; Chaudhuri et al., 1998]; either the binding site was abolished [Chaudhuri et al., 1998] or the on-rate/off-rate constant was affected [Mukhopadhyay et al., 1990]. The only exception is phomopsin A; the cleavage of the C-termini does not affect the binding of phomopsin A to tubulin [Chaudhuri and Luduena, 1997].

We also examined whether griseofulvin and phomopsin A shared the same site on tubulin. We found that the binding sites of both griseofulvin and phomopsin A were distinctly different as determined by the tryptophan fluorescence quenching assay (data not shown). This is consistent with our earlier observation that the effects of griseofulvin and phomopsin A on tubulin are quite different [Chaudhuri and Luduena, 1996, 1997]. We studied the effect of griseofulvin on the exposure of the hydrophobic areas of αβ and $\alpha_s\beta_s$ tubulin (Fig. 2). We found that although restricted digestion by subtilisin significantly suppressed the exposure of the hydrophobic areas of tubulin, griseofulvin had no influence on the hydrophobic areas of both normal and subtilisin-treated tubulin. This is consistent with our earlier observation that griseofulvin has no effect on the exposure of the hydrophobic areas of αβ tubulin [Chaudhuri and Luduena, 1996]. We also studied the binding of griseofulvin to αβ and $\alpha_s\beta_s$ fluorometrically (Fig. 3). Earlier, we determined the apparent K_d (79 μM) of griseofulvin for tubulin by the tryptophan fluorescence quenching assay [Chaudhuri and Luduena, 1996]. Here, we found that the cleavage of the C-termini did not affect the K_d of griseofulvin. The K_d values of griseofulvin for αβ and $\alpha_s\beta_s$ tubulin were 91 μM and 83 μM, respectively (Fig. 2A,B); this suggested that the altered conformation of tubulin because of the cleavage of the C-termini did not affect the tryptophan residues that are either located at or close to the griseofulvin binding site or those that are affected by griseofulvin. Moreover, because the cleavage did not change the apparent K_d of griseofulvin, it is likely that the altered structure did not affect the kinetics of griseofulvin binding. It therefore appears that the binding site of griseofulvin is unique and that the site is not influenced by the C-terminal-induced conformational changes.

Because the affinity of griseofulvin to tubulin is low and the binding site neither overlaps with any of the known antitubulin drugs nor is influenced by the C-termini of α- and β-subunits, it is promising that the detection of the binding site of griseofulvin will help in designing and developing more specific and potent griseofulvin derivatives against the fungal tubulin with least side effects to the host cells. Moreover, the knowledge of the binding site will also help in understanding the significance of the site regarding microtubule assembly and dynamics.

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