The Tumor Suppressor Protein Fhit
A NOVEL INTERACTION WITH TUBULIN*

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FHIT (fragile histidine triad) is a candidate human tumor suppressor gene located at chromosome 3p14.2, a location that encompasses the FRA3B chromosomal fragile site. Aberrant transcripts have been detected in a variety of primary tumors, and homozygous deletions in the FHIT locus have been detected in different tumor cell lines. The gene product Fhit in vitro possesses the ability to hydrolyze diadenosine 5',5''-P1-P3-triphosphate (Ap3A). The mechanism of action of Fhit as a tumor suppressor is unknown. Because the tubulin-microtubule system plays an important role in cell division and cell proliferation, we investigated the interaction between wild-type Fhit or mutant Fhit (H96N) and tubulin in vitro. The mutant form of Fhit (H96N) lacks Ap3A hydrolase activity but retains tumor suppressor activity. We found that both wild-type and mutated forms of Fhit bind to tubulin strongly and specifically with $K_d$ values of 1.4 and 2.1 $\mu M$, respectively. Neither wild-type nor mutant Fhit cause nucleation or formation of microtubules, but in the presence of microtubule-associated proteins, both wild-type and mutant Fhit promote assembly to a greater extent than do microtubule-associated proteins alone, and the microtubules formed appear normal by electron microscopy. Our results suggest the possibility that Fhit may exert its tumor suppressor activity by interacting with microtubules and also indicate that the interaction between Fhit and tubulin is not related to the Ap3A hydrolase activity of Fhit.

Multiple deletions in the short arm of chromosome 3 have been frequently found in different human cancers (1–4), implying the presence of tumor suppressor genes. Recently the FHIT gene was mapped by positional cloning to chromosome region 3p14.2, a location encompassing FRA3B, the most active constitutive chromosomal fragile site known. Aberrant transcripts have been detected in a variety of primary tumors, and homozygous deletions in the FHIT locus have been detected in different tumor cell lines (2–4). FHIT encodes a 147-amino acid protein that has a HIT (histidine triad) sequence motif (positions 94–99) identified by the presence of a histidine triad, HXHXHX, where $X$ is a hydrophobic residue (1). Fhit has Ap3A hydrolase activity in vitro (5). FhitH96N, generated by site-directed mutagenesis of FHIT, has negligible Ap3A hydrolase activity, which suggests that the central histidine of the triad is essential for hydrolase activity (5). Suppression of tumors in nude mice injected with tumor cells transfected with either FHit or FHIT-H96N provides the strongest data that Fhit is a tumor suppressor (6). The results also indicate that the Ap3A hydrolase activity of Fhit is not necessary for its tumor suppressor activity (6). The mode of action of Fhit as a tumor suppressor and the relationship of the Ap3A hydrolase activity to tumor suppression are not yet understood. The Fhit gene and protein have been reviewed recently (7, 8).

Microtubules are ubiquitous cytoskeletal organelles found in most eukaryotic cells; they play critical roles in mitosis and other cellular processes (9). Since microtubules participate actively in mitosis, they are the prime target of taxol, vinblastine, and other anti-tumor drugs (10). These microtubules are composed of the protein tubulin, consisting of two subunits called $\alpha$ and $\beta$.

We decided to explore the relationship between microtubules and Fhit. Most tumor suppressor proteins are DNA-directed and function at the transcriptional level (11). APC is the only tumor suppressor protein known to interact directly with microtubules and to promote microtubule assembly. A domain of APC has sequence homology with the microtubule-associated protein Tau (12). Mutations in APC cause the dissociation of APC from the microtubule cytoskeleton (13). Since the mechanism of Fhit as a tumor suppressor is still unknown, we examined the interaction of Fhit and tubulin in vitro. In this paper, we demonstrate for the first time that both Fhit and FhitH96N interact specifically with unfractionated tubulin with $K_d$ values of 1.4 and 2.1 $\mu M$, respectively. Fhit and FhitH96N do not promote microtubule assembly of tubulin by themselves, but in the presence of MAP2 and Tau, both forms of Fhit induce assembly to a greater extent than do either MAP2 or Tau alone, as determined by turbidimetry, electron microscopy, and sedimentation. Thus, the relationship of Fhit with the tubulin-microtubule system may help to understand the role of Fhit in the suppression of tumorigenesis.

EXPERIMENTAL PROCEDURES

Materials—6-IAF was purchased from Molecular Probes, Inc. (Eugene, OR). Centricron-10 concentrators were purchased from Amicon, Inc. (Beverly, MA). All other materials were obtained or purchased as described previously (14).

Preparation of Microtubule Protein and Tubulin—Microtubules were purified from fresh bovine brain cerebra by assembly and disassembly according to the method of Fellous et al. (15). When experiments were to be done, microtubules were resuspended in 0.1 M MES, pH 6.4, 1 mM EGTA, 0.5 mM MgCl$_2$, and 0.1 mM EDTA and centrifuged at 12,000 × g at 4 °C for 30 min. The resulting supernatant is designated as micro-
FIG. 1. Analysis of the competitive binding of labeled and unlabeled Fhit to tubulin. Aliquots of tubulin (6 μM) were mixed with a single concentration of fluorescently labeled Fhit (1 μM) and a series of concentrations of unlabeled Fhit (0–20 μM) and incubated at 37 °C for 30 min. The fluorescence of the samples was measured at 515 nm. The excitation wavelength was 492 nm. The fluorescence of the labeled Fhit (1 μM) alone was also measured. The intensity of fluorescence of the labeled Fhit (1 μM) quenched by tubulin (6 μM) was considered 100% (calculated as the difference in fluorescence between labeled Fhit alone and labeled Fhit incubated with tubulin). The effect of the different concentrations of unlabeled Fhit on the fluorescence was measured, and the data were fitted to a power curve using cricket graph III version 1.01. Half-maximal reversal of fluorescence quenching by the unlabeled Fhit was also determined (inset).

FIG. 2. Analysis of the binding of Fhit and FhitH96N to tubulin. Fhit (panel A) and FhitH96N (panel B), each at 1 μM in buffer, were incubated with different concentrations of tubulin (0–6 μM) at 37 °C for 30 min. After incubation, the samples were excited at 492 nm, and the emission at 515 nm was measured. The difference in fluorescence value between fluorescent-labeled Fhit alone and fluorescent-labeled Fhit containing the tubulin at different concentrations was determined. Fluorescence data were fitted to a one-site binding model using the nonlinear curve-fitting MINLSQ software as described under "Experimental Procedures."

Analysis of the competitive binding of labeled and unlabeled Fhit to tubulin. Aliquots of tubulin (6 μM) were mixed with a single concentration of fluorescently labeled Fhit (1 μM) and a series of concentrations of unlabeled Fhit (0–20 μM) and incubated at 37 °C for 30 min. The fluorescence of the samples was measured at 515 nm. The excitation wavelength was 492 nm. The fluorescence of the labeled Fhit (1 μM) alone was also measured. The intensity of fluorescence of the labeled Fhit (1 μM) quenched by tubulin (6 μM) was considered 100% (calculated as the difference in fluorescence between labeled Fhit alone and labeled Fhit incubated with tubulin). The effect of the different concentrations of unlabeled Fhit on the fluorescence was measured, and the data were fitted to a power curve using cricket graph III version 1.01. Half-maximal reversal of fluorescence quenching by the unlabeled Fhit was also determined (inset).

For the one site model, \( F = F_m \times L/K_d \), where \( F \) is the fluorescence value at any ligand concentration, \( F_m \) is the maximum fluorescence, \( L \) is the ligand concentration, and \( K_d \) is the apparent dissociation constant for the tubulin-Fhit complex.

For the two-site model, \( F_1 = F_{m1} \times L/K_{d1} + L \), \( F_2 = F_{m2} \times L/K_{d2} \), where \( F_{m1} \) and \( F_{m2} \) are the maximum fluorescence values at any ligand concentration \( (L) \) for high and low affinity sites, respectively. \( F_{m1} \) and \( F_{m2} \) are the maximum fluorescence values for high and low affinity sites, respectively. \( K_{d1} \) and \( K_{d2} \) are the apparent dissociation constants for high and low affinity sites, respectively, and \( F \) is the total fluorescence value at any given ligand concentration.

Microtubule assembly—Tubulin in assembly buffer containing 1 mM GTP was mixed with either MAP2 or Tau and incubated at 37 °C in a cuvette in a Beckman DU 7400 spectrophotometer. Microtubule assembly was monitored by the increase in turbidity at 350 nm (17). Cold sensitivity of assembled microtubules was determined by measuring the decrease in turbidity at 350 nm after incubating microtubule samples in ice for 30 min.

Electron microscopy—Microtubule structures were examined on negatively stained grids using a JEOL 100 CX electron microscope at an accelerating voltage of 60 kV as described previously (18). Samples were mixed with an equal volume of glutaraldehyde (1%) followed by mounting on carbon-coated grids for 30 s. The grids were then washed se-
Interaction of Fhit with Tubulin

RESULTS

To study the interaction of Fhit with tubulin in vitro, we covalently labeled Fhit and FhitH96N with 6-IAF and studied the interaction between Fhit and tubulin fluorometrically. The fluorescence at 515 nm of both labeled forms of Fhit was quenched by increasing concentrations of tubulin when samples were excited at 492 nm. Unlabeled Fhit was able to reverse about 80% of the tubulin-induced quenching of fluorescence (Fig. 1). Since Fig. 1 shows hyperbolic behavior with a limiting plateau at about 80% of the tubulin-induced quenching of fluorescence at 515 nm of both forms of Fhit was studied fluorometrically. The interaction between Fhit and tubulin was monitored by the increase in turbidity at 350 nm in a Beckman DU 7400 spectrophotometer. For structural analysis by electron microscopy, samples of microtubule protein (2 mg/ml) containing either Fhit (12 μM) (C) or FhitH96N (12 μM) (D) were incubated at 37 °C for 30 min followed by preparation and examination by electron microscopy as described under “Experimental Procedures.” Magnification, ×29,900.

The fluorescence quenching of 6-IAF-labeled Fhit and FhitH96N in the presence of tubulin was enhanced significantly (Figs. 3, A and B), and the extent of microtubule assembly was a function of the concentration of Fhit (Fig. 3A). Microtubules formed in the presence of Fhit and FhitH96N appear to have typical microtubular structures as determined by electron microscopy (Figs. 3, C and D). The microtubules formed in the presence of Fhit and FhitH96N are also cold-sensitive (data not shown), which is a property of cold-sensitive tubulin in vitro.

Because tubulin forms cylindrical microtubular structures in the presence of its associated proteins in vitro, we studied the effect of Fhit and FhitH96N on microtubule protein assembly. We found that with both forms of Fhit, microtubule assembly was enhanced significantly (Figs. 3, A and B), and the extent of microtubule assembly was a function of the concentration of Fhit (Fig. 3A). Microtubules formed in the presence of Fhit and FhitH96N were incubated either alone (□) or in the presence of either Fhit (12 μM) (○) or both MAP2 (0.3 mg/ml) (■) or Tau (0.15 mg/ml) (□) or in the presence of both Tau (0.15 mg/ml) and Fhit (12 μM) (▲). Panel B, tubulin (10 μM) was mixed with either MAP2 (0.3 mg/ml) (□) or both MAP2 (0.3 mg/ml) and Fhit (6 μM) (●). Panel C, tubulin (10 μM) was incubated either alone (□) or with FhitH96N (12 μM) (○) or both MAP2 (0.3 mg/ml) (□) or both Tau (0.15 mg/ml) and FhitH96N (12 μM) (●). Panel D, tubulin was mixed with either MAP2 (0.3 mg/ml) (□) or both MAP2 (0.3 mg/ml) and FhitH96N (12 μM) (●). Assembly was monitored by turbidimetry at 350 nm.
normal microtubules formed in the absence of Fhit (22).

Neither Fhit nor FhitH96N initiate microtubule assembly of pure tubulin in the absence of MAPs as detected by turbidimetry (Fig. 4), electron microscopy (Fig. 5A and F), or sedimentation assay (Table I). However, in the presence of either Tau or MAP2, both Fhit and FhitH96N promote the assembly of tubulin more than do either Tau or MAP2 alone (Fig. 4). In all cases with either Fhit or FhitH96N, the assembled microtubules appear to be normal in structure as determined by electron microscopy (Figs. 5). Sedimentation analysis (Table I) shows that when Fhit and tubulin are incubated with either MAP2 or Tau, the presence of Fhit increases the polymer mass by 34% and 106%, respectively. These data are in good agreement with the data shown in Figs. 4, A and B, demonstrating that the effect of Fhit on Tau-induced assembly is greater than on MAP-2-induced assembly. In an analogous experiment using unfractionated microtubule protein, Fhit increases polymer mass by only 7%.2 The pelleted samples were analyzed by polyacrylamide gel electrophoresis (Fig. 6). The results demonstrated that some Fhit copolymerized with tubulin.

**DISCUSSION**

We found that both Fhit and FhitH96N bind to tubulin with similar apparent affinities (K<sub>D</sub> values for wild-type and mutant Fhit = 1.4 and 2.2 μM, respectively). This observation suggests that the mutation at histidine 96, which is the central histidine of the histidine triad, has no significant influence on the interaction between Fhit and tubulin. This mutation causes loss of the Ap<sub>3</sub>A hydrolase activity of Fhit (5) but not loss of the tumor suppressor capacity of Fhit (6). Binding of both wild-type Fhit and FhitH96N to tubulin is compatible with the tumor suppressor function of Fhit being independent of Ap<sub>3</sub>A hydrolysis. This finding also predicts that the Ap<sub>3</sub>A catalytic domain and the tubulin binding domain of Fhit are distinctly different and that they do not significantly influence each other.

Since tubulin forms microtubules in the presence of GTP and associated proteins, we studied the effect of the Fhit protein on...
the assembly process. We found that both Fhit and FhitH96N promote assembly in a concentration-dependent manner and that the assembled microtubules had normal structures as revealed by electron microscopy. These results are consistent with our observation that mutation at histidine 96 does not influence the binding of Fhit to tubulin. Our sedimentation assay also shows clearly that Fhit-mediated assembly of tubulin increases the tubulin microtubule mass and that Fhit is physically associated with microtubules. The data obtained using different methodologies to quantitate the increment of Fhit-induced microtubule assembly vary, but the phenomenon that Fhit promotes assembly by increasing microtubule mass is highly consistent. The data obtained by electrophoretic analysis indicates that Fhit probably behaves sub-stoichiometrically in promoting assembly of tubulin. Reversal of fluorescence quenching by Fhit and the cold-sensitivity of Fhit-induced microtubules also strongly suggest that the interaction between Fhit and tubulin is specific and that the increment of light scattering and the polymer mass by Fhit is due to formation of normal microtubules.

Our results suggest that Fhit has a unique binding site on tubulin and that the site probably does not overlap with any of the MAP binding sites as Fhit is copelleted along with other microtubule-associated proteins. Sequence comparisons using the FASTA3 program (23) of Fhit and proteins known to interact with tubulin also support this hypothesis as the analyses failed to find any significant sequence similarities among Fhit and such proteins. In contrast, the tumor suppressor protein APC, which is known to bind to tubulin, has sequence similarity with a particular region of Tau protein (12). There are distinct differences between APC and Fhit in terms of their interactions with tubulin. APC promotes the assembly of tubulin by itself (12) without requiring the presence of any MAPs. In contrast, neither Fhit by itself nor FhitH96N by itself induce the assembly of tubulin, but in the presence of Tau or MAP2, both Fhit and FhitH96N promote the assembly of microtubules more than do Tau or MAP2 alone. Since the C-terminal domains of both α and β subunits are flexible and exposed even on the surface of microtubules (24, 25), these regions can be selectively removed by limited proteolysis with subtilisin (26, 27).

The C terminus-cleaved tubulin exhibits MAP-independent microtubule assembly due to reduction of electrostatic repulsion and forms microtubule-like sheets (26, 27). Interestingly, Fhit alone can enhance the assembly of subtilisin-cleaved tubulin. 2 This observation is also consistent with the hypothesis that Fhit interacts at a unique site other than the C-terminal regions, where MAPs are thought to interact (26, 28). Because the electrostatic repulsion among the C-terminal regions of tubulin hinders microtubule assembly, Fhit cannot induce microtubule assembly by itself. It needs other proteins such as MAP2 or Tau that bind at the C-terminal regions to exhibit its microtubule-inducing property.

In view of the critical roles microtubules play in mitosis and other cellular processes, it is not unexpected that they would also interact with tumor suppressor gene products such as APC and Fhit. The precise nature of the physiological connection between our results and tumor suppression is, however, not clear. Both APC and Fhit enhance microtubule assembly. It is conceivable, therefore, that they may either diminish microtubule dynamic behavior or else interfere with microtubule disassembly, either of which effects could inhibit a process such as mitosis, which is a highly coordinated and complex interplay of microtubule growth, shrinkage, and dynamics. The tubulin binding domain of APC resembles part of the microtubule-associated protein Tau, a part that is not known to bind to tubulin directly but which regulates the Tau-tubulin interaction (12). One may speculate that this domain acts like a MAP to enhance assembly and inhibit dynamics; this could also explain why APC can induce microtubule assembly in the absence of MAPs. In contrast, Fhit can only enhance microtubule assembly in the presence of a MAP; for Fhit, the correlation between structure and function is still unclear. The fact that the H96N mutation, which markedly decreases the Apα hydrolase activity (5, 29), alters neither the tumor suppression nor the effect on microtubule assembly indicates that the hydrolase activity is not required for either of these properties. However, the connection between the tumor suppression and the binding to tubulin remains the subject for future investigation.

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