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13. ABSTRACT <i>(Maximum 200 words)</i> Microtubules are essential components of dividing cells as part of the mitotic spindle. The principal protein components of microtubules are the α - and β -tubulins, which exist as heterodimers. Disruption of the supply of functional heterodimers therefore offers a potential new route in cancer chemotherapy. This concept is distinct from existing approaches which depend on drugs that interfere with microtubule dynamics. The assembly of the α/β -tubulin heterodimer involves interaction of newly synthesized tubulin polypeptides with several tubulin-specific chaperones. These chaperones function by locking α - and β -tubulins together into the assembly-competent heterodimer. Our work is geared toward laying the groundwork for the discovery of reagents that would functionally disrupt the de novo assembly of tubulin heterodimers. Here we report progress in obtaining biochemically workable quantities of several of the tubulin-specific chaperones using various host/vector systems, as well as the results of initial biopanning experiments with a bacteriophage display library.			
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FOREWORD

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INTRODUCTION

Microtubules are essential and ubiquitous elements of the cytoskeleton, a complex network of filaments that exists in virtually all eukaryotic cells. The function of microtubules is remarkably diverse: they participate in maintaining the structural integrity of cells, in directing intracellular transport, and in mediating cell division as part of the mitotic spindle. The striking functional versatility of microtubules depends critically on their dynamic properties and the modulation of these properties via interaction with a class of proteins known as MAPs (Microtubule Associated Proteins) (Caceres and Kosik, 1990; Dinsmore and Solomon, 1991). The essential role of microtubules in cell division means that interfering with microtubule behavior is a potentially promising route in cancer chemotherapy. This is not merely a theoretical concept: the drug taxol, for example, which changes the dynamic behavior of microtubules, is in current clinical use as an effective treatment for breast and ovarian cancer (Holmes et al., 1991; Hortobagyi, 1995).

Two abundant soluble proteins (called α - and β -tubulin) are associated as a heterodimer which forms the subunit from which microtubules are assembled. Taxol interferes with microtubule behavior by forcing the polymerization of heterodimers into stable microtubules and suppressing their dynamic behavior, including their disassembly and reassembly into the mitotic spindle structures necessary for cell division. Although effective, the use of this drug is not without limitations: there can be problems with cytotoxicity, and the potential exists in a large tumor mass for the generation and selection of tumor cell lines that have acquired resistance to the drug. For these reasons, it would be desirable to develop new reagents that interfere with microtubule behavior.

Work performed in my laboratory over the past several years has established the existence of a complex pathway leading to the generation of the native (i.e. assembly-competent) tubulin heterodimer (reviewed in Lewis et al., 1996). In common with all proteins, α - and β -tubulins are synthesized on ribosomes as linear assemblies of amino acids. While many proteins can fold to their final three-dimensional structure spontaneously following their synthesis, tubulin polypeptides cannot: they must interact with a class of proteins termed molecular chaperones whose function is to assist in the correct folding of other proteins (reviewed in Hartl, 1996; Fenton and Horwich, 1997). The first chaperone in the complex pathway leading to the formation of the tubulin heterodimer is the cytosolic chaperonin, a ribosome-sized heteromeric double toroidal structure that contains a central cavity within which newly synthesized tubulin polypeptides bind (Gao et al., 1992; Frydman et al., 1992). This binding reaction effectively sequesters newly synthesized tubulin molecules from the crowded environment that prevails in living cells, thus preventing aberrant reactions with other protein molecules. The chaperonin undergoes dramatic shape changes that accompany ATP binding and hydrolysis (Roseman et al., 1996) such that the tubulin target proteins cycle between a chaperonin-bound state and a non-bound state where folding can take place within the central cavity (Lewis et al., 1996). If folding is successful, the native molecules no longer have any significant affinity for the chaperonin, and are discharged; if folding to the native state fails, the target molecule must rebind to the same or another chaperonin

molecule until it successfully partitions to the native state (Tian et al., 1995).

In some cases - for example actin - ATP-dependent interaction with chaperonin is all that is required for the molecule to acquire its native conformation (Gao et al., 1992). However, in the case of tubulin, interaction with chaperonin is necessary, but not sufficient: α - and β -tubulin molecules discharged from chaperonin are in a quasi-native state, but they are incapable of associating into functional heterodimers (Gao et al., 1993). To do so, they must follow a complex series of interactions with several different proteins (originally termed cofactors, but now renamed tubulin-specific chaperones). These proteins were identified, characterized and purified in my laboratory (Gao et al., 1994; Tian et al., 1996, 1997; Lewis et al., 1996, 1997). They bind to quasi-native α - or β -tubulin folding intermediates generated via ATP-dependent interaction of newly synthesized (or denatured) chains with chaperonin, and interact with one another in a complex pathway that ultimately leads to the generation of native tubulin heterodimers.

At the outset of the funding period of this grant, we had identified and purified four tubulin-specific chaperone proteins (termed A, C, D and E) that, together with chaperonin itself, are sufficient to generate assembly-competent β -tubulin in folding reactions done in vitro (Tian et al., 1996). However, we found that these four chaperone proteins were insufficient (in conjunction with chaperonin) to support the efficient in vitro folding of α -tubulin. In view of this observation, we felt it important to define the full complement of chaperones involved in the α - and β -tubulin folding pathways before beginning our analysis of tubulin-chaperone interactions. We therefore fractionated a crude extract of bovine testis tissue by anion exchange chromatography, and assayed the emerging proteins in in vitro α -tubulin folding reactions containing c-cpn, unfolded ^{35}S -labeled target protein, ATP, GTP and native bovine brain tubulin, included so as to provide a reservoir for the potential stabilization by exchange of any de novo folded α -tubulin (Gao et al., 1993). The reaction products were analyzed by non-denaturing polyacrylamide gel electrophoresis. To our surprise, a species comigrating with native tubulin was generated in these reactions without supplementation with cofactors A, C, D and E. We used this assay as a method to purify to homogeneity the new tubulin folding chaperone responsible for the generation of this product. The purified protein (which we termed tubulin folding chaperone B) is essential for the efficient in vitro folding of α -tubulin.

With the full complement of chaperone proteins involved in α - and β -tubulin folding in vitro now in hand, we were able to define the integrated α - and β -tubulin folding pathway (Fig. 1). We found that the α - and β -tubulin folding pathways converge via the formation of a multimolecular supercomplex (Tian et al., 1997). This supercomplex contains α - and β -tubulin and tubulin chaperones C, D and E; native tubulin heterodimers are then released from this complex in a GTP-dependent reaction. While it was long thought that α - and β -tubulins could freely exchange in the tubulin heterodimer (Dietrich and Williams, 1978), we now know that they cannot: the functional heterodimer can only be assembled by passage through the supercomplex and hydrolysis of GTP. Indeed, if the subunits of the a/b heterodimer are "pulled apart" in a backreaction in which native heterodimer is allowed to react with folding chaperone(s), α - or β -tubulins on their own each rapidly decay to a non-native state that cannot reassemble into native

heterodimer (Tian et al., 1997). These data thus define the function of tubulin-specific chaperones A-E as essential participants in a molecular machine (the supercomplex) that locks together α - and β -tubulins in an activated state, and explain why it has never proved possible to obtain native α - or β -tubulin polypeptides in mutual isolation.

Studies in fission yeast (*Saccharomyces pombe*) have shown that homologs of the tubulin-specific chaperones B, D and E are essential for life (Hirata et al., 1998), and it is almost certain that this is true for mammalian cells as well, given their dependence on microtubules; this will be tested by experiments we proposed for this grant. Whether the requirement for chaperones A and C is likewise absolute has yet to be tested, and also forms a part of our original proposal. The existence of a specialized and complex pathway in the generation of assembly-competent tubulin heterodimers in all eukaryotic cells thus offers an opportunity to uniquely interfere with it. This concept forms the basis of the work funded by the US Army Breast Cancer Initiative. The purpose of the proposed experiments is to lay the groundwork for possible approaches to the development of drugs that target the tubulin folding pathways.

BODY

1. Preparation of purified bovine brain microtubules and purification of tubulin by ion exchange chromatography

We found that tubulin itself is an essential component in our in vitro α - and β -tubulin folding reactions (Tian et al., 1997). For this reason, and as a prerequisite to protease digestion experiments (see below), it was necessary to develop methods for the preparation of highly purified tubulin. To do this, we used published procedures for the preparation of crude microtubule protein from bovine brain (Shelanski and Cantor, 1974). This method involves several successive cycles of GTP-dependent polymerization of microtubules from a soluble supernatant prepared from a total calf brain extract, isolation of assembled microtubules by centrifugation, and depolymerization of the resulting pellet. We found that contaminating MAPs were most effectively removed from this preparation by passage of solubilized microtubule protein over a column of DEAE-cellulose run in buffer containing 0.25M NaCl: under these conditions, only tubulin heterodimer binds to the resin, and can be eluted with higher concentrations of salt (Fig. 2). We found that tubulin prepared in this manner was capable of participating in productive in vitro folding reactions, and was a suitable substrate for protease digestion experiments (see below).

2. Purification of tubulin folding chaperones in milligram quantities

The yields of tubulin folding chaperones purified from crude extracts of bovine testis are quite low (typically of the order of 0.2-0.3mg, starting from 10g of soluble protein). The purification procedures used in these protocols are also extremely labor intensive. Since many of our planned experiments require significant quantities of these materials, it is important (both for

the experiments proposed here, and for those proposed in our N.I.H grant to study the function of the chaperones *in vitro*) to devise methods which yield larger amounts than are obtainable by purification from tissue extracts. To accomplish this, we tried to produce those folding chaperones that are essential for α - and β -tubulin folding (i.e. B, C, D and E) by expression in host *E. coli* cells. We therefore cloned full-length cDNAs encoding tubulin chaperones B, C, D and E into T7-driven expression vectors and introduced the recombinant plasmids into the host expression strain BL21DE3 (Studier et al., 1990). We found that two (chaperones B and C) were expressed as soluble, biologically active recombinant proteins (Fig. 3) that could be purified in good yield. However, chaperones D and E were produced exclusively in insoluble form (i.e. in inclusion bodies). Therefore, as an alternative to expression in *E. coli*, we attempted the expression of chaperones D and E in insect Sf9 cells using baculovirus vectors engineered for the expression of these proteins. We were able to express soluble chaperones D and E in this system. However, only chaperone E was found to be biologically active. It is not clear at the present time why chaperone D expressed in Sf9 cells is biologically inactive: one possibility is that it lacks some critical post-translational modification. We are currently engaged in experiments to express biologically active chaperone D in an alternative (i.e. mammalian) eukaryotic expression system.

3. Screening experiments using combinatorial phage display libraries

Because we were able to produce recombinant, biologically active tubulin folding chaperone C in good yield (see above), and because this chaperone functions in the supercomplex that is critical for heterodimer formation (see Fig. 1), we proceeded to screen a combinatorial M13 phage display library to see whether we could identify short peptide sequences that bind specifically to this chaperone. Purified tubulin folding chaperone C was bound to a polystyrene dish, and incubated with buffer containing about 10^{12} recombinant phage displaying randomly inserted heptameric peptide sequences. The plate was then washed several times with buffer, and bound phage were eluted by brief incubation with glycine buffer, pH 2.5. Eluted phage were then amplified and the chaperone C selection procedure repeated through several additional cycles. After either 5 or six such cycles, selected phage were plaque-purified, single stranded DNA was prepared, and the inserts were sequenced. Although the titer of phage selected by the solid-phase bound chaperone suggested that specific selection might be taking place, sequence analysis of the selected phage did not reveal any significant consensus sequences (Fig. 4A,B). Nonetheless, in several cases, the screening procedure did result in the independent isolation of the same heptameric peptides, suggesting that these sequences might be binding specifically to chaperone C. We therefore synthesized four peptides, in each case consisting of the heptameric sequence encoded by one of the selected bacteriophage, plus four residues (GGGS) encoded by sequences immediately upstream of the inserts so as to increase the probability that the peptides would be soluble. The purified peptides were added to *in vitro* tubulin folding reactions at 0.2mg/ml (i.e. in vast molar excess over endogenous chaperones), as well as to actin *in vitro* translations as a control for the general inhibition of the translational machinery. The data from these experiments (Fig. 5) showed that the synthetic peptides had little discernible effect on the production of tubulin

heterodimer.

CONCLUSIONS

1. Because tubulin-specific chaperones are essential to life in higher eukaryotes, the potential exists to develop reagents that block this pathway.
2. We have developed a protocol for preparing native tubulin free of detectable contaminating MAPs that can support in vitro tubulin folding reactions and that is a suitable substrate for proteolytic digestion experiments.
3. We have successfully produced biologically active chaperones B, C and E in milligram quantities by expressing them as recombinant proteins in prokaryotic or eukaryotic hosts. Experiments are ongoing to try to produce chaperone D using a mammalian host/vector expression system.
4. A bacteriophage display library was screened to identify sequences that interact specifically with tubulin folding chaperone C. Several identical sequences were obtained as independent isolates; however, the selected phage contained no significant consensus sequences. In addition, several synthetic peptides corresponding to selected sequences had little or no effect on tubulin synthesis in a cell-free system. These data suggest that no inhibitor of chaperone C will be found using this methodology. Therefore, we are turning our attention to the other proposed approaches to generating inhibitors of chaperone activity, namely the generation and assaying of tubulin fragments and the screening of libraries to search for binders/inhibitors of chaperones A, B, D and E.

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APPENDIX

FIGURE 1.

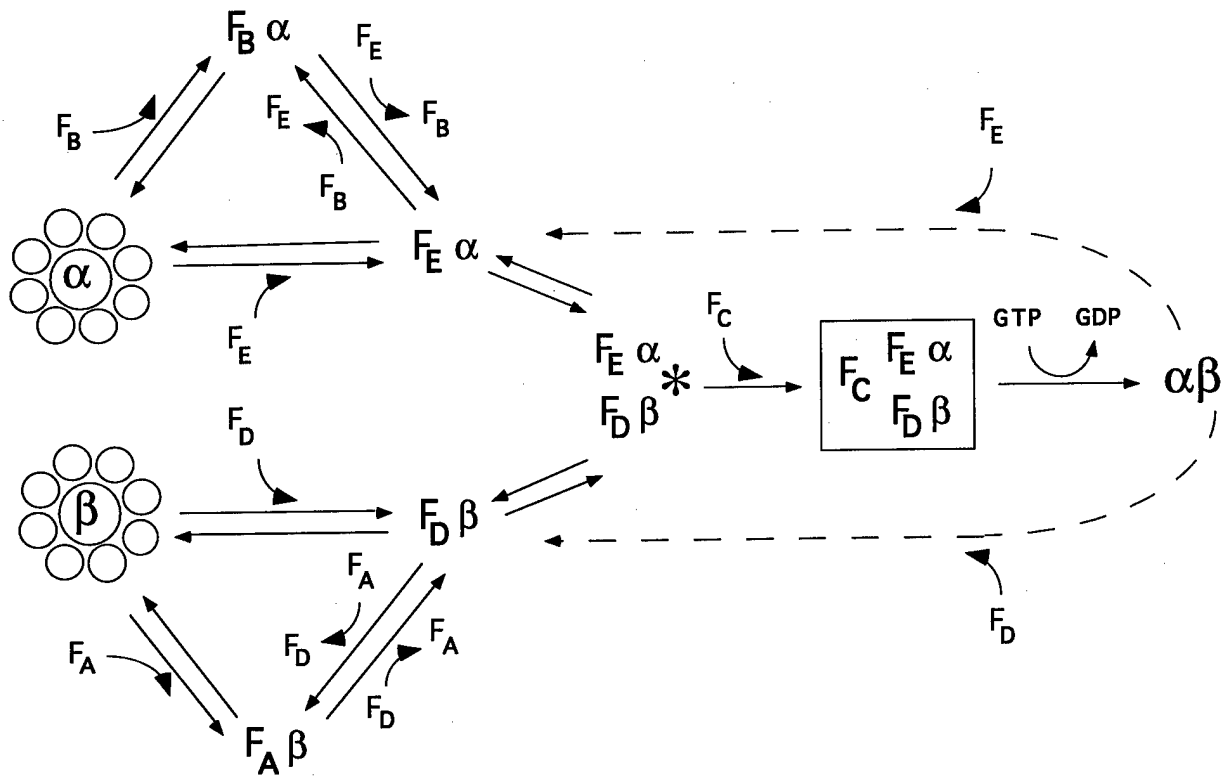


Fig. 1. Convergence and symmetry in the α - and β -tubulin folding pathways. Quasi-native folding intermediates produced through ATP-dependent interaction with cytosolic chaperonin (shown as eight-subunit toroids) interact with a series of proteins (tubulin-specific chaperones) designated F_A , F_B , F_C , F_D , F_E . The pathways converge through the formation of a complex containing α - and β -tubulin and chaperones D and E (marked with an asterisk). Entry of chaperone C generates the α - β supercomplex (boxed); GTP hydrolysis then results in the release of native heterodimer. Broken arrows show the backreaction between native tubulin and chaperones E or D.

FIGURE 2.

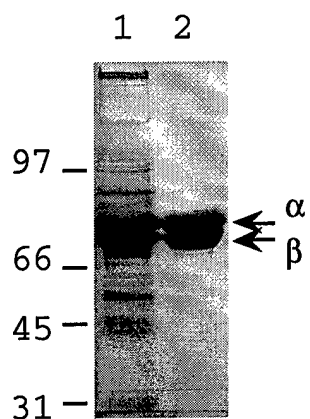


Fig. 2. Purification of native tubulin from crude extracts of calf brain. Analysis by SDS-PAGE of microtubule protein prepared by successive cycles of GTP-dependent polymerization and depolymerization (Lane 1) and of tubulin prepared from this material by DEAE-cellulose anion exchange chromatography (lane 2). Location of molecular weight markers (in kDa) is shown on the left.

FIGURE 3.

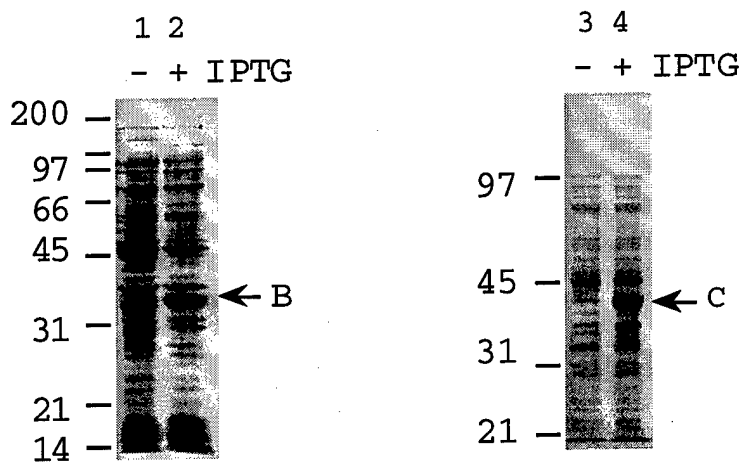


Fig. 3. Expression of recombinant chaperones B and C in host *E. coli* cells. SDS-PAGE analysis of whole extracts of *E. coli* control cells (lanes 1 and 3) and cells harboring plasmids engineered for the expression of tubulin folding chaperones B (lane 2) and C (lane 4). Location of molecular weight markers (in kDa) is shown on the left.

FIGURE 4.

A.

T	G	T	G	V	T	I	*
H	K	R	P	R	N	N	+
V	T	V	K	K	F	V	
K	R	T	P	Q	L	P	#
H	K	R	P	R	N	N	+
S	V	V	G	L	A	Q	
T	H	R	L	P	S	R	
K	R	T	P	Q	L	P	#
S	H	T	P	Y	V	S	
T	G	T	G	V	T	I	*

B.

T	G	T	G	V	T	I	*
H	S	S	K	I	T	S	&
S	H	I	R	R	T	N	
S	D	I	R	S	K	P	
H	S	S	K	I	T	S	&
A	T	R	H	A	K	S	!
I	P	S	T	V	F	A	\$
I	P	S	T	V	F	A	\$
A	T	R	H	A	K	S	!
A	V	S	L	R	L	P	
H	S	S	K	I	T	S	&

Fig. 4. Peptide sequences obtained as a result of biopanning of a bacteriophage display library using chaperone C after 5 (panel A) or 6 (panel B) consecutive rounds of selective binding to chaperone C. Identical sequences marked with symbols (*,#,+,!, \$) were obtained as independent isolates.

FIGURE 5.

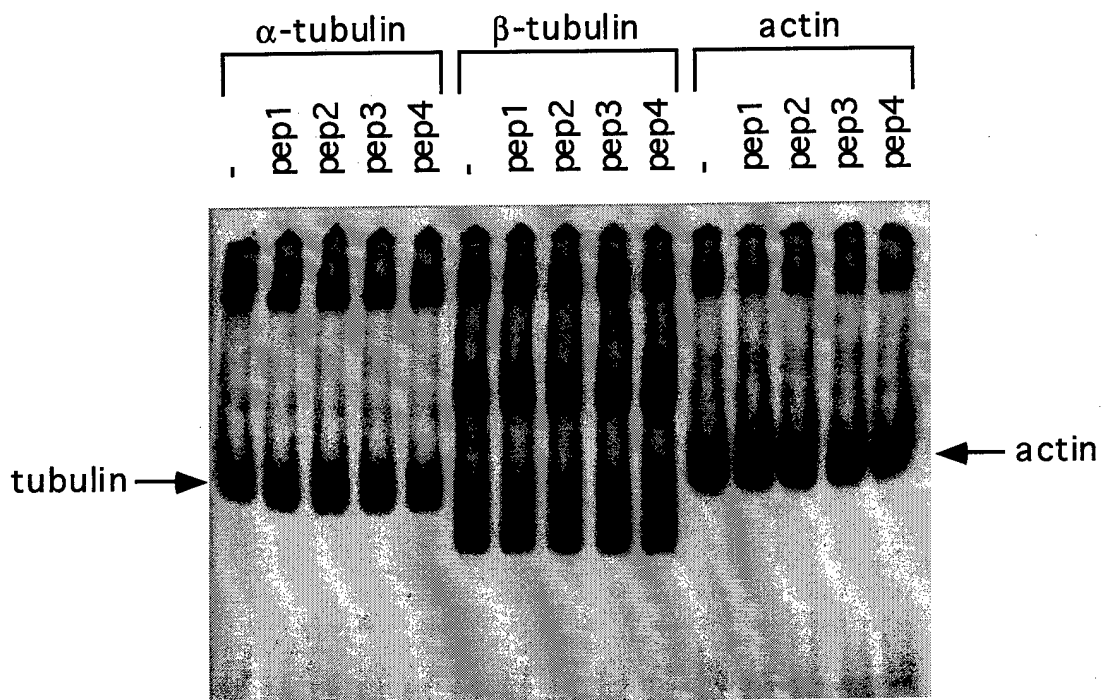


Fig. 5. Effect of four peptides (SHIRTNGGGS [pep1], HSSKITSGGGS [pep2], ATRHAKSGGGS [pep3], SDIRSKPGGGS [pep4] - see Fig. 4B) on the synthesis of native α -tubulin, β -tubulin and actin (as a control) in rabbit reticulocyte lysate in vitro.