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Chemically synthesized 182–235 segment of tau protein and analogue peptides are efficient in vitro microtubule assembly inducers of low apparent sequence specificity*

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A 54-amino acid peptide reproducing the first and second repeats and intervening spacer sequence of the tubulin binding motif (residues 182–235) of murine tau protein, and several congeners representing different degrees of sequence scrambling have been prepared by solid phase methods and fully characterized chemically. These double-repeat peptides have been shown to induce microtubule formation at concentrations about one order of magnitude lower than single-repeat controls, under conditions close to the critical concentration needed for tubulin self-assembly. On the other hand, partial loss of microtubule-inducing capacity was observed for peptides with primary structures increasingly disorganized with respect to the canonical peptide. These results call into question the assumption that a high degree of primary structure specificity is involved in the tau–tubulin interaction leading to in vitro microtubule formation.

Tubulin assembly; Tau sequence specificity; Solid phase peptide synthesis

1. INTRODUCTION

Microtubules are eukaryotic organelles involved in a wide variety of biological functions such as mitosis, motility, intracellular transport and establishment and maintenance of cell shape [1]. Microtubules are formed by assembly of $\alpha\beta$-tubulin, and a number of microtubule-associated proteins (MAPs) and microtubule-based motors, which bind to their surface and provide the regulation and functionalities to these organelles. High molecular weight MAPs include MAP1A, MAP1B, MAP1C (cytoplasmic dynein) MAP2A, MAP2B and MAPU/MAP4; lower molecular weight MAPs include low molecular weight forms of MAP2, and the tau factor [2,3].

The tau protein family consists of six isoforms generated by alternative splicing from one gene, which are highly conserved between mammalian species, and are subject to phosphorylation [4,5]. Modified tau protein is the main part of the characteristic paired helical filaments that appear during neuronal degeneration in Alzheimer disease [6,7]. An important feature in the sequence of tau protein is the presence of three or four 18-residue imperfect repeats, separated by 13- or 14-residue linkers, which are believed to constitute a tubulin motif [8]. The putative tubulin binding module of tau protein is shared by MAP2 [9] and MAPU/MAP4 [10,11].

An important problem in microtubule assembly, regulation and function consists in understanding how these MAP microtubule binding modules, which contain abundant basic residues, bind to the acidic tubulin molecules within the ordered surface lattice of the microtubule wall, in a multivalent interaction. This interaction should have a large electrostatic component, although hydrophobic contacts appear to be involved [12].

The role of the tau/MAP2 repeats has been studied by two different approaches, employing either genetically engineered constructs or MAP fragments prepared by chemical synthesis. Transfection of cells with deletion constructs of MAP2 and tau indicated the requirement of at least one repeat to bind to microtubules [13]. Employing tau cDNA clones from human fetal brain and an *E. coli* expression system it was shown that fragments containing three, two or one repeat were able to bind to in vitro-assembled microtubules, whereas the N-terminal part of the molecule did not [14]. Purified recombinant human tau isoforms containing four repeats promoted microtubule assembly faster than the three-repeat isoforms [15]. These studies have reinforced the crucial role of MAP repeat sequences in tubulin assembly. However, the degree of sequence specificity of these interactions has not been established. In the second approach, synthetic peptides encompassing

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either the second or the first repeats of tau and MAPU were reported to induce microtubule assembly, though peptide concentrations in the 0.1–0.8 × 10^{-3} M range were required, in contrast with typical 10^{-6} M protein concentrations [15,17]. Single-repeat, relatively inefficient peptides have been frequently employed to model the interactions of tau/MAP2/MAPU with microtubules [12,18–21].

The flexibility and good chemical definition of the synthetic peptide approach make it, in our opinion, particularly well suited to the study of this type of problem. In this paper we report the synthesis of a more efficient microtubule inducer, a 54-residue peptide encompassing the first repeat, the first linker, and the second repeat of murine brain tau, as well as a series of sequence variants designed to probe the specificity of the interaction, and a first characterization of their ability to induce microtubule assembly from purified tubulin.

2. MATERIALS AND METHODS

2.1 *Chemicals*

Boc-protected L-amino acids were from Propeptide (Vert-le-Petit, F), Bachem Feinchemikalien (Bubendorf, CH) or Novabiochem (Läufelfingen, CH). *p*-Methylbenzhydrylamine resin (0.81 mmol/g) was from Peptides International (Louisville, KY). All other chemicals for peptide synthesis were of the highest purity available and were used without further purification. GTP dilithium salt was from Boeringer-Mannheim; poly-L-lysine hydrobromide, average degree of polymerization 50, was from Sigma; glycerol and other products were of analytical grade from Merck.

2.2. *Peptide synthesis and purification*

All syntheses were performed stepwise in Applied Biosystems 430A synthesizers, using the standard solid phase [22] Boc protocols provided by the manufacturer, with minor modifications. Side-chain protections were benzyl (Ser, Thr), 2-bromobenzyloxycarbonyl (Tyr), 2-chlorobenzyloxy-carbonyl (Lys), cyclohexyl (Asp, Glu), 2,4-dinitrophenyl (His) and *p*-toluenesulfonyl (Arg). After 54 synthetic cycles (Tau 2.1 described here as an example), the fully protected peptide resin (300 mg, ca. 25 μmol) was treated with 5 ml of thiophenol/DIEA/DMF (3:3:4 by volume) (6 × 1 h) [23,24]. The Boc group was removed with trifluoroacetic acid (40% (v/v) in dichloromethane for 1 min; 100% for 5 min; again 40% for 20 min), the resin washed with dichloromethane, dried and treated with HF/cresol (9:1 v/v) for 1 h at 0°C. After HF evaporation, the residue was extracted with anhydrous ether, solubilized in 10% acetic acid and gel-filtered through Sephadex G-10 (2 × 100 cm; 1 M acetic acid), to give 85 mg peptide that was further purified by reverse phase chromatography on octadecylsilica (Vydac, 5 × 25 cm; 15–20 mm) using a 5–50% (v/v) linear gradient of acetonitrile in water (both with 0.05% trifluoroacetic acid), at a flow rate of 10 ml/min. Fractions appearing homogeneous by HPLC were pooled and lyophilized, to give 24 mg (4.2 μmol; 17% overall yield) of highly purified matrial. No residual Dnp groups were found in this material by HPLC analysis at 340 nm. Amino acid analysis (6 N HCl, 110°C, 24 h) of the purified peptide gave Asx 4.97 (5), Thr 2.09 (2), Ser 4.87 (5), Glx 2.93 (3), Pro 3.99 (4), Gly 9.59 (9), Val 4.59 (5), Ile 2.46 (3), Leu 4.11 (4), Tyr 0.99 (1), His 3.10 (3), Lys 8.31 (8), Arg 0.95 (1). The electrospray mass spectrum included peaks at m/z 567.1 [$(M+10H)^{10+}$], 629.9 [$(M+9H)^{9+}$] and 708.5 [$(M+8H)^{8+}$], consistent with the calculated mass of 5,662. Sequence determination (Applied Biosystems 477A protein sequencer) of the 34 N-terminal residues gave PDLKNVRSKIGS(T)ENLKH(Q)(P)G(G)KVQIVY-P--L (weak signals in parentheses; hyphens, no clear signal). Sequencing of Tau 1a (obtained from an aliquot of the same peptide-resin, after 20 synthetic cycles) gave: VYKPVDLS(K)VT(S)--G(S)(L)G(N)I.

2.3. *Microtubule assembly with synthetic peptides*

Tubulin was purified from bovine brain as described [25,26]. It was equilibrated by Sephadex G-25 chromatography (0.9 × 20 cm column) into 10 mM sodium phosphate, 6 mM $MgCl_2$, 3.4 M glycerol, 1 mM [ethylenebis(oxyethylenedinitrilo)]tetraacetic acid, 0.2 mM GTP buffer, pH 6.5 (glycerol assembly buffer). It was centrifuged at 12,000 rpm in a SS34 Sorvall rotor for 20 min at 4°C, and its concentration was measured spectrophotometrically, employing a scattering corrected extinction coefficient of 1.16 $g^{-1} \cdot l \cdot cm^{-1}$ [26]. Microtubule assembly was monitored by turbidity and electron microscopy [27].

3. RESULTS

The six 54-residue peptides of Table I and two shorter peptides used as controls were satisfactorily assembled by solid phase methods [28] using Boc chemistry on *p*-methylbenzhydrylamine resin. The 2,4-dinitrophenyl (DNP) [29] group was chosen for histidine protection, based on previous results [24]. After its thiolytic removal, peptides were released from the resin by HF acidolysis and purified by gel filtration and reverse phase liquid chromatography to give HPLC-homogeneous products, with amino-acid analyses and mass spectra consistent with theory. Global synthetic yields (chain assembly, cleavage and purification) were in the 15–20% range.

The ability of the synthetic peptides to induce tubulin assembly was determined by turbidimetry. The experimental curves (Fig. 1) were characteristic of cooperative nucleated polymerization of protein [27]. At 10^{-5} M concentration, Tau 2.1 clearly induced assembly from 10^{-5} tubulin in glycerol-containing assembly buffer. This tubulin concentration was close to the critical concentration required for self-assembly in this system, as shown by the large lag (nucleation) time, and marginal spontaneous assembly observed in the absence of peptide (tracing 3). Increasing the protein concentration well above the critical concentration led to spontaneous assembly (tracing 4). The two-repeat peptide Tau 2.1 induced assembly considerably more efficiently than the one-repeat Tau 1a at equal mass concentration (tracings 1 and 2, respectively). On the other hand, upon lowering the Mg^{2+} concentration from 6 to 2 mM in the same buffer, a condition where tubulin will not assemble at all below 3 × 10^{-5} M, Tau 2.1 (2 × 10^{-5} M) was still able to induce assembly, at one order of magnitude below Tau 1a (ca. 2.5 × 10^{-4} M). The turbidity measured in these experiments is simply due to the scattering of light by newly formed macromolecular aggregates in the protein solution. It does not tell what these aggregates are and is only valid to quantitate the amount of polymer under very restricted conditions [27]. Therefore, it was verified by electron microscopy that the polymers induced by Tau 2.1 and Tau 1a were indistinguishable from normal microtubules.

Table 1

Amino acid sequence of Tau tubulin-binding domain and synthetic related peptides

Name	Amino Acid Sequence	Pattern[a]	Comments
Tau (182–272)	...PDLKN **VRSKIGSTENLKHQPGGG** KVQIVYKPVDLSK **VTSKCGSLGNIHHKPGGG** QVEVKSEKLDEKDR **VQSKIGSLDNITHVPGGG** NKKIE...	N+1R+1S+2R+2S+3R+C	Partial sequence of murine Tau including the repeat domain[b]
Tau 2.1	PDLKN **VRSKIGSTENLKHQPGGG** KVQIVYKPVDLSK **VTSKCGSLGNIHHKPGGG**	N+1R+1S+2R	Reference synthetic sequence (positions 182–235)
Tau 1a	VYKPVDLSK **VTSKCGSLGNIHHKPGGG**	1S″+2R	Control (single repeat + part of linker)
Tau 2.2	DLKN **VRSKIGSTENLKHQPGGG** KYKVDLK **VTSKCGSLGNIHHKPGGG**	N+1R+1S′+2R	Overall charge distribution unchanged; linker contracted
Tau 2.3	DLKN **VRSKIGSTENLKHQPGGG** KVQIVYKPVDLSK **VTSKCGSLGPGGGNIHHK**	N+1R+1S+2R′	
Tau 2.4	KVQIVYKPVDLSKPDLKN **VRSKIGSTENLKHQPGGG** **VTSKCGSLGNIHHKPGGG**	1S+N+1R+2R	
Tau 2.5	PDLKN **PGGGIGSTENLVRSKKHQ** KVQIVYKPVDLSK **PGGGCGSLGNIVTSKHHK**	N+1R′+1S+2R′	Increasing structural disorganization
Tau 2.6	KVQIVYKPVDLSKPDLKN **PGGGIGSTENLVRSKKHQ** **PGGGCGSLGNIVTSKHHK**	1S+N+1R′+2R′	
Tau 1b	**PGGGCGSLGNIVTSKHHK**	2R′	Control (single disorganized repeat)

[a] Sequences can be envisioned as resulting from the juxtaposition of the following fragments: N, N-terminal fragment; 1R, first repeat; 2R, second repeat; 3R, third repeat; 1S, first spacer; 2S, second spacer; 1S′, shortened spacer; 1R′, first repeat scrambled; 2R′, second repeat scrambled; C, C-terminal fragment.
[b] Ref. [8].

The synthetic constructs of altered sequence (Tau 2.2 to 2.6) promoted different patterns of assembly in glycerol assembly buffer (Fig. 2). The turbidity observed

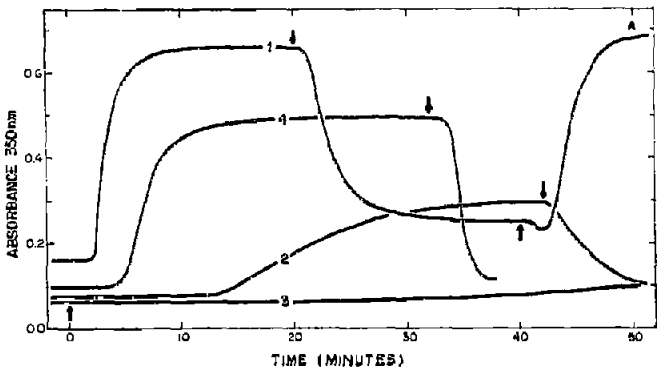


Fig. 1. Time courses of reversible assembly of purified tubulin induced by synthetic peptides Tau 2.1 and Tau 1a in glycerol assembly buffer, monitored turbidimetrically. The different curves correspond to 10^{-5} M tubulin with 10^{-5} M Tau 2.1 (tracing 1, same with 2×10^{-5} M Tau 1a (tracing 2, same tubulin without added peptide (tracing 3), and 2×10^{-5} M tubulin (tracing 4). The assembly reaction was started by raising the temperature from 4°C to 37°C at time 0. The arrows pointing downwards and upwards indicate re-cooling and re-heating of the samples, respectively.

was possibly due not only to microtubules but to microtubule bundles frequently observed under the electron microscope (not shown). Microtubules were apparently more abundant with the Tau 2.1 and Tau 2.2 peptides than with the other, increasingly disorganized sequences. In the case of these sequences, a substantial part of the turbidity could be also due to the formation of disordered aggregates, which cannot be properly quantified by electron microscopy (see below). The time courses of several thermally-induced reversible polymerizations (Fig. 1) allowed a qualitative estimation of the assembly-inducing abilities of the six double-repeat peptides. Thus, the low initial turbidity and the degree of cold reversibility shown by Tau 2.1, Tau 2.2 and, to a lesser extent, Tau 2.5 seems to suggest specific tubulin assembly by these peptides. On the other hand, the high initial turbidity observed for peptides Tau 2.4, 2.6, and 2.3, and their poor cold reversibility are suggestive of non-specific tubulin precipitation. Actually, at 5×10^{-6} M tubulin, the turbidity induced by 10^{-5} M Tau 2.3 was roughly twice larger than that induced by Tau 2.1 or Tau 2.2, but disordered aggregates were evident under the electron microscope (not shown). This non-specific effect hampered any attempt to quantitate the relative

microtubule assembly-inducing activity of each peptide. Polymers induced by poly-lysine were nearly temperature-intensive, and their morphology appeared to be similar to polycation-induced polymers previously described [30].

The tubulin superstructures induced by Tau 2.1 were shown to be microtubules and tubulin rings by electron microscopy. However, even peptide sequences widely diverging from the canonical Tau 2.1 were able to promote to a certain extent the formation of apparently indistinguishable microtubules and rings, in clear contrast with poly-lysine-induced morphologies (Fig. 2, panels B and C).

4. DISCUSSION

Currently accepted views about the tau–microtubule interaction have indicated its cooperative and highly specific character [13–15], and implied that the size of the spacer sequences could be critical for effective induction of assembly. The purpose of this work was to test the first of these assumptions by comparing the in vitro tubulin assembling ability of a synthetic peptide reproducing the first and second repeats of tau (Tau 2.1) vs. that of a single repeat peptide (Tau 1a). Secondly, we wished to examine the specificity of such induction at the level of primary structure. Since it is known that practically any polycation may induce the association of tubulin by preferential binding to the aggregated form of this acidic protein [31], we synthesized a series of peptides with increasing structural deterioration relative to Tau 2.1 (Table I). Peptides Tau 2.2 and 2.4 were primarily designed to examine the role of spacer size. Thus, Tau 2.2 has a 6-amino acid first spacer, about half as long as the original one. As tau–tubulin interaction is thought to be mainly electrostatic, charged amino acids (Lys, Asp) were preserved, together with some arbitrarily chosen non-polar residues. In designing Tau 2.4, it was decided to remove entirely the spacer region between the repeats, and place it at the N-terminus. The high conservation of repetitive sequences in MAPs seemed to suggest an important functional role for such repeats. This hypothesis was tested by means of peptides Tau 2.3, where the NIHHK and PGGG sequence blocks of the second repeat were shifted, and Tau 2.5, where more substantial scrambling of both first and second repeats took place. Our final attempt at sequence degradation was peptide Tau 2.6, bearing little resemblance (except for size and amino acid content) to the original Tau 2.1; the spacer is found at the N-terminus (as in Tau 2.4), and both repeats are highly scrambled (as in Tau 2.5).

Our experimental results with synthetic peptides confirmed previous reports [13–15,32,33] on the first of the above assumptions, namely the multivalent character of tau–tubulin interaction. The higher assembly-promoting ability of two-repeat vs. one-repeat peptides (Fig. 1) clearly indicates the multivalent nature of the interaction of the tau repeat sequences with the tubulin molecules in the microtubule wall lattice.

On the other hand, our present data do cast some doubt upon the validity of the other two assumptions. Thus, the fact that peptides such as Tau 2.3, Tau 2.5 or Tau 2.6, only remotely resembling the original tau tubulin binding domain, can promote microtubule formation to a significant degree (Fig. 2), seems to call for a reconsideration of the concept of specific tau–tubulin recognition. In a similar way, the observation that peptides Tau 2.2 and Tau 2.4, with no spacer sequence between the first and second repeats, are still capable of inducing low albeit significant levels of microtubule formation, puts into question the supposedly critical role of such spacer sequences and favors a flexible interaction, in agreement with recently reported results with genetically engineered tau fragments [33]. Our results provided only a qualitative gradation of the microtubule-inducing abilities of the peptides. Since microtubules could be observed by electron microscopy in all cases (except with poly-lysine), the degree of specificity of each sequence cannot be conclusively established except by more detailed, quantitative studies. Current work at our laboratories is directed to determine the

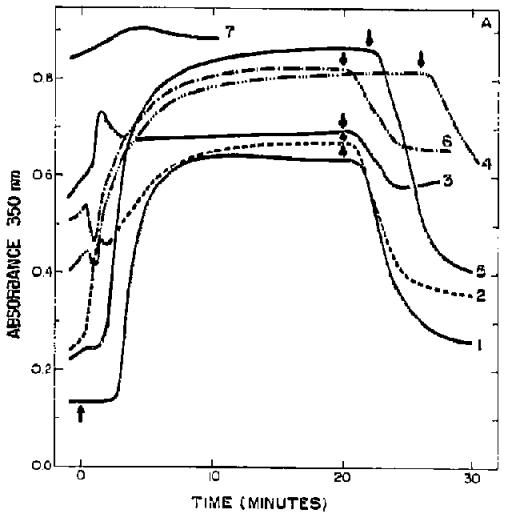
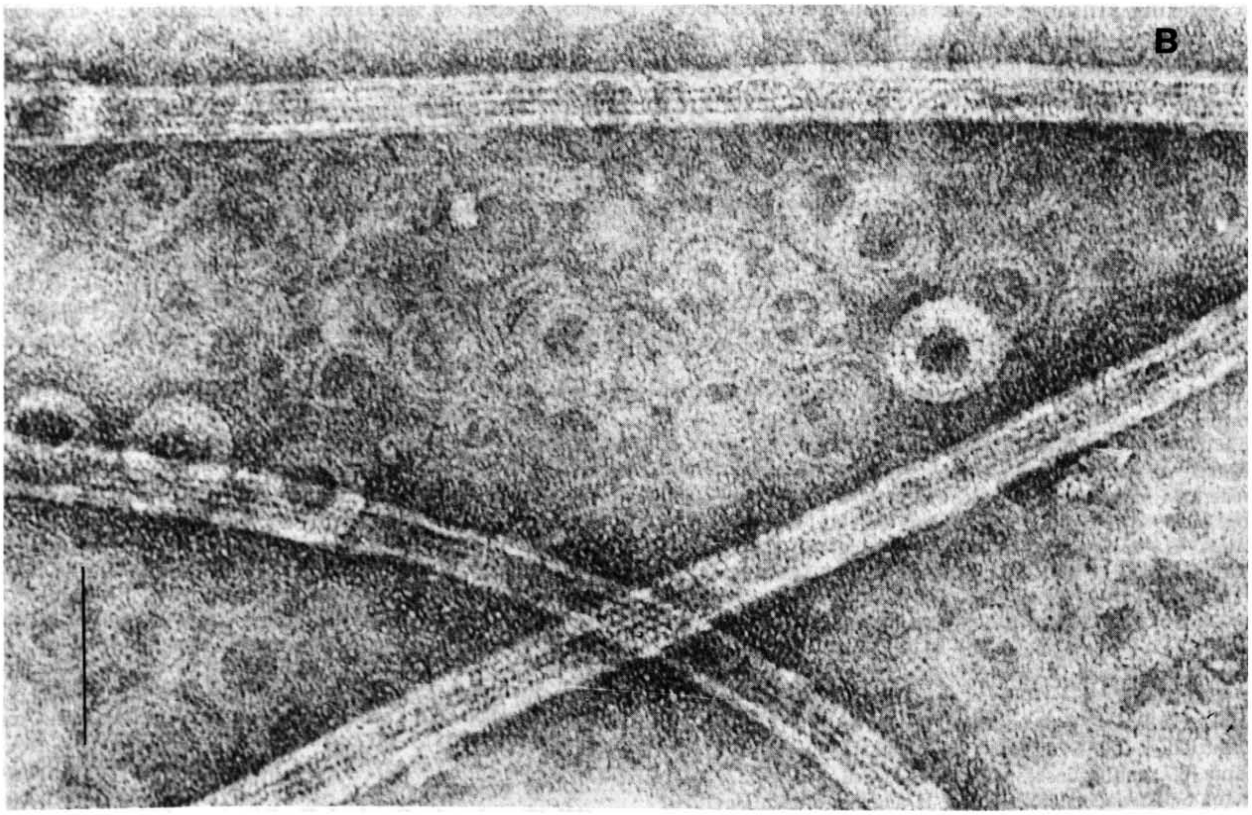


Fig. 2. (A) Turbidity time courses of tubulin assembly induced by Tau 2.1 in comparison with other synthetic constructs of altered primary structure. Tubulin and peptide concentration were 10^{-5} M each, in glycerol assembly buffer. Tracings 1–6 correspond to peptides Tau 2.1, 2.2, 2.3, 2.4, 2.5 and 2.6, respectively. Tracing 7 was obtained with poly-L-lysine of an average chain length of 50 residues (this absorbance recording is offset by −0.3). Samples were warmed from 4°C to 37°C at time 0, and re-cooled at the times indicated by the downward-pointing arrows. (B) Characteristic electron micrograph of the microtubules and rings induced by two-repeat tau synthetic peptides. Conditions are as in panel (A), and the actual sample shown corresponds to peptide Tau 2.6, i.e., the more disorganized sequence. The bar indicates 100 nm. The apparent external diameter of the partially flattened microtubules in the specimen is approximately 29–36 nm. The diameter of the middle ring of the triple rings, or the outer ring of the double rings, is about 50 nm. (C) Double-wall polymers induced by poly-lysine. The outer diameter of these structures is ca. 55 nm.



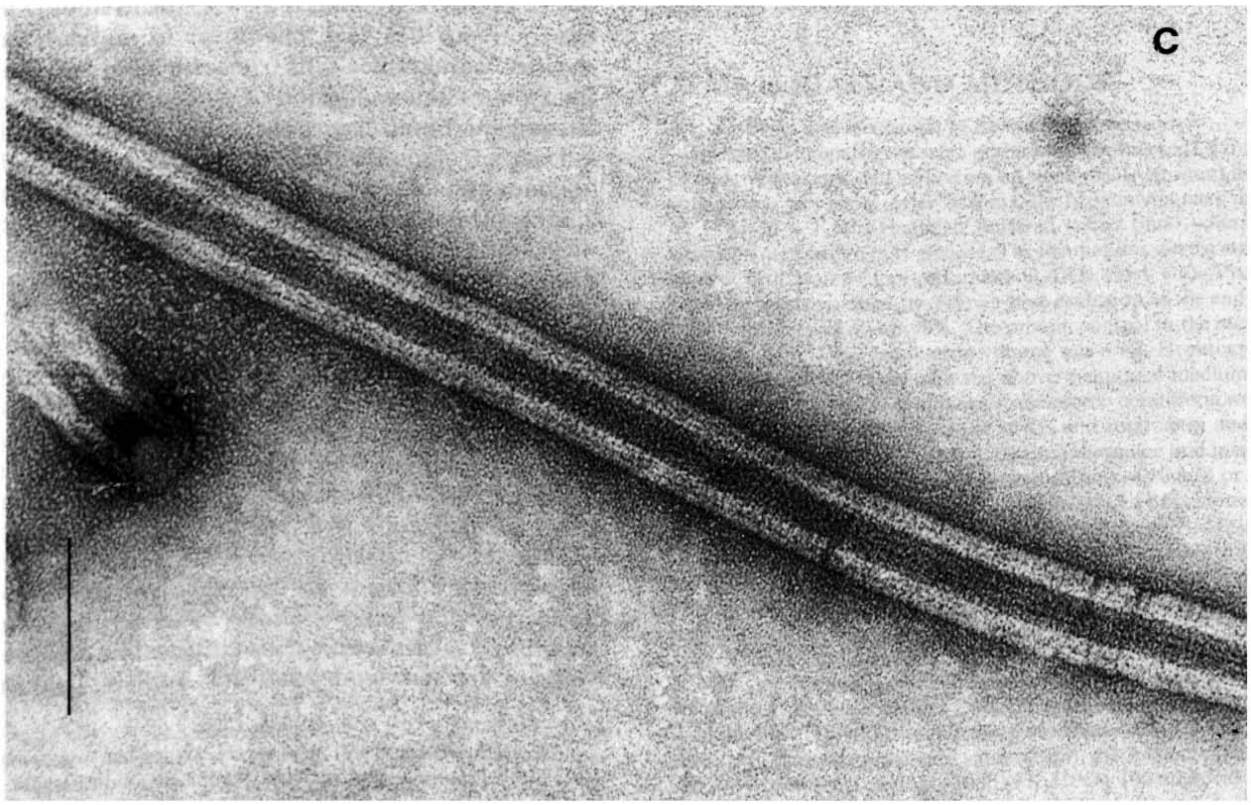


Fig. 2B and C.

relative binding affinities of the six double-repeat peptides, as well as their ability to displace MAPs from assembled microtubules.

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