# MAP-1 and MAP-2 Binding Sites at the C-Terminus of β-Tubulin. Studies with Synthetic Tubulin Peptides†

Daniel Cross,‡ Jorge Dominguez,§ Ricardo B. Maccioni,*,‡ and Jesûs Avila*,§

*International Center for Cancer and Developmental Biology (ICC), Casilla 70111, Santiago 7, Chile, and Centro de Biologîa Molecular, Universidad Autônoma-CSIC, Cantoblanco 28049, Madrid, Spain*

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ABSTRACT: The interaction of microtubule-associated proteins MAP-1 and MAP-2 with different peptides containing sequences covering the C-terminal region of β-tubulin isoforms has been analyzed. Our results indicate that MAP-1 and MAP-2 bind to a common sequence within the variable C-terminal region of the different β-tubulin isoforms, while MAP-2 also interacts with the subdomain β(422–434) of the constant region, in agreement with previous results (Maccioni, R. B., Rivas, C., & Vera, J. C. (1988) *EMBO J. 7*, 1957–1963). The productive interaction of MAP-2 with the latter domain appears to be involved in the assembly of microtubules.

The most well-known microtubule-associated proteins, MAP-1, MAP-2, and tau, copurify with tubulin through cycles of assembly and disassembly. They stabilize assembled microtubules, induce bundles of these polymers, and are involved in modulating tubulin polymerization into microtubules (Olmsted, 1986; Maccioni & Arechaga, 1987; Drubin et al., 1986; Lewis et al., 1989; Kanai et al., 1989). These functions are mediated by the selective interaction of MAPs with tubulin, and therefore, it is important to identify the tubulin sequences involved in the binding of different MAPs. Macromolecular interaction between microtubular components is a critical aspect in understanding the functions of microtubules and the regulation of their assembly.

MAP-1, MAP-2, and tau interact with the C-terminal domain of tubulin subunits (Serrano et al., 1984, 1985; Maccioni et al., 1985; Bhattacharyya et al., 1985; Rodionov et al., 1990). There is also indication of the interaction of cytosolic dynein MAP-1C with this tubulin moiety (Paschal et al., 1989). By the use of synthetic peptides defined by C-terminal tubulin sequences, it was demonstrated that MAP-2 and tau bind preferentially to β-tubulin (Littauer et al., 1986; Maccioni et al. 1988; Rivas et al., 1988, Vera et al., 1988). MAP-2 and tau share common sequences for their binding to tubulin (Lee et al., 1988; Lewis et al., 1988; Goedert et al., 1988; Maccioni et al., 1989), a finding that is correlated with the similarities in the interactions of these proteins with tubulin (Maccioni et al., 1988). However, a distinct MAP-1 sequence for its binding to tubulin has been described (Noble et al., 1989), suggesting a different mechanism for MAP-1 binding to tubulin with respect to MAP-2.

The C-terminal domain of β-tubulin is composed of two subdomains, one with a sequence that is constant among isoforms (amino acid residues 406–430) and a second region containing the C-terminal residue, which exhibits variability among β isoforms (Figure 1). Current evidence indicates that a synthetic peptide containing residues of the constant region and bearing the sequence β(422–434) from the tubulin βII isotype (Cleveland et al., 1990) binds to MAP-2 and tau and inhibits MAP-dependent polymerization of tubulin (Maccioni et al., 1988). The roles of both constant and variable regions were suggested in experiments using larger C-terminal peptides (Littauer et al., 1986). On the other hand, Paschal et al. (1989) suggested the involvement of a tubulin C-terminal sequence, rich in acidic amino acid residues within the variable region, as a site for MAP-1C and MAP-2 interactions. Here, we use synthetic peptides of C-terminal sequences within both constant and variable regions of β-tubulin isoforms, in order to shed further light onto the analysis of MAP-interacting tubulin sequences.

## Materials and Methods

*Protein Purification.* Microtubule protein was isolated from bovine brain by three temperature-dependent cycles of assembly–disassembly (Shelanski et al., 1973). Microtubule pellets were frozen in liquid nitrogen after the second cycle and stored at −80 °C. Before use, microtubule pellets were resuspended in 0.1 M morpholinoethanesulfonic acid (Mes), pH 6.5, 0.5 mM $MgCl_2$, and 2 mM EGTA (buffer A) and a third cycle was performed. MAPs were isolated as described (Serrano et al., 1984).

*Peptide Synthesis.* Tubulin peptides from different β isoforms, βI(408–426) FTEAESNMNDLVSEYQQYQ, βI(431–444) EEEEDFGEEAEEEA, βII(422–434) YQQYQDATADEQG, βII(434–440) GEFEEEG, and βIII(435–450) EMYEDDEDESESQGPK, were synthesized on an automatic solid-phase peptide synthesizer (Type 430A, Applied Biosystems) and purified by reversed-phase HPLC on a NOVAPACK C-18 column, with a Waters 501 apparatus. The purified peptides were lyophilized and dissolved in buffer A.

*Polymerization Assay.* Microtubule protein from bovine brain (1 mg/mL) was incubated in buffer A, in the presence of 0.5 mM GTP and in the presence or absence of tubulin peptides. Tubulin assembly was assayed by the sedimentation of polymers by use of a Beckman air-driven ultracentrifuge for 15 min at 140000*g* (Maccioni et al., 1988). The sedimented protein was characterized by polyacrylamide gel electrophoresis under the conditions described (Laemmli, 1970) and stained with Coomassie blue. The percentage of each protein fractionated by electrophoresis was determined by densitometric scanning of the Coomassie blue stained gels.

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* Address correspondence to these authors.

‡ International Center for Cancer and Developmental Biology.

§ Universidad Autônoma-CSIC.

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410 420 430 440

βI mefteaesnmndlvseyqqyqdataEEEEDFGEEAEEEA

βII mefteaesnmndlvseyqqyqdataDEQGEFEEEGEEDEA

βIII mefteaesnmndlvseyqqyqdataEEEGEMYEDDEDESESQGPK

FIGURE 1: Carboxyl-terminal sequences of mammalian brain β-tubulin isotypes. The residues present in the constant region are indicated in lower case letters and those of the variable domain are indicated in capitals. The underlined sequences correspond to the synthetic peptides used in the experiments described in the text. Sequence data were obtained from Sullivan (1988).

*Binding Assay.* Binding of the phosphorylated βIII-tubulin peptide to MAPs was performed by incubation of the peptide ($10^5$ cpm/μg) with MAPs immobilized in nitrocellulose paper for 1 h at 30 °C. Incubation was followed by a fast washing with buffer A, and the bound peptide was identified by autoradiography. In vitro phosphorylation of the synthetic tubulin peptides was performed essentially as indicated by Diaz-Nido et al. (1990). The peptides at a final concentration of 1 mM were incubated at 30 °C with 1 mM [$^{32}$P]ATP and casein kinase II. It was shown that Ser 444 in βIII(435–450) was the main phosphorylated residue. For the binding experiments of peptide βI(431–444) to MAPs, we proceeded as follows: (a) a peptide with an identical sequence but with an N-terminal tyrosine (YEEEEDFGEEAEEEA) was labeled by iodination with $^{125}$I; (b) the binding of the iodinated peptide was analyzed after transfer of MAPs components to nitrocellulose by use of overlay and autoradiography with the radioactive peptide as indicated above.

*Electron Microscopy.* Samples were obtained from the assembly mixture (both before and after the assembly the mixture was cooled to 0 °C), fixed in warm (32 °C) 0.1 M Mes, pH 6.8, 2% glutaraldehyde, 1 mM $MgCl_2$, 1 mM EGTA, and 1 mM GTP, stained with uranyl acetate, and observed in a Philips 300 electron microscope.

## RESULTS

To analyze the binding of MAP-1 and MAP-2 to different sequences within the C-terminus of the β-tubulin subunit, three peptides were synthesized whose sequences and localization in the C-terminal primary structure of β-tubulin are indicated in Figure 1. These peptides cover constant and variable sequences within the 4-kDa C-terminal tubulin domain defined from the subtilisin cleavage site at phenylalanine 408 (Figure 1) to the C-terminus of the β isoforms (Maccioni et al., 1985). The regions containing amino acids from the subtilisin cleavage site to alanine 430 contain the constant domain present in each β-tubulin isoform, while the domain from glutamic 431 (aspartic in βII isoform) to the C-terminal end contains the variable region for each isoform.

The method followed to study the binding of the tubulin peptides to MAPs was to analyze the effect of those peptides on microtubule polymerization and on the association of MAPs to microtubules. Figure 2A shows that the addition of 160 μg of peptide βI(431–444) to microtubule protein resulted in a 60% decrease in the assembly of microtubules. A similar inhibition was observed in the presence of the fragment βII-(422–434) (Figure 2B) in agreement with Maccioni et al. (1988), while the peptide βI(408–426), containing only residues of the constant region, did not affect at all the assembly of microtubules (Table I). Although βII(422–434) inhibited tubulin assembly induced by MAP-2, the peptide did not affect significantly MAP-1-induced assembly. It is worth pointing

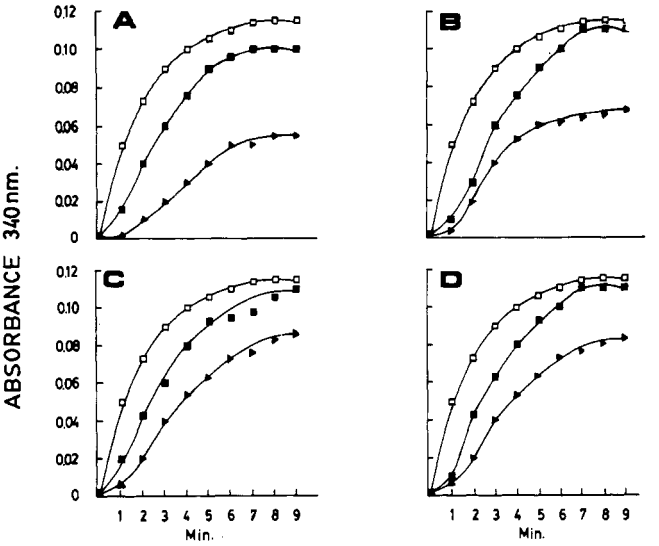


FIGURE 2: Effect of peptides on microtubule assembly. The figure shows the effects of peptide fragments βI(431–444) (A), βII(422–434) (B), βIII(435–450) (C), and βII(434–440) (D). In each graph, □ represents microtubules polymerized from microtubular protein (1 mg/mL) in the absence of any peptide, ■ represents polymerization in the presence of 80 μg of the respective peptide, and ▲ represents polymerization in the presence of 160 μg of each synthetic peptide. Polymerization was monitored at 340 nm in a Shimadzu spectrophotometer with a thermoregulated incubation chamber.

Table I: Effects of C-Terminal Tubulin Peptides on Tubulin/MAPs Assembly

| tubulin peptide | concn (μM) | extent of assembly[a] $\Delta A_{340}$ |
|---|---|---|
| none (control) | | 0.117 ± 0.008 |
| βI(408–426) | 160 | 0.121 ± 0.012 |
| βI(431–444) | 80 | 0.097 ± 0.017 |
| | 160 | 0.051 ± 0.006 |
| βII(422–434) | 80 | 0.108 ± 0.010 |
| | 160 | 0.057 ± 0.005 |
| βIII(435–450) | 80 | 0.110 ± 0.002 |
| | 160 | 0.091 ± 0.010 |
| βII(434–440) | 80 | 0.115 ± 0.014 |
| | 160 | 0.086 ± 0.008 |

[a] Samples of microtubular protein (1.1 mg/mL) cycled three times were adjusted to 1 mM GTP, and the assembly was assayed by the turbidimetric method either in the presence or in the absence of the synthetic tubulin peptides. The net change of absorbance at 340 nm ($\Delta A_{340}$) corresponds to the mean value of three experiments, including the respective mean errors.

out that the N-terminal moiety of βI(431–444) from the variable sequence and the C-terminal moiety of βII(422–434) have overlapping sequences (EEEE in the βI peptide and DEQG in the βII peptide). A decrease in the assembly of around 20% was observed in the presence of 160 μg of either peptide βIII(435–450) (Figure 2C) or peptide βII(434–440) (Figure 2D), which do not share overlapping sequences with βII(422–434). Table I summarizes the data in terms of extent of polymerization. The action of peptides on the assembly was also assayed by electron microscopy. A significant decrease in the amount of polymers was observed upon addition of the βI peptide (Figure 3A), while the number of microtubules found in the presence of the βIII peptide was not significantly lower than the controls (Figure 3C). Polymers assembled in the presence of the βI peptide exhibited a smoother surface (Figure 3B) as compared with controls (inset) and those obtained with the βIII-tubulin fragment (Figure 3D).

The direct binding of one of the previous peptides, βIII-(435–450), to MAP-1 and MAP-2 was studied by incubation of the $^{32}$P-labeled peptide (label at serine 444) with the respective MAP immobilized in nitrocellulose. Figure 4 shows

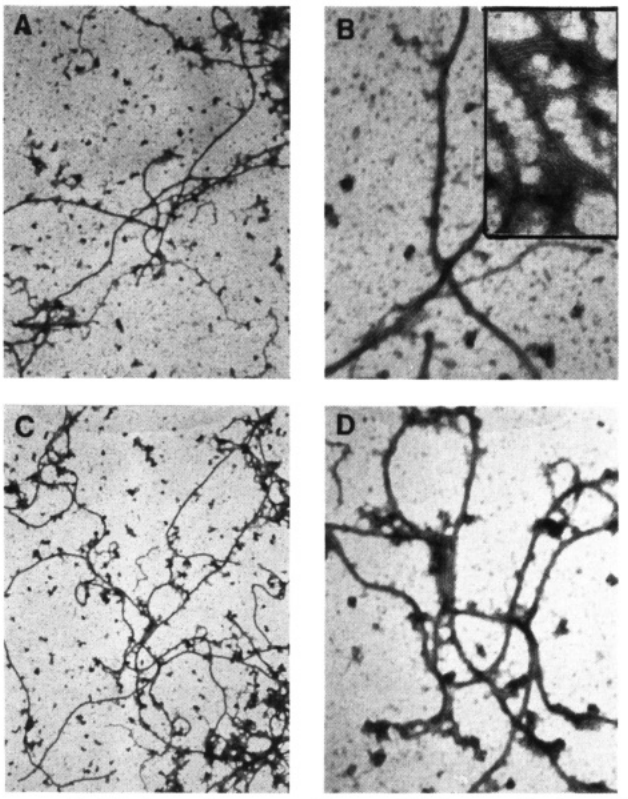


FIGURE 3: Electron microscopy at low and high magnifications of assembled products obtained in the presence of peptide βI(431–444) (A and B) and in the presence of peptide βIII(435–450) (C and D). Control polymers are shown in the inset. Magnification is 21000× (B, D, and inset) or 7000× (A and C).

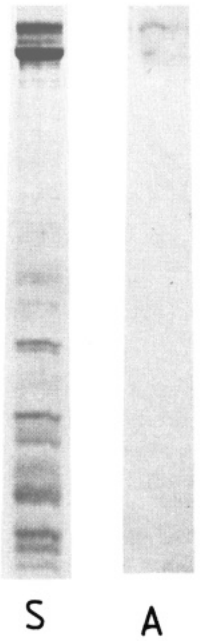


FIGURE 4: Interaction of the peptide βIII(435–450) to MAP-1 and MAP-2. A mixture of bovine brain MAPs were fractionated by gel electrophoresis and transferred to nitrocellulose paper. The paper was incubated with $^{32}$P-βIII(435–450) peptide under the conditions indicated in Materials and Methods, and the binding of the labeled peptide to MAPs was characterized by autoradiography. The left lane (S) indicates the Coomassie blue stained gel containing MAPs. The right lane (A) indicates the autoradiography of nitrocellulose paper containing the radioactive peptide associated to MAPs components.

that $^{32}$P-βIII(435–450) binds to both MAP-1 and MAP-2. Binding determinations of βI(431–444) with the same overlay procedure showed its interaction with both MAP-1 and

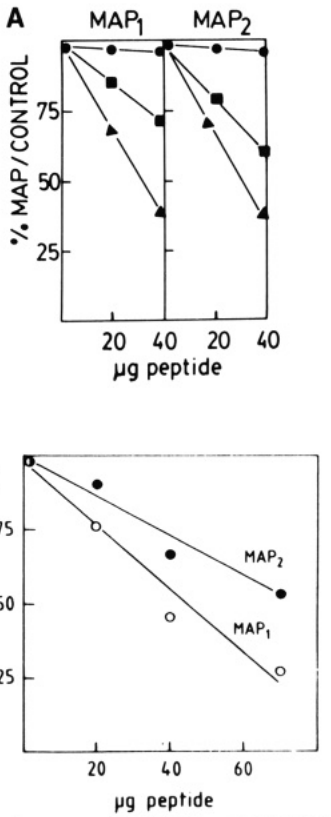


FIGURE 5: (A) Displacement of MAP-1 and MAP-2 from microtubules assembled in the presence of C-terminal β-tubulin peptides. Microtubular protein (1 mg/mL) in the absence of any peptide or in the presence of variable amounts of βI(408–426) (●) in the presence of βI(431–444) (▲), or in the presence of βIII(435–450) (■) was induced to assemble by incubation for 25 min at 30 °C in the presence of 1 mM GTP. The assembled polymers were sedimented in the Airfuge at 140000*g*, the pelleted fractions were subjected to gel electrophoresis, and the protein bands of stained gels were quantitated by densitometric analysis. Plots represent the percentage of MAPs remaining in the pellets of microtubules assembled in the presence of β-peptides with respect to that found in controls assembled in the absence of tubulin peptides. (B) Association of MAP-1 and MAP-2 to microtubules assembled in the presence of βII(434–440), GEFEEEG. The assembled protein was isolated by centrifugation, and the pellet fractions were subjected to electrophoresis; O represents the proportion of MAP-1 and ● represents the proportion of MAP-2 present in the polymers assembled in the presence of 20, 40, and 60 μg of peptide GEFEEEG with respect to that found in the absence of the peptide.

MAP-2 (data not shown). On the other hand, the preferential binding of βII(422–434) to MAP-2 and its effects in blocking tubulin–MAP-2 interaction have been already shown (Maccioni et al., 1988).

To test if the addition of peptides βI(431–444) and βIII-(435–450) results in a decrease in the amount of microtubules formed or in a differential decrease in the proportion of MAPs associated to microtubules, an experiment to determine the competition between those peptides and tubulin for their binding to MAPs was carried out. The protein polymerized in the presence or absence of the peptides was characterized by gel electrophoresis. The data of Figure 2 indicated a significant decrease in the amount of polymer formed in the presence of βI(431–444). Furthermore, the data of Figure 5A, on the basis of densitometric analysis, indicate that the addition of βI(431–444) also resulted in a decrease in the proportion of MAP-1 and MAP-2 in the total microtubular protein as compared with controls. Under the assembly conditions the amount of MAP-2 (and also MAP-1) was below the necessary amount to saturate all MAPs sites on microtu-

bules, considering that saturation is attained with a molar ratio of MAP2 (or MAP-1) to tubulin of 1:6 (Amos, 1977; Gottlieb & Murphy, 1983). An effect on MAPs displacement was also produced by peptide βIII(435–450), although in this case a lower decrease of MAPs with respect to total microtubular protein was found (Figure 5A). The data of Figure 5, along with the data of Figure 2, indicate that this peptide decreases the content of MAPs without affecting significantly the polymerization of microtubules. However, no effect on the amount of MAPs associated to microtubules is seen upon the addition of peptide βI(408–426) to microtubular proteins (Figure 5A).

Previous work demonstrated that the βII(422–434) peptide interacts with MAP-2 (Maccioni et al., 1988), binding that according to Figure 2B appears to be involved in tubulin assembly. Peptide βI(431–444) shares a subdomain with βII-(422–434), which appears to be the same domain involved in the productive interaction of MAP-2 with tubulin. On the other hand, peptides βI(431–444) and βIII(435–450), with overlapping sequences (residues 434–444), appear to share a common domain for the interaction of both MAP-1 and MAP-2. Since Paschal et al. (1989) recently suggested that the short sequence EEGEE may be involved in MAPs binding, we also assayed the effect of peptide βII(434–440), containing the acidic subdomain near the C-terminus β-tubulin, on microtubules polymerization. The addition of this peptide did not result in a significant inhibition of the assembly (Figure 2D). However, densitometric analysis of gel electrophoresis of microtubule pellets showed that this peptide produces a decrease in the amounts of MAP-1 and MAP-2 associated to microtubules (Figure 5B).

## Discussion

The results shown in this report suggest the presence of at least two binding subdomains for MAP-2 within both the constant and the variable C-terminal moieties of β-tubulin isoforms. These two β-tubulin subdomains were characterized as follows: (a) An interacting site is present within the constant domain that ends in a short, less homologous sequence of β isoforms, including amino acid residues 431–434 (Figure 1). This domain appears to be involved in the productive interaction of MAP-2 with tubulin as evidenced by the effects of peptides βI(431–444) and βII(422–434) on microtubule assembly and on MAPs displacement from microtubules (Figures 2 and 5). The short segment containing residues 431–434 corresponds to an overlapping sequence of these two peptides. (b) A common site for MAP-1 and MAP-2 interaction is present within the acidic subdomain of the variable moiety (GEFEEEGEE). Tubulin peptides sharing this acidic C-terminal segment interacted with both MAPs components. These two subdomains indicated in (a) and (b) may be discrete C-terminal segments contributing to the topographic domain for MAP-2 interaction or be parts of a continuous sequence covering constant and variable subdomains involved in the binding of MAP-2.

Despite the role of this acidic segment in the interaction of MAP-1 and MAP-2, the results also suggest that the more acidic sequence within the variable region does not appear to be directly involved in the MAPs-driven microtubule assembly. Actually, peptides βII(434–440) and βIII(435–450), sharing the acidic moiety, decreased the amount of MAPs incorporated into microtubules as shown in Figure 5. As evidenced from the Results section and the data of Figure 4, these peptides bind to both high molecular weight MAP-1 and MAP-2 but did not have a significant effect on tubulin assembly (Figures 2 and 3). Thus, an integrated analysis of the experiments points to the role of these two C-terminal tubulin subdomains in the interaction of MAP-2.

Our present results are in agreement with previous reports that indicate the selective role of the constant C-terminal tubulin sequence in the differential interaction of both MAP-2 and tau (Maccioni et al., 1988; Vera et al., 1988) and with those showing no alterations of MAP-2 interaction after removal of at least six residues from the tubulin C-terminus by carboxypeptidase Y (Vera et al., 1989). Additional support for the selective role of the constant sequence in MAP-2 interaction comes from recent studies that indicate the binding of monoclonal anti-idiotypic antibodies, produced by mice immunization with βII(422–434), to MAP-2 but not to MAP-1 (Cross et al., submitted for publication). The effects of MAP-reacting polyclonal anti-idiotypic antibodies on the assembly system were previously shown by Rivas et al. (1988). On the other hand, our data at the level of the variable domain are also compatible with those of Paschal et al. (1989) suggesting the role of the tubulin sequence EFEEEG in the interaction of both MAP-1 and MAP-2. It has been suggested that binding of a particular MAP-1, the cytosolic dynein MAP-1C, to tubulin may occur through a different mechanism (Rodionov et al., 1990).

The tubulin sequences shown to interact with MAP-2 are present in peptides that have been described as MAP-2 binding peptides (Maccioni et al., 1988; Cleveland et al., 1990; Paschal et al., 1990). The minimal homology between those peptides is the presence of two Glu (or Gln) residues adjacent to a glycine (or Ser as in the βIII peptide). This type of sequence is present in peptide βII(422–434) from the constant domain and in peptides βI(431–444), βII(434–440), and βIII(435–450) of the variable C-terminal region of β-tubulin. However, such a sequence is not found in peptide βI(408–426), which does not interact with MAPs, or in the rest of the β-tubulin molecule. Similar sequences were found in specific segments of other MAP-2 or tau binding proteins: TDSEEEI in calmodulin (Padilla et al., 1990), QEYDEAG in actin (Correas et al., 1990), and DEDGE in S-100 protein (Baudier & Cole, 1988).

Interestingly, it has been shown that MAP-2 as well as tau has more than one tubulin binding site (Lee et al., 1988; Lewis et al., 1988), although the repetitive MAPs sequences appear to bind to tubulin with a differential affinity (Joly et al., 1989; Maccioni et al., 1989). The presence of at least two acidic residues in MAP-2 binding sequences on tubulin may be complementary to the presence of two basic residues adjacent to the PGGG motif of the repetitive sequences of MAP-2 and tau, which appear to be implicated in their binding to tubulin (Joly et al., 1989; Maccioni et al., 1989; Padilla et al., 1990).

In regard to the binding sequence responsible for MAP-1 interaction, two or more adjacent acidic residues appear to be required. The data are in agreement with the work of Fujii et al. (1990) indicating that the addition of a homopolymer composed of acidic residues removes MAP-1 from microtubule protein. This finding suggests that the interaction of tubulin with MAP-1 is essentially of electrostatic nature, while tubulin interaction with MAP-2 appears to be mediated by both electrostatic forces and conformational effects (Maccioni et al., 1985, 1988; Yamauchi & Purich, 1987).

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