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Common and distinct tubulin binding sites for microtubule-associated proteins

(microtubule-associated protein 2/τ factors/carboxyl-terminal domain)

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ABSTRACT **A specific binding assay was developed that monitors the interaction of ^{125}I-labeled microtubule-associated proteins (MAPs) with tubulin or its fragments bound to nitrocellulose membrane. To identify the tubulin-binding domains for MAPs we have examined the binding of rat brain ^{125}I-labeled MAP2 or ^{125}I-labeled τ factors to 60 peptides derived from porcine α- and β-tubulin. MAP2 and τ factors specifically interacted with two peptides derived from the carboxyl-terminal region of β-tubulin, which are located between positions 392–445 and 416–445. In addition, there is a distinct τ-binding site at the amino-terminal region of α-tubulin. τ factors but not MAP2 displayed strong interaction with a peptide derived from the amino-terminal domain of α-tubulin between positions 1 and 75. To narrow down the location of the β-tubulin binding site that is common to MAP2 and τ factors, we have synthesized five peptides that are homologous to the corresponding sequence from the porcine or rat carboxyl-terminal region. Binding studies with the synthetic peptides suggest that amino acid residues 434–440 of β-tubulin are crucial for the interaction of MAP2 and τ factors.**

Microtubules are involved in a number of cellular processes, such as chromosomal movement, cell migration, intracellular transport, formation of cell shape, and secretion (1, 2). The major protein constituent of microtubules is tubulin, which is a heterodimer composed of α- and β-subunits, each having a molecular mass of 50 kDa (3–5). In addition to tubulin, microtubules contain a heterogeneous class of proteins, known as microtubule-associated proteins (MAPs), which copolymerize with tubulin and have been shown to be potent promoters of tubulin assembly *in vitro*. The diversity of microtubule functions has stimulated the idea that not all microtubules are alike and that different microtubules with distinct functions are present in different cells and even within a single cell. This may result from the assembly of microtubules from different isotubulin and MAP forms. Thus, brain α- and β-tubulins display extensive microheterogeneity when compared with tubulin from other tissues (6, 7). Brain microtubules are particularly rich in the variety of MAPs they contain and >20 distinct polypeptide bands have been identified by polyacrylamide gel electrophoresis (8, 9). The best-studied brain MAPs are the high molecular mass MAPs termed MAP1 (350 kDa) and MAP2 (270 kDa) (10, 11) and, more recently, MAP3 (180 kDa) and MAP4 (220 kDa) (12, 13) as well as a number of proteins in the molecular mass range of 48–62 kDa, known collectively as τ proteins (14). Although MAP2 and τ factors appear to be differentially compartmentalized in individual cells, they can compete *in vitro* for the same binding sites on microtubules (15, 16). It also appears that in living cells the microtubule binding sites for MAP2 are conserved regardless of the presence or absence of this protein in the cells (17). Several reports have suggested that the highly charged carboxyl-terminal region of tubulin (3, 4) may be involved in the binding of both MAP2 and τ factors (18–23). We have developed a specific binding assay that enabled us to monitor the interaction of MAP2 and τ factors with tubulin peptides and thus localize precisely the MAP binding sites of tubulin.

MATERIALS AND METHODS

Materials. Nitrocellulose membrane filters were obtained from Schleicher & Schuell; $NaDodSO_4$ and bovine serum albumin were from Sigma. Crude brain extracts were prepared from 12-day-old rats. Freshly dissected brains were homogenized for 20 sec at 0°C in buffer A [0.1 M 2-(*N*-morpholino)ethanesulfonic acid, pH 6.4/0.5 mM $MgCl_2$/0.1 mM EDTA/1.0 mM EGTA/1.0 mM dithiothreitol] containing 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, aprotinin (10 international units/ml), pancreatic RNase (100 μg/ml), and pancreatic DNase (100 μg/ml). The extracts were centrifuged at 100,000 × *g* for 60 min. Microtubule proteins were purified by two cycles of assembly and disassembly in the presence of 4 M glycerol and 1 mM GTP (24). Tubulin was isolated by phosphocellulose column chromatography (PC tubulin) (14). Heat-stable MAPs were prepared from microtubule pellets (25). MAP2 was separated from τ proteins by chromatography on an LKB Ultrogel AcA34 polyacrylamide/agarose column. Total MAPs, MAP2, or τ proteins were labeled with ^{125}I with *N*-succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate (26) obtained from Amersham. The ^{125}I-labeled protein solutions were passed through Sephadex G-100 columns equilibrated with buffer A containing 1% bovine serum albumin and eluted with the same buffer.

Preparation of Peptides. Porcine brain α- and β-tubulin were cleaved with cyanogen bromide, trypsin, mouse submaxillary gland protease, and *Pseudomonas fragi* protease (3, 4). The peptides were separated by reversed-phase high-pressure liquid chromatography and characterized by amino acid composition and sequence analyses (27). The concentrations were calculated from these analyses. Peptide synthesis was carried out by the solid-phase method according to Merrifield (28). The synthesized peptides were purified by gel filtration thrugh Sephadex G-25 (fine) column equilibrated in 10% acetic acid or in 0.01 M HCl. The peptide-containing fractions were lyophilized and then dissolved in 0.01 M HCl. Composition of the peptide was confirmed by amino acid analysis.

Binding of ^{125}I-Labeled MAPs (^{125}I-MAPs) to Tubulin or Its Fragments Bound to Nitrocellulose Membrane. Rat brain PC

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Abbreviations: MAPs, microtubule-associated proteins; PC tubulin, tubulin isolated by phosphocellulose chromatography; ^{125}I-MAP, ^{125}I-labeled MAP.

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tubulin in 3 μl of buffer B [25 mM 2-(*N*-morpholino)-ethanesulfonic acid, pH 6.4/0.5 mM $MgCl_2$/0.1 mM EDTA/1.0 mM dithiothreitol] (0.05–0.2 nmol per spot) or various peptides in 0.01 M HCl (0.1–0.4 nmol per spot) were spotted on a nitrocellulose membrane and then air-dried. The membrane was soaked in buffer A containing 3% bovine serum albumin for 30 min at room temperature with gentle shaking. The buffer was removed and the membrane was incubated with buffer A containing 3% bovine serum albumin, 1 mM GTP, and ^{125}I-MAPs (1–3 × 10^6 cpm/ml) for 60 min at room temperature with gentle shaking. The membrane was washed twice for 30 min each with buffer A containing 3% bovine serum albumin, air-dried, and subjected to autoradiography.

RESULTS

Identification of Specific Tubulin Binding Sites to MAPs. Analysis of ^{125}I-MAP2 and τ fractions by gel electrophoresis indicated a complete separation of the two groups of proteins. The MAP2 fraction displayed several additional high molecular mass bands, which are the products of limited endogenous proteolysis (9), while the major τ band had an apparent molecular mass of 52 kDa. A specific binding assay of ^{125}I-MAPs to tubulin immobilized on nitrocellulose membrane was developed. The specificity of the assay was examined in preliminary experiments in which tubulin or crude rat brain extracts were subjected to gel electrophoresis and then transferred by blotting onto nitrocellulose membrane. The nitrocellulose membrane was incubated with ^{125}I-MAP2 or τ proteins and after washing, the dried membrane was subjected to autoradiography. The ^{125}I-MAPs were mainly found to bind to tubulin, while the other brain proteins were almost unlabeled (Fig. 1).

A similar procedure was applied to examine the binding of ^{125}I-MAPs to tubulin peptides attached to nitrocellulose membrane. Initial experiments showed that only a few peptides were capable of binding strongly to the ^{125}I-MAPs. The above results encouraged us to assay the binding properties of the isolated ^{125}I-MAP2 and ^{125}I-labeled τ proteins to a series of peptides. Fig. 2*a* shows the autoradiogram of the binding of ^{125}I-MAP2 to 46 peptides derived from porcine α- and β-tubulin. Peptides were applied at 0.2 nmol per spot and their position in the sequence of tubulin and location on the autoradiogram are given in Fig. 2*c*. Location

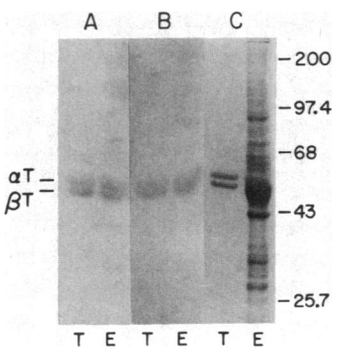

FIG. 1. Specificity of ^{125}I-MAP2 and τ binding to tubulin. PC tubulin (lanes T) and crude rat brain extract (lanes E) were subjected to slab $NaDodSO_4$/10% polyacrylamide gel electrophoresis (29, 30) and then transferred to nitrocellulose membrane by electrophoretic blotting. The nitrocellulose membrane was cut into two strips, which were incubated with ^{125}I-MAP2 (strip A) and τ factors (strip B), then washed, dried, and subjected to autoradiography. A Coomassie brilliant blue staining of a gel strip is shown in *C*. αT and βT represent the positions of migration of α- and β-tubulin. The positions of migration of the molecular size markers (in kDa) are indicated on the right margin.

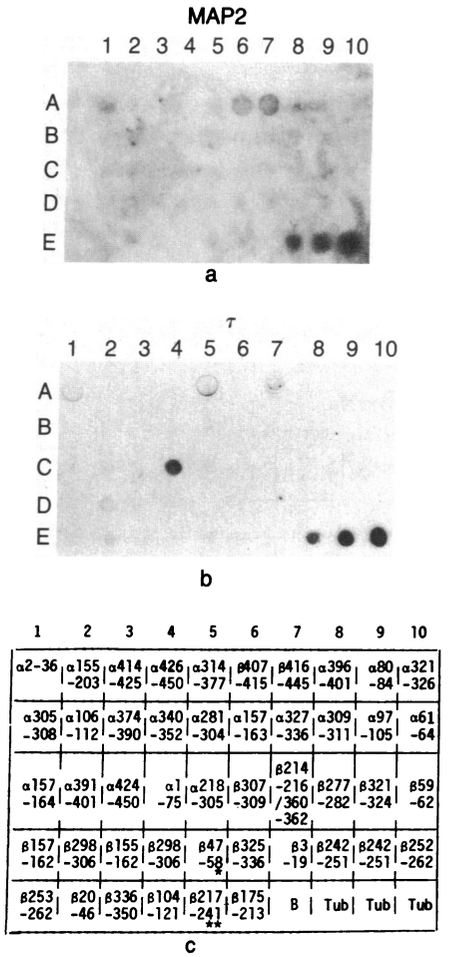

FIG. 2. Autoradiogram of the binding of ^{125}I-MAP2 and τ factors to peptides derived from porcine α- and β-tubulin. Each peptide (0.2 nmol per spot) was applied on the nitrocellulose membrane, and the binding of MAP2 (*a*) and τ (*b*) was assayed as described. The corresponding positions of the peptides on the tubulin subunit are depicted in *c*. Coordinate location E7 is blank (B) and at E8–E10, PC tubulin (0.05, 0.1, and 0.15 nmol per spot) was applied (Tub). *Peptide 47–58 (D5) is derived from the major variant of β-tubulin corresponding to the following amino acid sequence I N V Y Y N E A A G N K. **β-Tubulin peptide 217–241 (E5) showed weak binding to ^{125}I-labeled τ factors. However, in several other experiments, intermediate or strong binding was observed, including those shown in Fig. 4 (L1–L3).

E7 served as a blank. At E8, E9, and E10, increasing amounts of tubulin were applied (0.05, 0.10, and 0.15 nmol, respectively), which served as positive controls for the binding assay. Binding to long peptides was observed as compact spots, while binding to short peptides appeared as rings. The results show that ^{125}I-MAP2 strongly interacts with two peptides derived from β-tubulin, located at the carboxyl-terminal region between positions 407–415 and 416–445 (locations A6 and A7, respectively). On the other hand, none of the peptides derived from α-tubulin showed strong binding with MAP2. Weak binding was apparent with α2–36, α1–75, and α314–377 (A1, C4, and A5, respectively) as well as β20–40 (E2). Several other β-tubulin peptides, β217–241 and β298–306 (E5, D2, and D4) showed weak or, in other experiments, inconsistent binding.

Similar experiments were carried out with ^{125}I-labeled τ factors (Fig. 2*b*). The results also demonstrate strong binding of ^{125}I-labeled τ factors to a peptide derived from the carboxyl-terminal region of β-tubulin between positions 416 and 445 (A7). However, unlike MAP2 the τ proteins did not bind to β407–415 (A6). In addition, τ factors, but not MAP2,

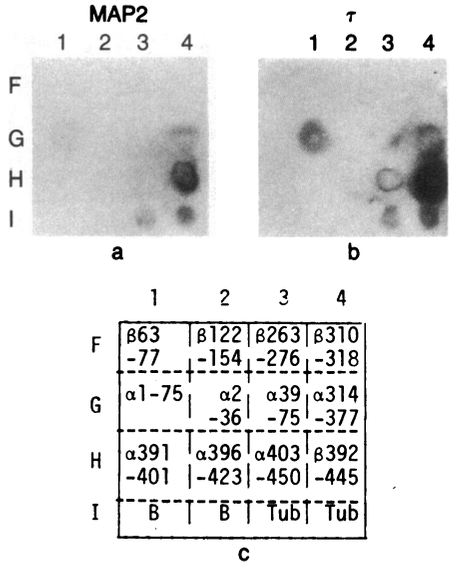

FIG. 3. Binding of MAP2 and τ factors to peptides. Peptides were applied at 0.2 nmol per spot. Coordinate locations I1 and I2 are blanks (B); at I3 and I4, PC tubulin was applied at 0.05 and 0.1 nmol per spot, respectively (Tub).

showed strong interaction with a peptide from the amino-terminal region of α-tubulin, α1–75 (Fig. 2*b*, C4; Fig. 3, G1; Fig. 4, J1–J3). In contrast to α1–75, two peptides derived from sequence positions α2–36 (Fig. 2*b*, A1; Fig. 3, G2) and α39–75 (Fig. 3, G3) displayed only weak binding. Weak binding was also found for β20–46 (Fig. 2*b*, E2) and β298–306 (Fig. 2*b*, D2 and D4). α-Tubulin peptide 314–377 showed inconsistent results. Thus, in some experiments it showed strong or intermediate attachment to ^{125}I-labeled τ factors (Fig. 2*b*, A5; Fig. 3, G4), while in others weak binding was observed (Fig. 4, J4–J6). Similarly, β-tubulin peptide 217–241 showed weak binding in experiments depicted in Fig. 2*b* (E5), and in several other experiments it showed intermediate or strong binding, including those depicted in Fig. 4 (L1–L3). As

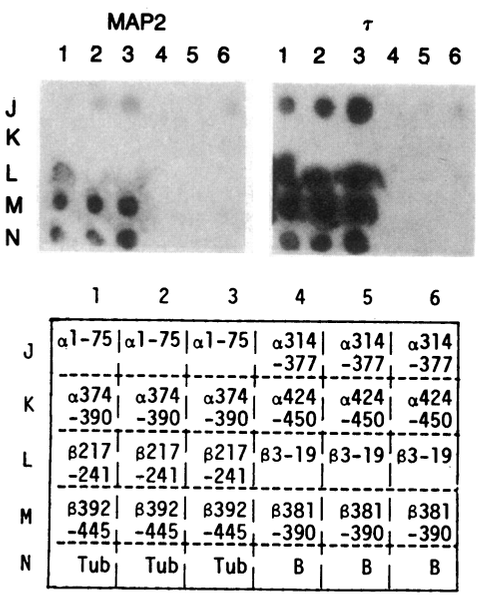

FIG. 4. Binding of MAP2 and τ factors to increasing concentrations of peptides. Peptides were applied at 0.1 (lanes 1 and 4), 0.2 (lanes 2 and 5), and 0.4 (lanes 3 and 6) nmol per spot. At coordinate locations N1–N3, PC tubulin was applied at 0.05, 0.1, and 0.2 nmol per spot (Tub), while N4–N6 served as blanks (B).

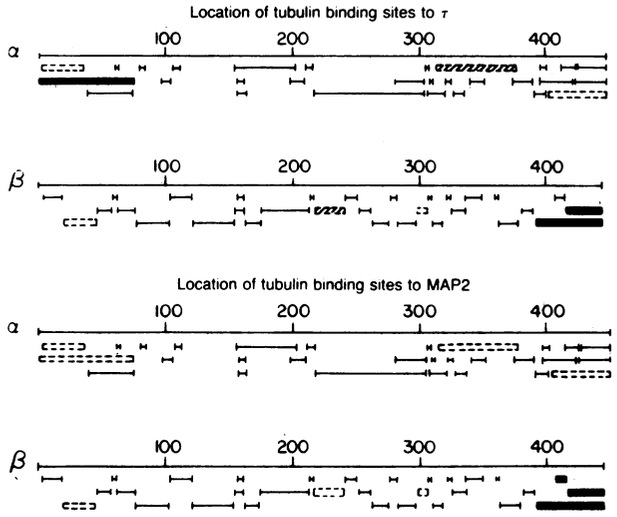

FIG. 5. The location of MAP2 and τ binding sites on the cleavage map of α- and β-tubulin. Filled bars and hatched bars denote peptides that show strong and intermediate binding, respectively, to ^{125}I-labeled τ factors and ^{125}I-MAP2. Open bars signify weak or inconsistent binding sites, while inactive peptides are indicated by lines. The latter include binding experiments with several peptides that are not shown in Figs. 1–4.

shown in Fig. 3, both MAP2 and τ factors display very strong binding to β-tubulin peptide 392–445 (H4). In addition, τ factors and perhaps MAP2 weakly interacted with α-tubulin peptide 403–450 (H3), while α391–401 (H1) and α396–423 (H2) were inactive. The binding of MAP2 and τ factors to increasing concentrations of a number of peptides is shown in Fig. 4. The results serve to illustrate again the unique binding of τ proteins to α-tubulin peptide 1–75 (J1–J3) and the strong binding of MAP2 and τ protein to β-tubulin peptide 392–445 (M1–M3). Several other peptides including α424–450 (K4–K6) were inactive. All these experiments are summarized in Fig. 5, which depicts the location of the MAP2 and τ binding sites on the cleavage map of α- and β-tubulin.

Binding of ^{125}I-MAP2 and ^{125}I-Labeled τ Factors to Synthetic Peptides Corresponding to Part of the Carboxyl-Terminal Domain of Brain β-Tubulin. To narrow down the location of the β-tubulin binding site that is common to MAP2 and τ factors, we have synthesized five peptides that are homologous to the corresponding sequence from porcine (4) or rat (31) carboxyl-terminal region (see Fig. 6 for the detailed sequence). Binding experiments were carried out with 0.2

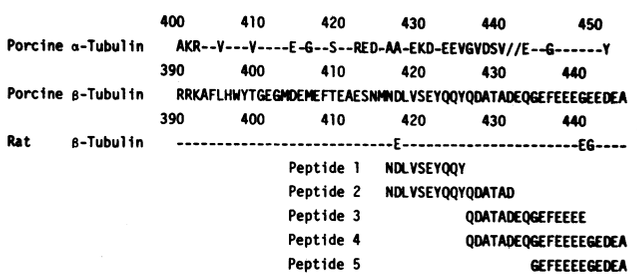

FIG. 6. Comparison of the amino acid sequence from the carboxyl-terminal region of porcine β-tubulin to that of porcine α-tubulin and rat β-tubulin. Gaps (/) were introduced at positions 441–442 in α-tubulin to obtain maximal homology (32). The rat β-tubulin amino acid sequence was derived from the nucleotide sequence of cDNA clones Tβ15 (31). Only positions that differ from porcine β-tubulin are indicated. Dashes (-) indicate identical residues. It should be noted that the carboxyl-terminal region of rat α-tubulin sequence (33, 34) is identical to the porcine sequence (3). The sequence of the synthetic peptides is also indicated. Amino acids are identified by the single-letter code.

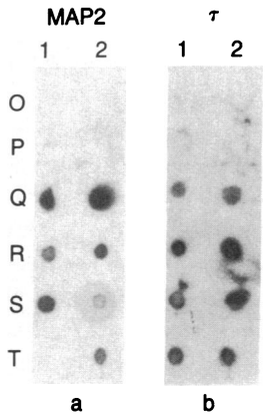

FIG. 7. Binding of ^{125}I-MAP2 and τ factors to synthetic peptides corresponding to the carboxyl-terminal region of β-tubulin. Peptides were applied at 0.2 and 0.4 nmol per spot (lanes 1 and 2). At coordinate locations Q1 and Q2, PC tubulin was applied (0.1 and 0.2 nmol per spot). Coordinate location T1 (*a*) is blank. Peptides 1 (O1, -2) and 2 (P1, -2) are homologous to positions: 416–425 and 416–431 from porcine β-tubulin. Peptides 3 (T2), 4 (S1, -2), and 5 (R1, -2) are homologous to positions: 426–440, 426–445, and 434–445 from rat β-tubulin, respectively.

and 0.4 nmol for each peptide (Fig. 7, lanes 1 and 2, respectively), except for tubulin, which was applied at a concentration of 0.1 and 0.2 nmol, while location T1 (Fig. 7*a*) served as a blank. As shown, peptide 1 and 2 corresponding to porcine β-tubulin positions 416–425 and 416–431 (O1, -2 and P1, -2) were inactive. On the other hand, peptides 3, 4, and 5 corresponding to rat β-tubulin positions 426–440, 426–445, and 434–445, showed strong binding to both ^{125}I-MAP2 (T2; S1, -2; and R1, -2, respectively) and τ factors (T1, -2; S1, -2; and R1, -2). These results would indicate that amino acid residues 434–440 of β-tubulin are crucial for the binding of MAP2 and τ factors.

DISCUSSION

The data presented in this study demonstrate that a common binding site for MAP2 and τ factors is located at the carboxyl-terminal region of β-tubulin. There is, however, another τ binding site at the amino-terminal region of α-tubulin. In addition, several fragments derived from α- and β-tubulin displayed intermediate or weak binding sites for MAP2 and/or τ factors (Fig. 5). More quantitative parameters could not be obtained from the results, since estimates of each peptide remaining attached to the nitrocellulose membrane were difficult to obtain. The use of nitrocellulose membranes for the binding assay appears to be specific since only a few peptides did interact with MAPs. The binding of the peptides is not related to net charge. For example, peptides derived from the carboxyl-terminal region of β-tubulin bind both MAP2 and τ factors, while a similar acidic peptide derived from α-tubulin has a low binding affinity for MAPs (Fig. 5). Moreover, strong binding to α-tubulin peptide 1–75 was only observed with τ factors. Thus, the binding is specific and the method may be used to map binding sites of other proteins. In addition to the strong binding of MAP2 to the carboxyl-terminal region of β-tubulin, several weak binding sites were revealed with peptides from α-tubulin. These may contribute to the binding of MAP2 to α-tubulin as observed in the experiment in which the interaction of α- and β-tubulin were assayed after separation by gel electrophoresis and transfer to nitrocellulose membrane (Fig. 1). It should be noted that we have not covered the entire α- and β-tubulin sequences and that several gaps still exist in our analysis that could serve as potential binding sites for MAP2 and τ proteins. In addition, it is possible that the cleavage of the protein caused the inactivation of some potential binding sites. This possibility is supported by the observation of strong binding of τ to α1–75, while it bound only weakly to α2–36 and not at all to α39–75. Evidence from other groups indicated that the carboxyl-terminal regions of both α- and β-tubulin are involved in the binding to MAP2. Protection of α-subunit and β-subunit against subtilisin digestion has been observed when proteolysis was performed in the presence of added MAP2 (21, 22, 35). Thus the carboxyl-terminal region of tubulin appears to serve as a regulatory domain in the polymerization of microtubules.

The binding experiments with the synthetic peptides would indicate that amino acid residues 434–440 of β-tubulin are crucial for the binding of MAP2 and τ factors. This suggestion is unexpected since a peptide derived from α-tubulin residues 403–450 showed a weak binding (Fig. 3, location H3), and α-tubulin peptide 424–450 was inactive (Fig. 2, location C3; Fig. 4, location K4–K6). These α-tubulin-derived peptides contain the amino acid sequence G E G E E E G E E at positions 442–450 that is very similar to the sequence G E F E E E G E E found in porcine β-tubulin at positions 434–442 and G E F E E E E G E in rat β-tubulin (Fig. 6). It is noteworthy that the adjacent sequences toward the amino-terminal end of the α- and β-subunits are completely different. This may suggest that the sequences preceding this acidic region modify its binding properties to MAPs. It would therefore appear that the synthesis of more peptides homologous to the carboxyl-terminal region may widen our understanding of the modulation of the properties of the MAP binding sites.

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