The Neuronal Cytoskeleton

The phosphatidylinositol-binding site of microtubule-associated protein MAP2
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Synopsis
Recent evidence [Surridge and Burns, Biochemistry (1994) 33, 8051–8057] on the interaction of native and recombinant tau, recombinant MAP2c, and native MAP2 with vesicles prepared from phosphatidylinositol (PtdIns) and other phospholipids demonstrate that MAP2 differs from MAP2c and from tau in having a high-affinity PtdIns-binding site. The location of this site within the MAP2-specific insert peptide, coupled with considerations of the nature of the MAP2 tubulin-binding site, suggests that PtdIns-binding induces a conformational change which alters the MAP2 tubulin-binding domain. Furthermore, the restricted cellular distribution of MAP2 implies that the MAP2-PtdIns interaction may play a central role in modulating the dendritic cytoskeleton.

Introduction
Immunological and other cytological studies have established that axons and dendrites contain different subsets of the microtubule-associated proteins (MAPs). In particular, the 200 kDa MAP2 is present in dendrites and dendritic processes while MAP2c, a differential splice product of the MAP2 gene which is selectively expressed during early development, is present in both axons and dendrites [1–3]. Furthermore, members of the tau family of MAPs are preferentially localized within the axons and are largely absent from dendrites and dendritic processes (e.g. [3]). Consequently, properties exhibited by MAP2 but not by either MAP2c or the tau proteins may be important as far as the dendritic cytoskeleton of MAP2c differs from MAP2 by the deletion, for the mouse sequence, of a 1372-residue internal peptide ([4–6], Figure 1). MAP2 and MAP2c therefore share common N- and C-terminal peptides (1–147 and 1519–1828 for the mouse sequence). The C-terminal peptide includes the tubulin-binding domain, which is highly homologous to the equivalent peptides of the tau proteins and the widely expressed MAP4 (see [7,8]). These tubulin-binding sequences are characterized by (a) being highly cationic; (b) having, as a result of differential gene splicing, either three or four 18-residue quasi-repeats which are highly homologous to each other [7,8]. These repeats are separated by somewhat more divergent 'spacer' peptides. In addition, MAP2 (and MAP2c) and tau each have a (non-identical) proline-rich peptide N-terminal to the quasi-repeats, and a protease-sensitive site. Cleavage at this site divides MAP2, MAP2c and tau into two structural domains, the C-terminal of which binds to assembled microtubules [28,33,36,37]. Most of the phosphorylation sites labelled by the cAMP-activated protein kinase map to the C-terminal fragment [43], which also contains the binding sites for estramustine phosphate [21,24].

Figure 1
The primary structures of MAP2, MAP2c and tau, showing that MAP2 and MAP2c share common N- and C-terminal peptides, but that MAP2c lacks the extensive MAP2 insert peptide

<table>
<thead>
<tr>
<th>N-</th>
<th>MAP2 Insert Peptide</th>
<th>C-</th>
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<tbody>
<tr>
<td>MAP2</td>
<td>N-</td>
<td>C-</td>
</tr>
<tr>
<td>MAP2c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tau</td>
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Abbreviations used: MAP, microtubule-associated protein including such proteins as MAP2, MAP2c, and the tau isoforms; PtdCho, phosphatidylcholine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine.

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interactions which are dispersed along about 140 residues (for the 4-repeat isoforms, and including contributions from the proline-rich peptide) of the C-terminal peptides (e.g. [9]), although there is some dissent about which peptides are the most important (e.g. [9] versus [10]). Furthermore, this model is consistent with the incremental reduction in the affinity of MAP2 for the microtubule lattice by increasing levels (0–12 mol·mol⁻¹) of phosphorylation by the co-puriﬁcating cyclic AMP-activated protein kinase [11,12], and by the effects of this phosphorylation on the promotion of microtubule assembly by both MAP2 and tau [12,13]. Such studies, together with the variation in the number of tandem repeats, have suggested that MAP2, tau, and MAP4 promote microtubule assembly by ‘lacquering’ the surface lattice [10], so reducing the anionic surface charge associated with the C-terminal peptides of the α- and β-tubulin subunits. This low-affinity ‘lacquering’ mechanism may also account for the observed gelation of actin ﬁlaments [14–17], even though actin and tubulin share minimal sequence similarity. Indeed, gelation is effected by MAP2, tau, and one of the shared tandem repeats [18], and it is inhibited, in common with microtubule assembly, by the level of phosphorylation of MAP2 by the cyclic AMP-activated protein kinase [16,19].

MAP-dependent microtubule assembly is speciﬁcally inhibited by two ligands: estramustine phosphate [20–24] and phosphatidylinositol (PtdIns) [25–28]. Microtubule assembly studies and direct ligand-binding assays indicate that estramustine phosphate binds to multiple (3–6) sites on MAP2 with an apparent Kₐ of ~20 μM [22,23]. These sites may correspond to the tandem repeats of the C-terminal peptide [22,24]. The effects of estramustine phosphate are therefore similar to those resulting from the multiple phosphorylation by the cyclic AMP-activated protein kinase, and support the general model that the promotion of microtubule assembly by the MAP C-terminal peptide is due to charge neutralization, and that any reduction in the cationic nature of the C-terminal peptide reduces its assembly-promoting ability.

It is more difficult to reconcile this model with the evidence that PtdIns inhibits microtubule assembly, and actin gelation [27], by binding to a single high-affinity site on MAP2 [26]. MAP-dependent microtubule assembly is also inhibited by other phospholipids [e.g. 25,26], but studies of the inhibition by phosphatidylcholine (PtdCho) vesicles doped with such phospholipids have clearly shown that only PtdIns binds to a specific high-affinity site (Kₐ < 1 μM MAP2, [26]). More recent work, which is summarized here, has established that there is a low-affinity interaction (apparent Kₐ 1.5–2.5 μM) between PtdIns and the highly cationic C-terminal peptide of MAP2, MAP2c and tau, and an additional high-affinity (apparent Kₐ ~ 221 nM) interaction between PtdIns and the MAP2 insert peptide, such that the apparent MAP2·PtdIns Kₐ is reduced to ~ 51 ± 6 nM MAP2 [28]. These binding studies strongly suggest that the MAP2 tubulin- (and actin-)binding site may have a more complex structure than previously proposed and may undergo a conformational change in response to PtdIns-binding, and that PtdIns-binding may be of considerable importance to the physiological control of the dendritic cytoskeleton.

Methods

Native MAP2 was puriﬁed from bovine microtubule protein by phosphocellulose chromato- graphy while native bovine tau was puriﬁed directly from bovine brain by heat denaturation and DEAE-cellulose chromatography [28]. Clones expressing murine MAP2c or human tau (pET NCO/MAP2c and pRK172 4RT/383; generous gifts from Drs. Andrew Matus and Michel Goedert) were grown in Escherichia coli, and the over-expressed proteins puriﬁed by fractionation on carboxymethyl-Sepha- rose [28,29]. The binding of these puriﬁed MAPs to phospholipid vesicles was assayed by incubating (10 min, 20°C) increasing concentrations (0–50 nM) of the clariﬁed (100000 g, 30 min) proteins with vesicles in MEM (100 mM Mes, 2.5 mM EGTA, 0.5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, pH 6.4, with KOH) buffer containing a ﬁnal concentration of 67 mM NaCl, and then pelleting the vesicles (100000 g, 30 min). The bound protein was quantiﬁed by colloidal gold dot blotting (see [28]), and used to derive Scatchard plots from which the apparent dissociation constants and the attached S.D. values were calculated by weighted least-square regression.

Results

The apparent Kₐs of native bovine tau and recombinant human tau383 to PtdIns vesicles are 2.4 ± 0.6 and 1.6 ± 0.2 μM tau respectively (Table 1). The small difference between the apparent Kₐs probably reﬂects the isoform heterogeneity of the native protein compared with the four-repeat recombinant isoform [29], or to phosphorylation of the native protein by the neuronal cyclic AMP-activated protein kinase. The apparent Kₐ of PtdIns binding to recombinant MAP2c is 1.4 ± 0.1 μM MAP2c (Table
The apparent $K_d$ values for the binding of native MAP2, native or recombinant (pRK1724RT/383), or recombinant MAP2c (pET NCO/MAP2c) to lipid vesicles prepared from either pure PtdIns, pure PtdSer, or PtdCho doped (1:500 or 1:9 mol mol$^{-1}$) with PtdIns

<table>
<thead>
<tr>
<th>Protein</th>
<th>Lipid vesicles</th>
<th>Apparent $K_d$</th>
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<tbody>
<tr>
<td>Native tau</td>
<td>PtdIns</td>
<td>2.4 ± 0.6 µM</td>
</tr>
<tr>
<td>Recombinant tau</td>
<td>PtdIns</td>
<td>1.6 ± 0.2 µM</td>
</tr>
<tr>
<td>Recombinant MAP2c</td>
<td>PtdIns</td>
<td>1.4 ± 0.1 µM</td>
</tr>
<tr>
<td>Native MAP2</td>
<td>PtdIns</td>
<td>51 ± 6 nM</td>
</tr>
<tr>
<td>Native MAP2</td>
<td>PtdSer</td>
<td>1.3 ± 0.3 µM</td>
</tr>
<tr>
<td>Recombinant MAP2c</td>
<td>PtdSer</td>
<td>1.8 ± 0.2 µM</td>
</tr>
<tr>
<td>Native MAP2</td>
<td>1:500 PtdIns/PtdCho</td>
<td>221 ± 25 nM</td>
</tr>
<tr>
<td>Native tau</td>
<td>1:9 PtdIns/PtdCho</td>
<td>Undetectable</td>
</tr>
<tr>
<td>Recombinant MAP2c</td>
<td>1:9 PtdIns/PtdCho</td>
<td>Undetectable</td>
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1) i.e. it closely resembles the values for the native and recombinant tau. By contrast, the apparent $K_d$ for PtdIns binding to native bovine MAP2 is 51 ± 6 nM MAP2 (Table 1), i.e. it binds approx. 30-fold more tightly than MAP2c or the tau isoforms.

The similarity of the $K_d$s for PtdIns binding to tau and MAP2c (1.4–2.4 µM) suggests that these values reflect an interaction between the pure PtdIns vesicles and the highly homologous C-terminal peptides, and that the tighter binding of the native MAP2 (51 nM) involves an additional interaction with a site located within the MAP2-specific peptide (see Figure 1). Such a model would be consistent with the previous studies [26] which had shown (a) that vesicles prepared from various pure phospholipids, such as phosphatidylserine (PtdSer), inhibit microtubule assembly, but that this binding required a high local concentration of the phospholipid; and (b) that only PtdIns is inhibitory at the low local concentrations obtained by doping PtdCho vesicles.

The binding of native bovine MAP2 and recombinant murine MAP2c to PtdSer vesicles yielded apparent $K_d$ values of 1.3 ± 0.3 and 1.8 ± 0.2 µM respectively (Table 1). This strongly suggests, as pure PtdSer vesicles inhibit microtubule assembly [25,26], that the low-affinity, non-specific interaction involves the cationic C-terminal peptide, and that the comparable apparent $K_d$s for the observed binding of tau and MAP2c to PtdIns vesicles reflects this non-specific interaction. Indeed, the C-terminal peptide of MAP2c, prepared by thrombin digestion of the recombinant protein, binds to PtdIns vesicles [28]. As this low-affinity interaction requires a high local concentration of the phospholipid, it would not be detected using the doped vesicle assay. Indeed, no binding of either tau or MAP2c was detected to PtdCho vesicles doped with PtdIns to a ratio of 1:9 PtdIns/PtdCho. By contrast, native MAP2 bound to PtdIns-doped PtdCho vesicles (1:500 PtdIns/PtdCho) with an apparent $K_d$ of 221 ± 25 nM MAP2 (Table 1). Lower levels of doping resulted in apparent $K_d$s which were intermediate between this and the 51 nM values, while higher levels of doping (up to 1:1000) resulted in no additional increase in the apparent $K_d$. Consequently, the observed binding of MAP2 to the PtdIns/PtdCho-doped vesicles (1:500) reflects the $K_d$ of the second, high-affinity, PtdIns-specific site which is peculiar to the native MAP2.

**Discussion**

The binding of tau and MAP2c to pure PtdIns vesicles and the binding of MAP2c and MAP2 to PtdSer vesicles have apparent $K_d$s in the 1.2–2.4 µM range, while MAP2 binds to pure PtdIns vesicles with an apparent $K_d$ of 51 ± 6 nM and to PtdIns-doped PtdCho vesicles with an apparent $K_d$ of 221 ± 25 nM (see Table 1). These results, together with the binding of the C-terminal peptide of MAP2c to pure PtdIns vesicles, strongly suggests that the observed binding of MAP2 to PtdIns vesicles reflects the cumulative effects of two interactions: (a) a non-specific interaction associated with the highly cationic C-terminal peptide.
common to tau, MAP2 and MAP2c, which contributes a binding energy equivalent to a $K_d$ of approx. 1.5–2.5 μM; and (b) a PtdIns-specific interaction at a site which is localized within the MAP2-specific insert peptide (see Figure 1), which has an apparent $K_d$ of 221 ± 25 μM (Table 1), and which is detected when PtdIns is dispersed within a non-binding environment. PtdIns binding to this site may account for the selective inhibition by PtdIns of MAP2-, but not tau-, induced actin gelation [27]. It is however surprising that actin gelation is apparently unaffected by the interaction between the pure PtdIns vesicles and the cationic C-terminal peptide of the tau protein.

One unexpected feature of this dual interaction of MAP2 with PtdIns is that the apparent $\Delta G$ for binding to pure PtdIns vesicles ($-41.6$ kJ mol$^{-1}$) is considerably less than the additive contributions from the two separate sites ($-37.9$ kJ mol$^{-1}$ and $-32.5$ kJ mol$^{-1}$). This contrasts with the additive $\Delta G$ contributions of the individual tandem repeats to the total free energy of tau binding to microtubules [9]. The dual nature of the interaction does, however, present a conundrum, since PtdIns binding to the MAP2-specific insert peptides appears to over-ride the tubulin-binding activity dispersed along the C-terminal peptide. This problem might be resolved if the geometry of the association between this MAP2 site and the PtdIns vesicles resulted in the steric inhibition of the binding of tubulin subunits to the C-terminal peptide. This seems unlikely since the equilibrium between the two competing processes (microtubule assembly and PtdIns binding) will be biased in favour of the microtubule assembly. Specifically, the $K_d$ for MAP2 binding to taxol-stabilized microtubules is lower than that of the PtdIns-MAP2 interaction (15–50 nM MAP2 in a highly co-operatived process [30] versus 220 nM MAP2, Table 1) and the concentration of tubulin is significantly higher than that of the PtdIns (typically >2 μM tubulin for the assembly and ~30 nM PtdIns for the 1:500 PtdIns-doped PtdCho vesicles, assuming that they are monolayered and that only the exposed surface is available for MAP2 binding).

An alternative explanation might be for the PtdIns binding to the MAP2-specific insert peptide to induce a conformational change within the MAP2 molecule which reduces the affinity of the microtubule-binding site for tubulin subunits. The dispersed nature of the microtubule-binding site means that such a conformational change would have to specifically affect that part of the microtubule-binding site which contributes most to the tubulin-binding activity. The target of this conformational change may be one of the 18-residue tandem repeats or a ‘spacer’ peptide, since the second repeat of MAP2 has the greatest effect in promoting microtubule assembly [31,32] while the first repeat of tau (or the R1/R2 ‘spacer’ peptide) contributes most to the binding energy [9,10] and the phosphorylation of tau:Ser$^{205}$ (of repeat 1) markedly affects the promotion of microtubule assembly [33]. The proposed change in the conformation of MAP2 may be related to the extraordinary elasticity of the tau protein, as illustrated by the 3-fold variation in the paracrystalline periodicities [34], since the reported length of the MAP2 molecule varies widely (90–185 nm [35–37]).

The measured PtdIns–MAP2 dissociation constants provide some indirect support for this conformational interpretation. The $\Delta G$ from two independent, but spatially adjacent, PtdIns-binding sites should equal the sum of the contributions from the two sites, yet the observed $\Delta G$ for binding to pure PtdIns vesicles ($-41.6$ kJ mol$^{-1}$) differs markedly from the summed value ($-70$ kJ mol$^{-1}$). Knowledge of the independent dissociation constants permits the distance between the independent sites to be calculated, assuming that they are separated by a flexible linkage [38]. Calculating this distance for the two PtdIns-binding sites of MAP2 implies that they are separated by 580 nm, i.e. 3-fold further apart than the highest estimates of the length of the MAP2 molecule. The contribution of the C-terminal peptide is therefore much less than that predicted from the apparent $K_p$ (1.5–2.5 μM MAP2 for the binding to pure PtdIns or pure PtdSer vesicles, Table 1). Further evidence that a simple 'lacquering' mechanism fails to account for the observed properties of MAP2 comes from the finding that the salt sensitivity of the binding to MAP2 to immobilized actin or tubulin depends upon whether the salt is added before or after the MAP2 is bound to the target proteins [17].

The mechanism by which PtdIns binding to the high-affinity site influences the binding of tubulin (and actin) remains to be elucidated, but may prove to be central to any understanding of the physiological role of the PtdIns–MAP2 interaction. Such a physiological role is clearly indicated by both the endogenous character of the ligand and by the tightness of the PtdIns–MAP2 interaction, while the MAP2 localization studies indicate any functional role will involve dendrites rather than axons. The in vitro studies imply that a fraction of the dendritic MAP2 may be associated in vitro with one or more classes of membrane, yet inspection of the
The Neuronal Cytoskeleton

Figure 2
A possible model for the physiological role of the MAP2–PtdIns interaction

A transmembrane receptor is shown traversing the dendritic plasmamembrane which contains some PtdIns. A fraction of the dendritic MAP2 is shown bound to this membrane PtdIns. Activation of the transmembrane receptor results, via a G-protein cascade, in the stimulation of a PtdIns-specific phospholipase, which then hydrolyses the PtdIns, thereby releasing the bound MAP2. This increase in the concentration of free MAP2 leads to an enhancement in the extent of actin cross-linking and in the promotion of microtubule assembly.

Available immunofluorescence images fail to demonstrate any MAP2–membrane association. This suggests that either the in vitro observations cannot be extrapolated to the in vivo state, or that the immunological method fails to preserve a MAP2–membrane interaction. Such immunofluorescence studies have also failed to detect a specific association of MAP2 with actin filaments, even following transfection of tissue-culture cells, despite the unambiguous in vitro evidence that MAP2 binds to and gelates actin filaments [14–19]. This discrepancy between the in vitro and in vivo evidence may not be peculiar to MAP2. The over-expression in Chinese hamster ovary cells of the genes for MAP4, the Drosophila homologue, and tau had little effect on the in vivo microtubule assembly dynamics, and led to the proposal [39] that such MAPs may not play a critical role in microtubule assembly in vivo, in contrast with the in vitro evidence.

We are left, until such discrepancies between the in vivo and the in vitro observations have been reconciled, with the possibility that PtdIns may modulate the ability of MAP2 to promote microtubule assembly and to gelate actin filaments. This may be analogous with the modulation resulting from the interaction between PtdInsP2 and various actin-binding proteins, including profilin and gelsolin (e.g. [40]). Studies of both amoeboid movement and phagocytosis show that the activation of a transmembrane receptor leads, via a G-protein cascade, to the activation of a phospholipase, which in turn hydrolyses PtdInsP2, the release of the bound proteins, and the consequential effects on the subcortical actin gel. By analogy, the activation of a transmembrane receptor might lead, via a G-protein cascade, to the activation of a phospholipase, to the hydrolysis of PtdIns, and to the release of MAP2 from the plasmamembrane (Figure 2). The restricted distribution of MAP2 would mean that this process would be restricted to dendrites and to dendritic processes, while the in vitro evidence would suggest that an elevation in the concentration of available MAP2 would lead to enhanced actin gelation and to the promotion of microtubule assembly. Consequently, the activation of a trans-
membrane receptor would lead to a reorganization of the dendritic cytoskeleton. This reorganization would be likely, within the confines of the brain, to be a response to a signal originating from a second neuronal cell. The MAP2-PtdIns interaction may contribute to the process of learning.


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