Interactions of Microtubule-Associated Protein MAP2 with Unpolymerized and Polymerized Tubulin and Actin Using a 96-Well Microtiter Plate Solid-Phase Immunoassay†

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ABSTRACT: A solid-phase immunoassay is used to study the protein–protein interactions between microtubule-associated protein MAP2 and the cytoskeletal proteins tubulin and actin. The assay can be performed on 96-well microtiter plates and can be used to study the interactions with both subunit proteins and their respective polymers, microtubules and microfilaments. The microtiter format allows a large number of samples to be processed, and a number of conditions can be varied. In this solid-phase immunoassay MAP2 bound to microtubules/microfilaments and tubulin dimers/G-actin in a concentration-dependent manner. However, the bound MAP2 was not dissociated from the filaments even at high NaCl concentrations, while simultaneous addition of NaCl diminished MAP2 binding to these proteins. $MgCl_2$ was 1 order of magnitude more efficient in decreasing MAP2 binding compared with NaCl, suggesting that MAP2 may act by "screening" the electrostatic repulsion between tubulin dimers. The role of MAP2 in cross-linking microfilaments and microtubules was also examined. Microtubule/tubulin-bound MAP2 showed a diminished ability to bind to both microfilaments and G-actin, while microfilament/G-actin-bound MAP2 was able to bind efficiently to both microtubules and tubulin dimers. These differences in MAP2 behavior, depending on the initial binding partner, may be physiologically important in the cellular coordination of filament distribution. Although the solid-phase assay has been used to study MAP2 interactions, it is felt that the assay could be generally applied to other MAPs.

All eukaryotic microtubules are composed of a highly conserved heterodimeric structural protein called tubulin (Burns & Surridge, 1990). A number of accessory non-tubulin proteins termed microtubule-associated proteins (MAPs)[1] (Wiche, 1989; Vallee, 1986) can bind and modify the dynamic behavior of microtubules (Horio & Hotani, 1986; Farrell et al., 1987; Wallis et al., 1993) and have been colocalized with microtubules in vivo (Bloom et al., 1984; Peng et al., 1986; Matus & Riederer, 1986). Several high molecular weight MAPs have been observed to form protruding side arms from the microtubule surface (Chen et al., 1992; Erickson & Voter, 1976) which may play an important role in the interconnections between the major filament systems in the cytoskeleton (Yamauchi & Purich, 1993; Wiche, 1989; Wolosewick & Porter, 1979). These proteins may therefore regulate microtubule behavior in vivo both directly by altering microtubule dynamics and indirectly through interactions with other cytoplasmic components.

Different criteria have been used to identify putative MAPs. In the temperature-dependent cycles of assembly/disassembly MAPs cosediment under defined conditions in the warm but not in the cold, and for several MAPs a stoichiometric ratio is achieved after repeated cycles of purification (Shelanski et al., 1973; Borisy et al., 1975; Burns & Islam, 1984). However, other MAPs, for example, MAP1B, fail to fulfill this criterion (Bloom et al., 1985b). In the taxol procedure a protein is considered a MAP if it sediments under defined centrifugation conditions in a taxol-dependent manner (Vallee, 1982), and this procedure has proved extremely useful for the identification of several non-neuronal MAPs (Vallee & Bloom, 1983; Bloom et al., 1985a). Most of the MAPs identified using the taxol procedure commonly share a further property, namely that they can be selectively removed under defined salt conditions (Vallee, 1982; Burns et al., 1984). Other criteria include detergent extraction of whole cell cytoskeletons, selective removal of microtubules and the associated MAPs (Brown et al., 1976; Solomon, 1986), and affinity chromatography methods for the identification of proteins that bind to microtubules and actin (Miller et al., 1991). Immunological methods, using western blots, have also been used to identify putative MAPs, using monoclonal antibodies to tubulin and overlay techniques with either tubulin or taxol-stabilized microtubules (Diaz-Nido & Avila, 1989).

MAP2 is perhaps one of the most highly characterized of the high molecular weight MAPs [for a review, see Wiche (1989)]. MAP2 is neuron specific, predominantly associated with dendritic microtubules (Matus et al., 1982; Caceres et al., 1983), concentrated in the distal regions of developing dendrites, often preceding the appearance of microtubules (Bernhardt & Matus, 1982). This protein may therefore be involved in the coordination of several cellular functions. While

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; GTP, guanosine 5'-triphosphate; MAP(s), microtubule-associated protein(s); Mts, microtubules; Mfs, microfilaments; MES, 2-(*N*-morpholino)-ethanesulfonic acid; PES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SPI, solid-phase immunoassay; Tris, Trizma base; PMSF, phenylmethanesulfonyl fluoride; DTT, DL-dithiothreitol.

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there is strong morphological evidence that MAPs may interact with other components (Letterier et al., 1982; Matus, 1988; Wiche, 1989; Hirokawa et al., 1985; Wolosewick & Porter, 1979; Heuser & Kirschner, 1980), most of the biochemical evidence has been obtained using MAP2. This MAP has been shown to interact with neurofilament proteins (Letterier et al., 1982), secretory granules (Suprenant & Dentler, 1982), and microfilaments (Griffith & Pollard, 1982; Selden & Pollard, 1986; Sattilaro, 1986).

The interactions with actin have been studied in some detail, and MAP2 is classified as an actin binding and cross-linking protein (Pollard & Cooper, 1986). The specific MAP2–actin interactions have been studied by centrifugation techniques both for binding and for cross-linking activity (Sattilaro et al., 1981); the latter property has also been studied by low shear viscometry (Griffith & Pollard, 1982; Selden & Pollard, 1986). Arakawa and Frieden (1984) have also used fluorescence recovery after photobleaching to show that MAPs immobilized actin filaments as a cross-linked network, and Sattilaro (1986) demonstrated that MAP2 organizes actin filaments into bundles of 26-nm diameter. The cross-linking and gelling activity of MAP2 suggests that there are at least two actin binding sites on MAP2. Recently, using synthetic peptides corresponding to the tubulin binding domain of the MAP2 molecule, it has been shown that these peptides are also capable of binding G-actin (Correas et al., 1990), suggesting that at least one of the actin binding sites resides in the C-terminal domain of MAP2.

The most frequently used methods for studying MAP–microtubule/tubulin or MAP–microfilament/actin interactions generally require centrifugation techniques and consequently are limited by the number of samples that can be handled at one time and by the number of conditions that can be varied. We describe a solid-phase immunoassay for studying these interactions using 96-well microtiter plates which overcomes these limitations. Although we have used this technique to study the protein–protein interactions of MAP2 with microtubules/tubulin and microfilaments/actin, it may also prove useful for defining interactions of putative MAPs with tubulin or microtubules and potential interactions with other proteins.

EXPERIMENTAL PROCEDURES

The following buffers were used in this study: MES buffer [0.1 M 2-(*N*-morpholino)ethanesulfonic acid, 2.5 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM EDTA, pH 6.1]; Tris buffer (2 mM Tris, 0.5 mM dithiothreitol, 0.2 mM ATP, 0.2 mM $CaCl_2$, pH 8.0); PBS buffer (0.05 M sodium phosphate, 0.15 M NaCl, pH 7.3); PBS–Tween buffer [0.05 M sodium phosphate, 0.15 M NaCl, 0.025% polyoxyethylenesorbitan monolaurate (Tween 20 detergent), pH 7.3].

Purification of Microtubule Protein. Microtubule protein was purified from calf brain through two cycles of temperature-dependent assembly and dissassembly essentially as described by Pedrotti et al. (1993). Polymerization of microtubule protein was routinely monitored in a Shimadzu spectrophotometer at 350 nm as described previously (Pedrotti et al., 1993).

Taxol-stabilized microtubules were prepared by polymerization of pure tubulin (see below) in MES buffer containing 500 μM GTP, 1 mM DTT, and 20 μM taxol and incubation at 37 °C for 15 min.

Purification of MAP2 and Tubulin. MAP2 was prepared either by heat treatment according to the method of Herzog and Weber (1978) or in the native form according to the method of Gaskin and Roychowdhury (1986). Tubulin was purified by chromatography of twice-cycled microtubule protein by FPLC on a Mono-Q resin as described in Gaskin and Roychowdhury (1986).

Purification of Actin. Actin was prepared from rabbit muscle according to the procedure of Spudich and Watt (1971) as modified by MacLean-Fletcher and Pollard (1980). Prior to use, the actin was extensively dialyzed against three changes of Tris buffer for 2 days at 4 °C. After dialysis, any aggregated material was removed by centrifugation at 100000*g* for 60 min at 4 °C. G-Actin, in Tris buffer containing 100 mM KCl and 2 mM $MgCl_2$, was polymerized into microfilaments by incubation at 37 °C for 30 min.

Determination of Protein Concentration and Composition. Protein concentration was determined using the Bio-Rad protein estimation kit according to the manufacturer's instructions. Bovine serum albumin was used as the protein standard. The protein composition of MAP2, tubulin, and actin was analyzed by SDS–PAGE on 4–15% acrylamide gels using the Pharmacia Phast system. The gels were stained with Coomassie Brilliant Blue R-250 and after destaining were scanned on an LKB laser densitometer equipped with a peak integrator to determine the relative amounts of individual proteins (Pedrotti et al., 1993).

Solid-Phase Immunoassay (SPI). Microtubules, tubulin, and MAP2 were suspended in MES buffer while actin and microfilaments were suspended in Tris buffer in all experiments described below. One hundred microliters of substrate protein solution was added to wells in Greiner microtiter plates and incubated for 2 h to allow binding to the plastic surface. After removal of the protein solution, by aspiration, residual protein binding sites were saturated by incubating overnight with 300 μL of the blocking agents. The blocking solution was then aspirated, and the wells were washed and then challenged with MAP2 by addition of 100 μL of MAP2 solution. After incubation for 2 h, the plates were washed and further incubated with the primary (anti-MAP2 or anti-tubulin) antibody for 2 h. The primary antibody was removed, and the plates were washed prior to the addition of 100 μL of secondary IgG mouse horseradish peroxidase-labeled antibody (dilution 1:1000) and incubated for 1 h. After washing, binding was detected using 150 μL of a solution containing OPD (1 mg/mL *o*-phenylenediamine and 0.03% H_2O_2 in 0.1 M citric acid, pH 5) as a substrate for the peroxidase enzyme and the reaction terminated by addition of 50 μL of 4.5 M sulfuric acid. The absorbance was read at 492 nm on a Titertek MCC340 microtiter plate reader.

Monoclonal antibodies against MAP2 and tubulin (alpha-356) as well as the anti-mouse IgG labeled with horseradish peroxidase were all purchased from Amersham. All biochemicals were purchased from the Sigma Chemical Co. All chemicals used were of analar grade.

RESULTS

The protein composition of purified MAP2, actin, and tubulin (see Experimental Procedures) was examined by fractionation on 4–15% acrylamide gradient gels (Figure 1). Densitometric scanning of Coomassie-stained gels and determination of the integrated peak areas showed that all proteins were over 95% pure.

Determination of Conditions for an Optimal Signal with Low Background Noise. A good signal to noise ratio requires that the background noise, i.e. the nonspecific binding to plates, be low and the specific binding signal, i.e. the specific MAP2/microtubule protein–protein interaction, be high and depends

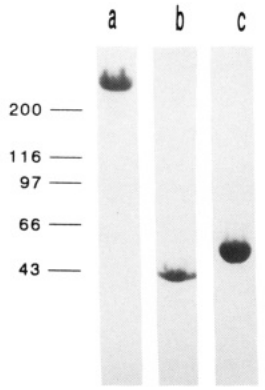

FIGURE 1: Protein composition of purified MAP2, actin, and tubulin. Purified proteins (see Experimental Procedures) were fractionated by SDS–PAGE on 4–15% acrylamide gradient gels and stained with Coomassie Blue: (a) MAP2, (b) actin, and (c) tubulin. Molecular mass standards are indicated in kilodaltons.

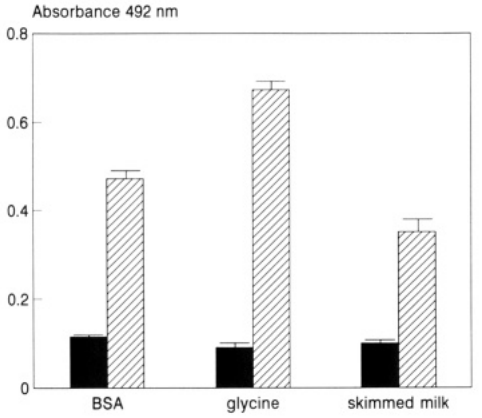

FIGURE 2: Effect of blocking conditions on MAP2/microtubule protein–protein binding. One hundred microliters of either MES buffer (solid bar) or MES buffer containing 0.2 mg/mL taxol-stabilized microtubules (slashed bar) was loaded into microtiter wells, and the plates were incubated at room temperature for 2 h. After coating, the plates were blocked with either BSA, 5% glycine, or skimmed milk prior to being challenged with 100 μL of a 0.15 μM MAP2 solution. MAP2 binding was detected with an anti-MAP2 monoclonal antibody (Experimental Procedures). The specific MAP2:microtubule signal with glycine is higher than that with BSA or skimmed milk. Bars represent the mean ± SD of five or six wells.

on the detection method, blocking agents, and washing conditions. Preliminary experiments showed that a good signal was observed at a 1:10000 dilution of the anti-MAP2 antibody (about 86% of that observed at a 1:1000 dilution) and that BSA, glycine, and skimmed milk were all equally effective at blocking the nonspecific protein binding to the plates. The effect of two different washing conditions, PBS or PBS–Tween, was also determined and showed that the background noise was high in the absence of Tween detergent, irrespective of the blocking agent used, and was reduced drastically when Tween was included in the wash buffer.

The specific MAP2 microtubule binding was therefore examined by coating the wells with 0.2 mg/mL taxol-stabilized microtubules prior to blocking and challenge with MAP2; PBS–Tween buffer was used for washing. Under these conditions, background noise was similar for all blocking agents, but the specific signal was lower when the plates were blocked with BSA or skimmed milk compared with glycine (Figure 2). All further experiments therefore employed glycine as the blocking agent, PBS–Tween for washes, and anti-MAP2 antibody at a dilution of 1:10000.

MAP2/Filament/Subunit Protein–Protein Interactions. The most suitable conditions for coating microtiter wells with the substrates for studying MAP2 interactions were also determined, since differences may exist in binding to the plastic surface for actin and tubulin. The wells were coated with different concentrations of the substrates prior to challenge with a fixed high concentration of MAP2. Binding of MAP2 was detectable at all substrate concentrations (0.025–1.0 mg/mL) tested, with a good signal being observed at 0.1–0.2 and 0.2–0.4 mg/mL, respectively, for tubulin/microtubules and actin/microfilaments. The interaction between MAP2 and the substrate proteins was therefore examined by coating the wells with either 0.1–0.2 mg/mL solution for microtubules/tubulin or 0.2 mg/mL solution for microfilaments/G-actin.

The wells were coated with either 0.2 mg/mL tubulin, taxol-stabilized microtubules, actin, or microfilaments and, after blocking with glycine, were incubated with increasing concentrations of MAP2. The unbound MAP2 was removed by washing, and the bound MAP2 was detected using the anti-MAP2 monoclonal antibody (see Experimental Procedures). MAP2 binding increased as the MAP2 concentration was raised and tended to achieve a plateau at high concentrations (Figure 3), suggesting concentration-dependent binding to these substrates. Maximal binding in this experiment was achieved at 0.15–0.2 μM MAP2 for microtubules and tubulin and about 0.1–0.15 μM MAP2 for microfilaments and G-actin (mean values for maximal MAP2 binding, determined from four different experiments, were 0.185 ± 0.038, 0.15 ± 0.045, 0.125 ± 0.011, and 0.125 ± 0.038 μM for microtubules, tubulin, microfilaments, and actin, respectively).

That the plateau level observed in the SPI represents the maximal binding of MAP2 was confirmed by centrifugation of assembled microtubules and determination of MAP2:tubulin stoichiometry. Taxol-stabilized microtubules (0.1 mg/mL) were incubated at three different MAP2:tubulin ratios and pelleted by centrifugation. The proteins in the pellets were fractionated by SDS–PAGE, and after staining destaining, the MAP2:tubulin stoichiometry was determined using the integrated peak areas and corrected for the molecular mass (Islam & Burns, 1981; Burns & Islam, 1984). A stoichiometry of 1 MAP2:18 tubulin dimers was observed at low MAP2 concentrations (0.05 μM) but increased to 1 MAP2:13 tubulin dimers and 1 MAP2:8 tubulin dimers at 0.1 and 0.3 μM MAP2, respectively.

Effect of Cations on MAP2 Interactions. MAP2–tubulin interactions involve charge interactions (Vallee, 1982; Burns & Surridge, 1990; Serrano et al., 1984; Burns et al., 1984), and the simultaneous addition of cations with MAP2 should therefore decrease the amount of MAP2 that binds to the substrate protein. The effect of simultaneous addition of salt and MAP2 on the binding to microtubules and tubulin was therefore examined using the SPI (Figure 4). Under these conditions, NaCl competed for MAP2 binding, and the amount of salt required to diminish the MAP2 binding by 50% was 210 mM for tubulin and 230 mM for microtubules. To further determine if MAP2 increases the electrostatic screening of surface charges and if NaCl can compete for this screening, according to the electrostatic Gouy–Chapman interactions, use of divalent as compared with monovalent ions should prove much more effective. The amount of magnesium chloride required to decrease MAP2 binding by 50% was therefore determined (Figure 4). As predicted by electrostatic screening type interactions, the divalent cation caused an equivalent decrease in MAP2 binding at an order of magnitude lower concentration compared with the monovalent cation (28 and 34 mM $MgCl_2$ compared with 210 and 230 mM NaCl for 50% decrease in MAP2 binding to microtubules and tubulin,

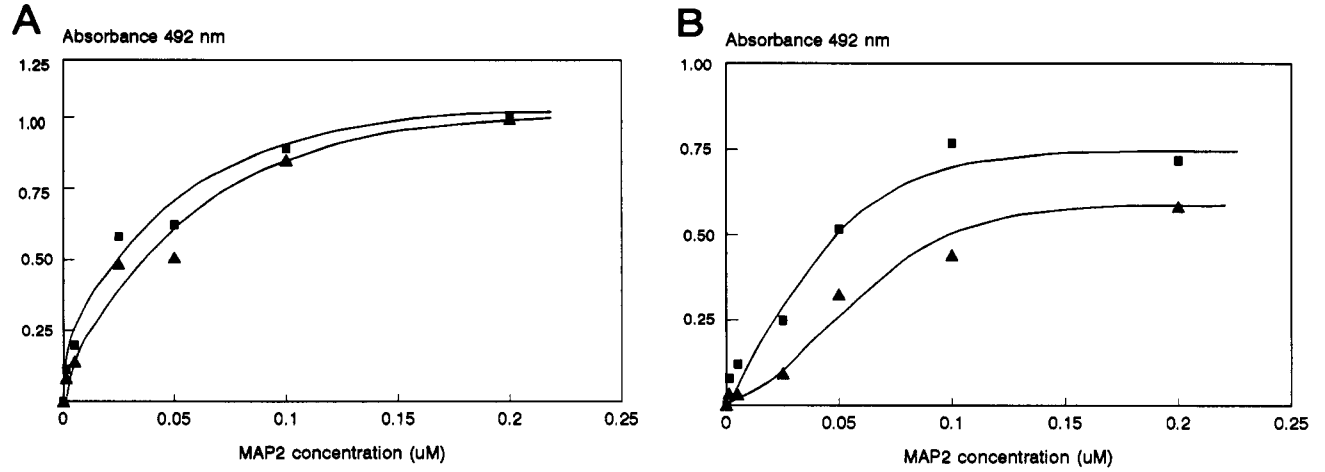

FIGURE 3: Effect of various MAP2 concentrations on protein–protein interactions. Wells were coated with either (A) 100 μL of a 0.2 mg/mL solution of tubulin (▲) or microtubules (■) or (B) G-actin (▲) or microfilaments (■). After blocking with 5% glycine, plates were challenged with 100 μL of the indicated concentrations of MAP2 solution. Binding of MAP2 to the different substrates was detected using an anti-MAP2 monoclonal antibody.

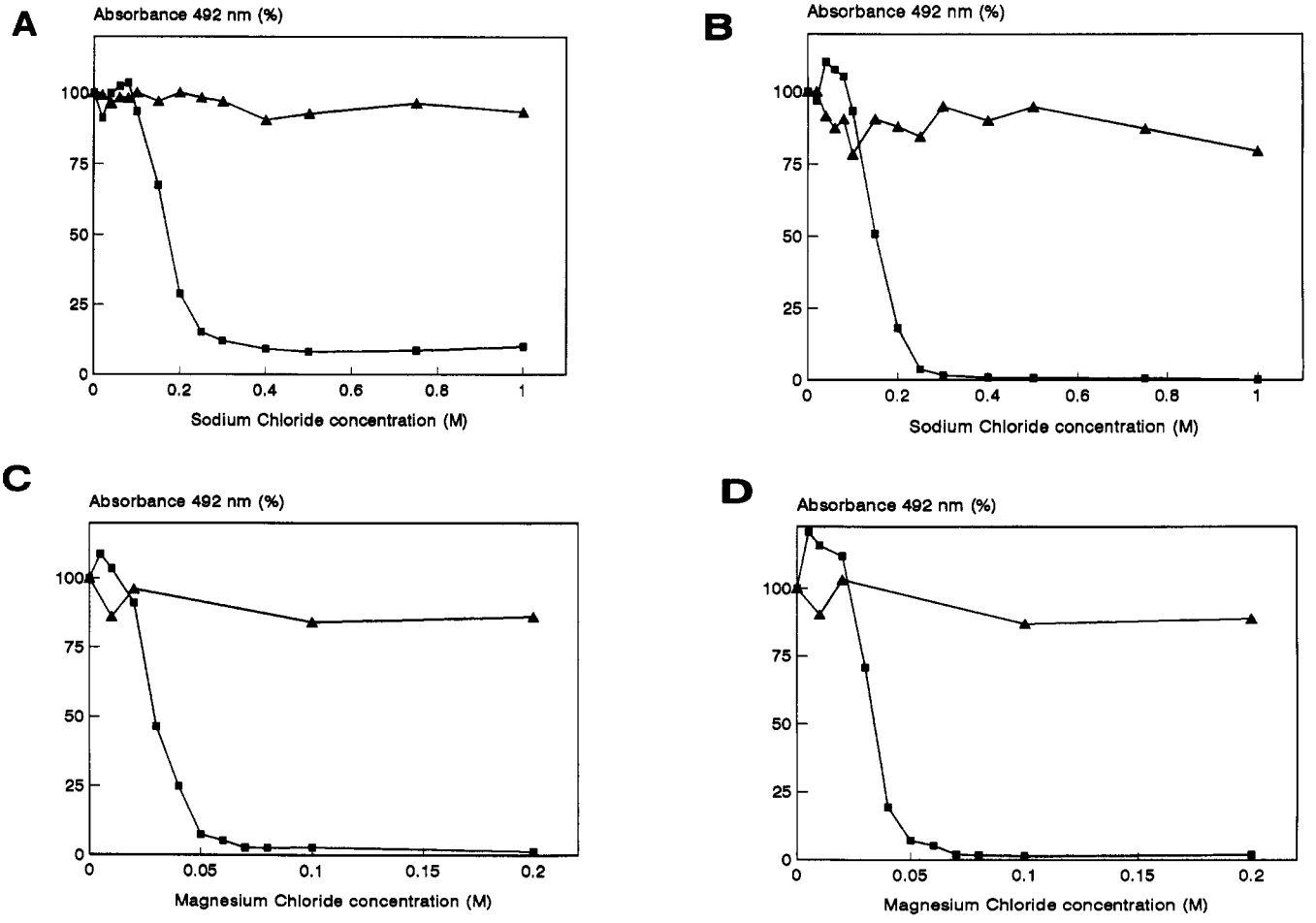

FIGURE 4: Effect of cations on MAP2–tubulin interaction. Increasing concentrations of NaCl (A, B) or $MgCl_2$ (C, D) were added either simultaneously with MAP2 (■) or after MAP2 had bound to the coated substrates (▲). Wells were coated with 100 μL of a 0.1 mg/mL solution of microtubules (A, C) or tubulin (B, D), blocked with 5% glycine, and challenged with a fixed amount of MAP2 (100 μL of a 0.15 μM MAP2 solution). The amount of MAP2 that remained bound to the coated substrates, under these two conditions, was expressed as a percentage of the control sample to which no salt was added.

respectively). The effect of NaCl and $MgCl_2$ on the interaction of MAP2 with actin and microfilaments was also determined (Figure 5). As observed with tubulin and microtubules, MAP2 binding to microfilaments/actin was decreased by 50% in the presence of 250/210 mM monovalent cation and only 22/18 mM divalent cation.

However, neither NaCl nor $MgCl_2$ caused MAP2 displacement in the SPI when MAP2 was first allowed to bind to microtubules or tubulin (Figure 4), although MAPs can be selectively removed from taxol-stabilized microtubules by the addition of NaCl (Vallee, 1982). This difference between taxol-stabilized microtubules and those bound to the solid phase suggested that the cation effects may be due to differences in the stability of the substrate proteins. Consequently, the effect of cations on the stability of taxol-stabilized microtubules was examined. Taxol-stabilized

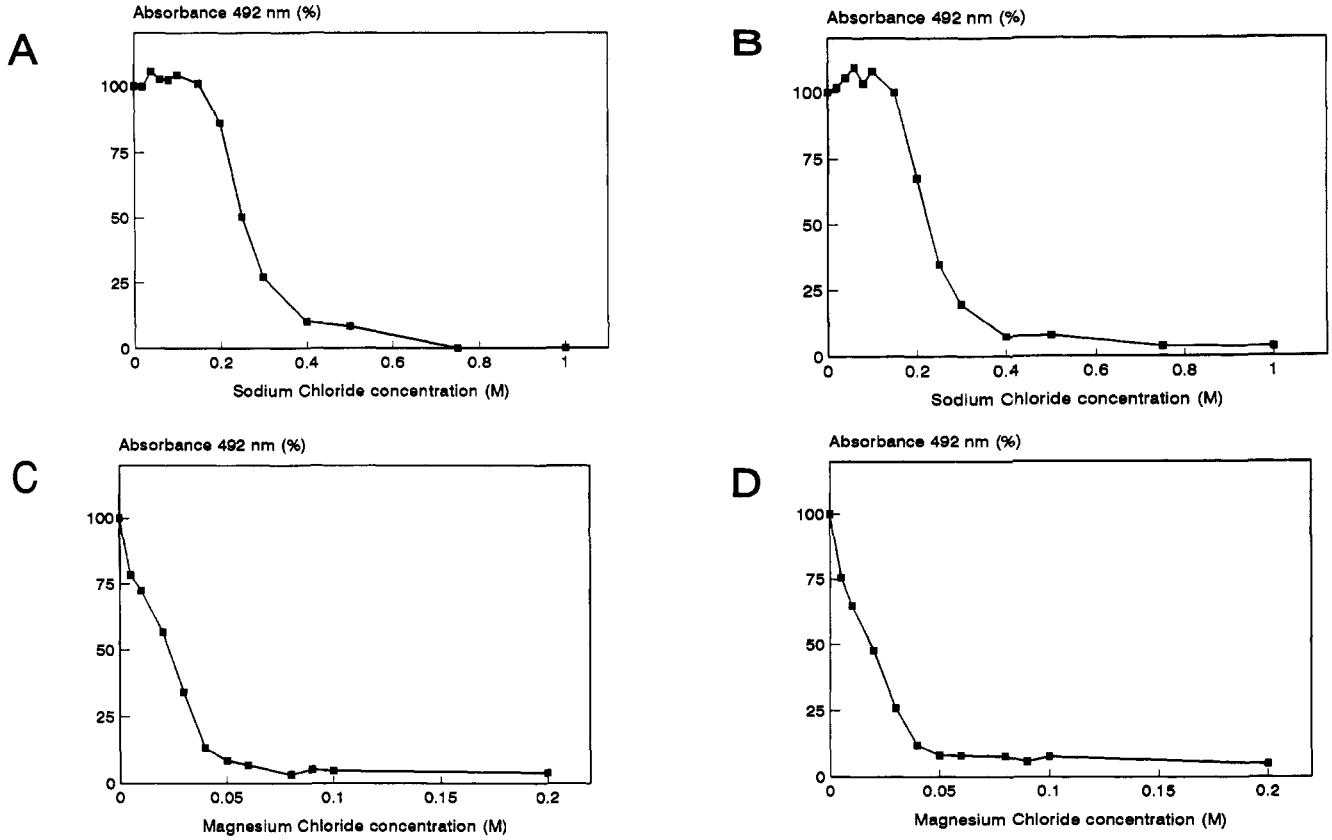

FIGURE 5: Effect of simultaneous addition of NaCl (A, B) or $MgCl_2$ (C, D) on MAP2–actin interaction. Wells were coated with 100 μL of a 0.2 mg/mL solution of microfilaments (A, C) or G-actin (B, D), blocked with 5% glycine, and challenged with a fixed amount of MAP2 (100 μL of a 0.15 μM MAP2 solution). The amount of MAP2 that remained bound to the coated substrates, under these two conditions, was expressed as a percentage of the control sample to which no salt was added.

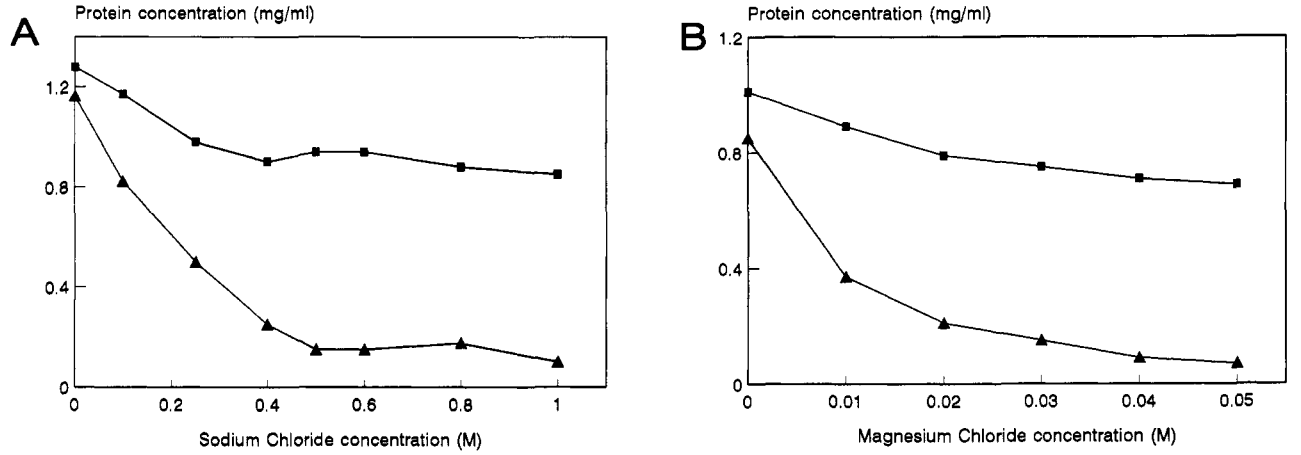

FIGURE 6: Effect of cations on the stability of taxol-stabilized microtubules. Twice-cycled microtubule protein (6 mg/mL) was polymerized in MES buffer, containing 500 μM GTP, 1 mM DTT, and 20 μM taxol, for 15 min at 37 °C. The protein was then diluted to a final concentration of either 1.4 (A) or 1.2 mg/mL (B) in warm polymerization buffer containing the indicated concentrations of NaCl (A) or $MgCl_2$ (B). The samples were subsequently incubated for a further 30 min at either 37 (■) or 4 °C (▲). At the end of the incubation period, samples were centrifuged at 100000*g* for 15 min and the protein concentration of the pellets was determined and plotted as a function of the salt concentration.

microtubules were suspended in MES buffer, containing taxol, adjusted to the indicated concentration of either NaCl or $MgCl_2$, and incubated at either 37 or 4 °C for 30 min and then centrifuged (100000*g*, 15 min at the appropriate temperature) to pellet the polymerized protein. The protein concentration of the pellets was determined and plotted as a function of the salt concentration (Figure 6). Whereas the addition of salt had a small effect on the amount of microtubule protein recovered at 37 °C, it dramatically reduced the amount of polymerized protein at 4 °C; a concomitant increase in the supernatant protein was also observed. SDS–PAGE analysis of proteins in the supernatants confirmed the presence of MAP2 and tubulin for microtubules incubated at 4 °C but only of MAP2 for microtubules incubated at 37 °C. The amount of NaCl or $MgCl_2$ required to displace the MAP at 37 °C is similar to the amount of salt that causes microtubule breakdown at 4 °C.

As sodium and magnesium ions destabilize taxol-stabilized microtubules, they should promote disassembly of "normal" microtubules. Twice-cycled microtubule protein was therefore assembled to steady state and challenged with either NaCl or $MgCl_2$; both cations caused a rapid disassembly of micro-

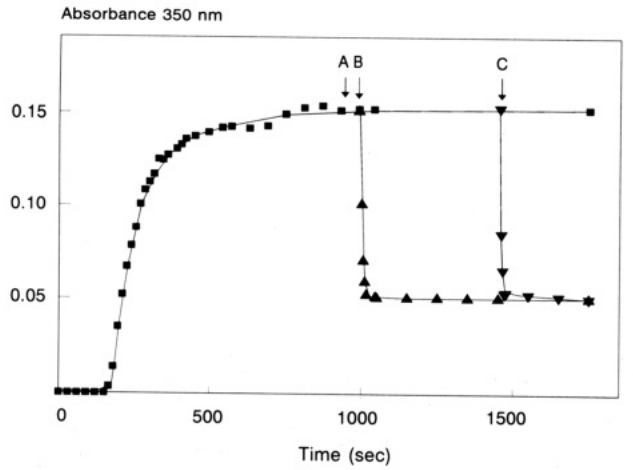

FIGURE 7: Effect of cations on polymerized microtubules. Twice-cycled microtubule protein (1 mg/mL) was incubated at 37 °C in MES buffer containing 500 μM GTP in a Shimadzu spectrophotometer, and polymerization (■) was monitored at 350 nm. Upon attainment of a steady plateau 14 μL of either MES buffer (arrow A, ■), 5 M NaCl (arrow B, ▲), or 0.5 M $MgCl_2$ (arrow C, ▼) was added and the absorbance monitored at 350 nm. The final salt concentrations were 100 mM NaCl (arrow B) and 10 mM $MgCl_2$ (arrow C).

tubules (Figure 7). Once more a 10-fold lower concentration of the divalent cation caused an equivalent change in the rate and extent of microtubule disassembly compared with the monovalent cation.

Competition Studies. The microtubule/tubulin binding site on the MAP2 molecule has been reported to reside in the 27-kDa C-terminal portion of the MAP2 molecule (Lewis et al., 1988). At least one of the two actin binding sites of the MAP2 molecule (Correas et al., 1990) is also located in the C-terminal portion and may be the same as the microtubule binding domain. We undertook competition studies using MAP2 that had been preincubated with tubulin/microtubules or actin/microfilament to determine its ability to bind to the coated substrates. As shown in Figure 8, microtubule-bound MAP2 exhibited a diminished ability to bind to coated microtubules, tubulin, G-actin, and microfilaments. A sample in which MAP2 was first allowed to bind to microtubules and was subsequently challenged with assembled microtubules showed no significant decrease in signal, confirming that there is no masking of the anti-MAP2 antibody epitope. In contrast to microtubule-bound MAP2, G-actin/microfilament-bound MAP2 bound efficiently to coated tubulin/microtubules and no drop in signal was detected (Figure 9); there was no significant change in the MAP2 signal when it was allowed to bind to the coated substrates prior to challenge with G-actin/microfilaments (Figure 9).

To further determine if tubulin or microtubules could bind to actin-bound MAP2, we determined the effect of preincubating MAP2 with actin prior to challenge with tubulin or microtubules. The amount of tubulin binding was detected by using an anti-tubulin antibody. As shown in Figure 10, tubulin bound efficiently to the actin-bound MAP2. Microtubules also bound to actin; the signal was lower compared with that with tubulin and may reflect either less efficient binding or differences in the ability of the antibody to recognize polymerized versus unpolymerized tubulin. By contrast, when MAP2 was preincubated with microtubules or tubulin prior to challenging the coated microfilaments or G-actin, the binding of tubulin or microtubules was greatly reduced (Figure 10), confirming the earlier observation using anti-MAP2 antibody (Figure 8).

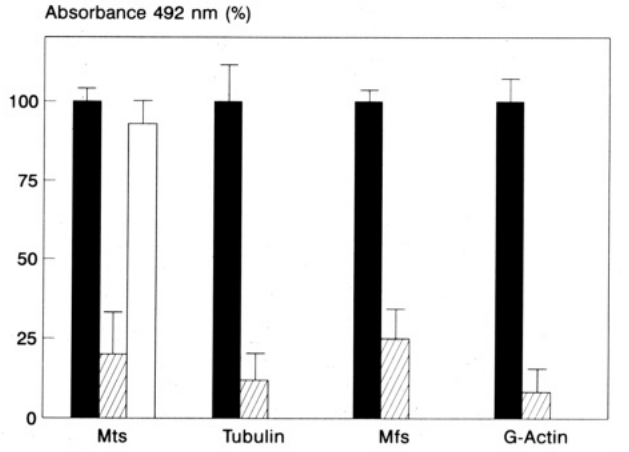

FIGURE 8: Binding of microtubule-bound MAP2 to coated substrates. Microtiter wells coated with 0.1 mg/mL of microtubules (Mts), tubulin, microfilaments (Mfs) or G-actin were challenged with either 0.15 μM microtubule-bound MAP2 (dashed bar) or 0.15 μM MAP2 (solid bar); a sample in which microtubules were added only after MAP2 had been allowed to bind to coated microtubules is also shown (open bar). Anti-MAP2 monoclonal antibody was used for signal detection, and absorbance values are expressed as percentage relative to the absorbance observed after challenge with 0.15 μM MAP2. Bars represent the mean ± SD of four wells. Microtubule-bound MAP2 was prepared by incubating MAP2 (0.15 μM) with taxol-stabilized microtubules (0.1 mg/mL) for 30 min at 37 °C before challenging the coated substrates.

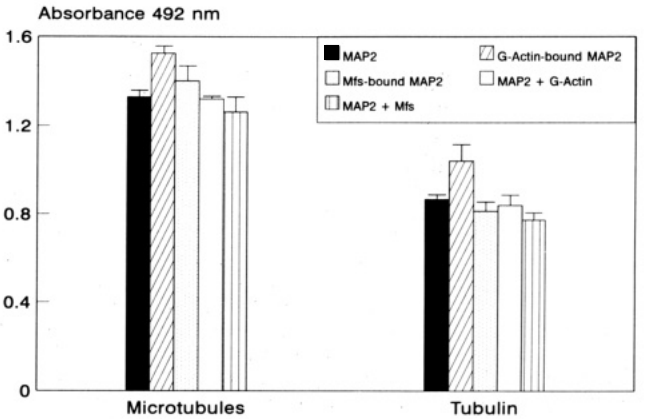

FIGURE 9: Binding of MAP2, microfilament-bound MAP2, or actin-bound MAP2 to coated microtubules or tubulin. Microtiter wells coated with tubulin or microtubules (0.1 mg/mL) were incubated with 0.15 μM MAP2 (solid, striped, and open bars), and MAP2 which bound to these substrates was subsequently challenged with either Tris buffer (solid bar), 0.1 mg/mL microfilaments (striped bar), or 0.1 mg/mL G-actin (open bar). Alternatively, microtubule- or tubulin-coated wells were challenged with either microfilament-bound MAP2 (dotted bar) or G-actin-bound MAP2 (slashed bar). Anti-MAP2 monoclonal antibody was used for signal detection. Bars represent the mean ± SD of four wells. Microfilament- or G-actin-bound MAP2 was prepared by incubating F-actin or G-actin (0.1 mg/mL) with MAP2 (0.15 μM) for 30 min at 37 °C.

DISCUSSION

We have developed a solid-phase immunoassay for studying protein–protein interactions between MAP2 and the cytoskeletal proteins actin and tubulin using 96-well microtiter plates. Compared with the more traditional methods, e.g. sedimentation techniques, used for studying protein–protein interactions, the results using the solid-phase immunoassay (SPI) are qualitatively and quantitatively similar with regard to MAP2/tubulin/microtubule interaction. MAP2 is known to bind to microtubules with a stoichiometry of 1MAP2:9 tubulin dimers (Burns & Islam, 1984; Wallis et al., 1993). The MAP2:tubulin ratio required for saturation in the SPI

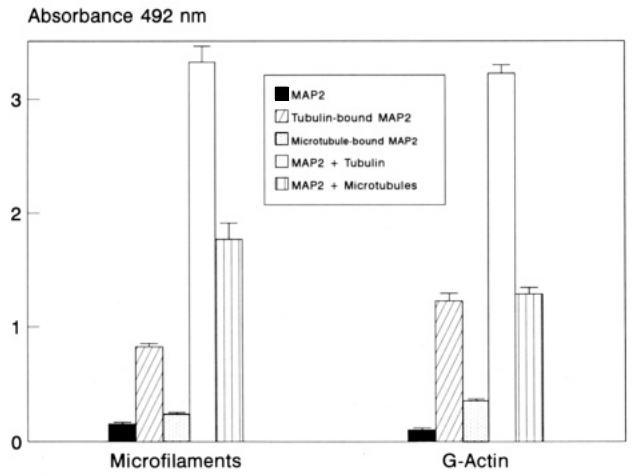

FIGURE 10: Ability of tubulin or microtubules to interact with G-actin/microfilament-bound MAP2 and of tubulin/microtubule-bound MAP2 to interact with G-actin/microfilaments. The anti-tubulin antibody was used to detect the amount of tubulin/microtubules that bound under these different conditions. Microtiter wells coated with microfilaments or G-actin (0.1 mg/mL) were incubated either with (solid, open, and striped bars) or without 0.15 μM MAP2 (slashed and dotted bars). Wells incubated with MAP2 solution were subsequently challenged with either MES buffer (solid bars) or tubulin (open bars) or microtubules (striped bars). Alternatively, the wells incubated in the absence of MAP2 were challenged with either tubulin-bound MAP2 (slashed bars) or microtubule-bound MAP2 (dotted bars). Bars represent the mean ± SD of four wells.

is similar to that for stoichiometric binding of MAP2 to taxol-stabilized microtubules (0.2 μM MAP2:1 μM tubulin in the SPI and 0.1–0.3 μM MAP2:1 μM tubulin by sedimentation of taxol-stabilized microtubules). Similarly, the concentration-dependent binding of MAP2 to tubulin observed in the SPI is in good agreement with that recently reported by Wallis et al. (1993) using immunoelectron microscopy.

SPI has several advantages over traditional methods used for studying MAP2 interactions: (a) the assay is sensitive and specific; (b) it requires only a small amount of sample; (c) it is amenable to screening a large number of samples. Furthermore, unlike conventional methods such as viscometry, immunological, and sedimentation techniques, the assay can also be used to study interactions with subunits and polymers. Affinity chromatography has been used to study subunit interactions (Miller et al., 1991), but compared with the SPI has the disadvantage that only a limited number of samples can be assayed. Although we have examined the interactions of MAP2, we feel that this assay method may prove useful in the identification of putative MAPs and novel interactions with other cytoplasmic components.

MAP2–tubulin interaction involves the charged domains on the two proteins, namely the MAP2 C-terminal positively charged domain, containing three imperfect repeats (Lewis et al., 1988), and the tubulin C-terminal glutamate-rich acidic domain (amino acids 433–440). NaCl has been demonstrated to lead to breakdown of tubulin oligomers and to selectively remove MAPs from taxol-stabilized microtubules, and the salt effect has generally been considered to represent competition between NaCl and MAP2 for the tubulin C-terminal domain (Burns & Surridge, 1990). However, the salt effects are complex and not only may affect the charge interactions between MAP2 and tubulin but may also directly affect tubulin for the following reasons: (a) Seventy millimolar NaCl can displace MAP2 from tubulin oligomers (Islam & Burns, 1986) and 100 mM NaCl can cause almost total breakdown of preassembled microtubules, yet at these concentrations NaCl leads to minimal displacement of MAPs from taxol-stabilized microtubules. (b) Since 0.35 M NaCl is required to displace MAP2 from taxol-stabilized microtubules, taxol stabilizes the lateral interactions between the tubulin molecules (Andreu et al., 1992), but at this concentration NaCl also causes destabilization of taxol-stabilized microtubules [see also Collins (1991)]. Clearly, stabilization of microtubules requires an increased amount of salt for MAP displacement, and consistent with this is the observation that salt addition cannot displace bound MAP2 in the SPI where the protein is additionally stabilized by binding to the plastic surface. Moreover, $MgCl_2$, at concentrations that lead to microtubule destabilization, results in displacement of bound MAP2 from taxol-stabilized microtubules but not in the SPI. Up to 48 weak binding sites for magnesium have been identified in the tubulin molecule (Frigon & Timasheff, 1975), and Gal et al. (1988) have clearly demonstrated that magnesium, at concentrations similar to those observed for microtubule destabilization and MAP2 release (Figures 6B and 7), increases the dissociation rate constant for microtubules and that this effect is independent of the presence of MAPs. We therefore propose that removal of bound MAP2 from the microtubule lattice by cations is due to destabilization of the microtubule lattice in addition to their effect on tubulin dimers. Recently, Wallis et al. (1993) have also shown that 1–2% MAP remains tightly bound to taxol-stabilized microtubules and resists dissociation in the presence of 0.6 M NaCl, perhaps representing a more stable subset of microtubules.

The association of tubulin dimers containing charged surface-exposed regions would require an altered balance between attractive van der Waals and electrical repulsion forces. The simultaneous addition of NaCl results in a decreased binding of MAP2 in SPI, and the amount of salt required is comparable to that reported previously (Burns et al., 1984; Vallee, 1982). This salt requirement was reduced about 10-fold when $MgCl_2$ replaced NaCl in the competition assay (230 mM NaCl vs 34 mM $MgCl_2$), suggesting that Gouy–Chapman type electrostatic interactions may be involved (Barber et al., 1977) as divalent cations are 1 order of magnitude more effective than monovalent cations in MAP2 displacement. The cations probably compete with MAP2 for screening the negative charged domains of tubulin dimers to reduce MAP2 binding, although the possibility that salt binding may alter the tubulin conformation cannot be excluded.

MAP2 promotes microtubule assembly by lowering the critical concentration for assembly in vitro specifically by altering the dissociation rate (Johnson & Borisy, 1977) and reduces dynamic instability and treadmilling of microtubules (Wilson & Farrell, 1986; Farrell et al., 1987). A reduction in the repulsive forces via screening would clearly increase the stability of subunit association and reduce dissociation, and any alterations in the balance between attractive and repulsive forces would dramatically affect microtubule behavior. In this context, it is important to mention that MAP2 acts as a substrate for several protein kinases both in vitro and in vivo (Therkauf & Vallee, 1983; Islam & Burns, 1981; Tsuyama et al., 1987), and it has been shown that the site of phosphate incorporation may alter MAP2 protein conformation (Brugg & Matus, 1991). Physiologically, phosphorylation/dephosphorylation of MAP2 may represent an ideal mechanism for modulating the MAP2–tubulin interaction. Indeed, phosphorylation of the C domain of the MAP2 polypeptide (Burns et al., 1984), introducing negative charges in close proximity to the negatively charged tubulin domains, results in an increase in the dissociation rate constant. Furthermore, highly phos-

phorylated MAP2 (46 mol of phosphate/mol of MAP2) has also been reported to no longer associate with microtubules in vivo (Tsuyama et al., 1987). Finally, an electrostatic screening model would also be predicted to lead to cooperative binding of MAP2 to the microtubule lattice.

The effects of monovalent and divalent cations on the MAP2–actin interaction are similar to that observed with tubulin, suggesting a similar electrostatic screening interaction. In fact, the MAP2 C domain has also been implicated in actin binding with amino acids 362–368 in the actin C terminus, showing a high degree of homology with the tubulin C terminus implicated in MAP binding (amino acids 433–440; Correas et al, 1990). Yamauchi and Purich (1993) have also shown that actin bundling by MAP2 is inhibited in the presence of 250 mM NaCl; this monovalent cation concentration is similar to that required to decrease MAP2 binding to microfilaments by 50% (Figure 5). In addition, phosphorylation of MAP2 has also been shown to affect the MAP2–actin interaction (Selden & Pollard, 1986). Despite these similarities, there are also important differences in the MAP2 interaction with these two filament types (see below).

MAP2 is an actin cross-linking and bundling protein and consequently must possess at least two sites for binding to actin (see introduction) and at least one of these sites must reside in the tubulin binding domain of the MAP2 molecule. There is a large body of cytological evidence that suggests the possibility of in vivo interactions between microtubules and microfilaments. In vitro mixtures of microfilaments and MAP2–microtubules have been reported to form gelled networks (Griffith & Pollard, 1978, 1982; Selden & Pollard, 1986). Contrasting results have been presented by Sattilaro (1986), who observed that addition of tubulin resulted in a dramatic disruption of MAP2–actin bundles in vitro. In agreement with Sattilaro (1986), we find that microtubule-bound MAP2 exhibits a diminished ability to bind to G-actin/microfilaments (Figure 8), and we have also confirmed the predicted disruption of actin gelling by MAP2 in the presence of tubulin by sedimentation (unpublished observation), with tubulin having a higher affinity for MAP2 compared with actin (Sattilaro, 1986). However, we have also shown that G-actin or microfilament-bound MAP2 is able to interact with tubulin and microtubules (Figure 9) and can cross-link the two protein types (Figure 10), agreeing with the findings of Pollard and co-workers (Griffith & Pollard, 1978, 1982; Selden & Pollard, 1986). Consequently, the previous contradictory reports can be easily reconciled as reflecting the assay conditions and the sequence of polymer addition.

Our results suggest that MAP2 function is dependent on the binding partner. This behavior of MAP2 may be physiologically important in the potential cross-links between cytoskeletal components and would depend on the spatial and temporal distribution of the binding partners. Several studies have demonstrated the immunochemical localization of actin and high molecular weight MAPs (Matus et al., 1982; Caceres et al., 1983) or MAP2 (De Camilli et al., 1984) in the dendritic processes. During the flexible phase of growth, MAP2 and actin are the major cytoskeletal proteins present, whereas tubulin is virtually absent in this phase (Bernhardt & Matus, 1982), and MAP2 may associate and form numerous cross-links with microfilaments. MAP2 may therefore play an important role in the gel–sol transformation of actin. Tubulin is normally present during the later stages of development, at which point MAP2 may dissociate from the gelled actin networks and cross-link microtubules with microfilament, giving the final stable structure of the developed dendritic tree (Matus, 1988). It is worth noting that actin-bound MAP2 can form cross-links much more easily than microtubule-bound MAP2 (Figures 8 and 9), suggesting a higher affinity for microtubules and possible masking of actin binding sites. Therefore, the relative affinities and the concentrations of actin and tubulin in diverse regions would determine the extent of cross-linking. Furthermore, once broken, an initial microfilament–microtubule bridge would be much more difficult to reform (Figures 8–10; Sattilaro, 1986) so that, with time, MAP2 would exhibit an altered distribution, initially actin-associated, leading to adult structure with MAP2 preferentially colocalized with microtubules.

In conclusion, we feel that the solid-phase immunoassay may provide a new and potent technique for examining MAP–protein interactions. Recently, other high molecular weight MAPs, namely MAP1A (Langkopf et al., 1992) and MAP1B (Noble et al., 1989), have been sequenced, and putative tubulin binding sites of these MAPs differ from those identified in the tau and MAP2 proteins. It would therefore be of interest to determine how these MAPs interact with tubulin and if they are also able to interact with other cytoskeletal proteins. The various differences and similarities between these high molecular weight proteins may shed new light on MAP function within the complex organization of the cytomatrix.

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