LETTERS TO THE EDITOR

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Microtubule Binding Proteins Are Not Necessarily Microtubule-Associated Proteins

Several studies have indicated the intriguing possibility of an interaction of the protein synthesizing machinery with the eukaryotic cytoskeleton. For example, ribosomes, polysomal mRNAs, and some translation initiation factors are found associated with the cytoskeletal framework from detergent-extracted HeLa cells (Lenk et al., 1977; Cervera et al., 1981; Howe and Hershey, 1984). Treatment of HeLa cells with the microfilament-disrupting drug cytochalasin releases ribosomes and mRNA from the cytoskeleton and inhibits protein synthesis (Ornelles et al., 1986). Polysomes, tubulin, and actin have been observed to cosediment with a particular membrane fraction from pea (Ito et al., 1994, and references therein). Collectively, these observations suggest that the translational machinery is associated with and possibly regulated by the cytoskeleton. Very little information exists on the interactions of specific proteins composing the cytoskeleton and the translational machinery. Recently, Durso and Cyr (1994) reported that a homolog of the protein synthesis elongation factor-1α (EF-1α) from carrot is a Ca^{2+}/calmodulin (Ca^{2+}/CaM) binding microtubule-associated protein (MAP). Unfortunately, this claim is premature on the basis of the experiments performed.

Durso and Cyr (1994) show that a carrot EF-1α homolog bound to microtubules (MTs) and bundled MTs in vitro (Figures 1 to 3 of Durso and Cyr, 1994). However, the EF-1α homolog was not shown by immunolocalization microscopy to be stably or transiently associated with bundled MTs in carrot cells, despite the availability of an antibody to the protein (Figures 5 and 6 of Durso and Cyr, 1994). In both the Introduction and Discussion, the terms "MT binding protein" and "MAP" were used indiscriminately, but these have very different meanings. The term "MAP" should be reserved for those proteins confirmed to be associated with MTs in cells fixed prior to their extraction (Sherline and Schiavone, 1977; Sheterline, 1978). This criterion for the identification of a MAP is equally applicable to the in vitro situation, in which proteins are initially discovered bound to MT cytoskeletons from lysed and extracted cells (Solomon et al., 1979) or protoplasts (Cyr and Palevitz, 1989). Cellular fixation minimizes the potential redistribution and fortuitous binding of non-MT proteins to MTs that may occur during extraction. In a recent review of MAPs, Cleveland (1993) reiterated that MAPs are identified by their immunolocalization to MTs in cells. To date, several MT binding proteins have been isolated from higher plants (e.g., Cyr and Palevitz, 1989; Cyr, 1991; Vantard et al., 1991; Yasuhara et al., 1992; Schellenbaum et al., 1993), but only one has been confirmed as an authentic plant MAP by its colocalization with cellular MTs (Chang-Jie and Sonobe, 1993). Therefore, the carrot EF-1α homolog cannot be confidently designated as a bundling MAP.

Because EF-1α has also been implicated in binding to polymerized plant actin in vitro (Yang et al., 1993), I sought to clarify the question of EF-1α localization in cells by performing immunolocalizations in maize cells using an affinity-purified polyclonal antibody to wheat EF-1α (Wick et al., 1981; Browning et al., 1990). Although bright staining was obtained throughout the cytosolic compartment of a variety of cell types from maize root, EF-1α did not appear to colocalize with either bundled MTs (stained with anti-tubulin antibody) or microfilaments (stained with phalloidin) in the same cells. Thus, I obtained no evidence that EF-1α is associated with the cytoskeleton in maize.

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In an approach to carrot MAP isolation, the authors used tubulin affinity chromatography (Kellogg et al., 1989) and found that a wide spectrum of carrot proteins bound to tubulin (Figure 1 of Durso and Cyr, 1994). These results are consistent with our observations on maize cell proteins. However, in our experience virtually none of the proteins is associated with MTs in cells. It is not surprising that the EF-1α homolog bound to the tubulin affinity column and to MTs (Figures 1, 2, 3, and 5 of Durso and Cyr, 1994), because EF-1α is a strong polycation, with a basic pI of 9.5 (Metz et al., 1992), and tubulin is a strong polyanion, having an acidic pI of 4.4–4.7 (Kopczak et al., 1992; Snustad et al., 1992). Tubulin is notorious for its nonspecific in vitro binding to cationic molecules having no physiological relation to MTs (Burton, 1981). Moreover, EF-1α is one of the most abundant cationic proteins in plant cells, comprising, for example, 5% of total protein in wheat germ cells (Browning et al., 1990). At low ionic strength, nonspecific binding of the EF-1α homolog and tubulin may have occurred via electrostatic interactions.

The authors also found that the EF-1α homolog bound to a CaM affinity column (Figure 3 of Durso and Cyr, 1994). However, a specific interaction between the Ca^{2+}/CaM complex and EF-1α was not demonstrated. The Ca^{2+}/CaM complex acts as an allosteric effector by binding to a specific site on its receptor molecule and inducing a conformational change that alters receptor activity (Allan and Hepler, 1989), but no solution binding experiments were done to demonstrate a saturable CaM site on the EF-1α homolog. Although specific binding of the Ca^{2+}/CaM complex to a protein may be distinguished from nonspecific binding, for example, by titration with paired calmodulin antagonists such as W compounds (e.g., Serlin and Roux, 1984), this was also not done. Because CaM binding to an EF-1α homolog has not been reported previously, it will be important to explore carefully the nature of the interaction. Thus, it is not yet clear that the EF-1α homolog has an authentic CaM binding site.

Although the authors detected binding of the pure carrot EF-1α homolog to taxol-stabilized bovine brain MTs by silver-stain SDS-PAGE analysis of cosedimentation (Figure 3 of Durso and Cyr, 1994), the results indicate an unusual stoichiometry of binding. MTs were combined with a 2.9-fold molar excess of purified EF-1α homolog prior to sedimentation; all of the EF-1α homolog appears to have cosedimented with MTs into the pellet fraction, because none was left in the supernatant fraction. It may be deduced, therefore, that 2.9 mol EF-1α homolog bound per mol polymerized brain tubulin. Because only one combination of protein concentrations was used, maximum binding may still not have been achieved. This binding stoichiometry, if correct, is very atypical, because mammalian MAPs that stabilize and bundle MTs bind substoichiometrically to polymerized tubulin (Wiche et al., 1991). For example, tau and MAP2 bind with a maximum of 1 mol MAP per 6 mol polymerized tubulin (Kim et al., 1986). Because plant MTs and mammalian MTs have similar distributions of MAP binding sites on their surface lattices (Hugdahl et al., 1993; Hugdahl and Morejohn, 1994), bundling MAPs from plants are also anticipated to bind substoichiometrically to MTs.

The EF-1α homolog was shown to bundle taxol-stabilized bovine brain and carrot MTs in vitro (Figures 2 and 5 of Durso and Cyr, 1994), but the conclusion that the Ca^{2+}/CaM complex specifically inhibited bundling is questionable, because the authors used concentrations of assay components that render the results uninterpretable. Bundling assays were performed with 1.2 μM MTs and an undetermined amount of EF-1α homolog in the presence or absence of 9 μM CaM and 1 mM free Ca^{2+}. These conditions provided a 7.5-fold molar excess of CaM to tubulin. Therefore, bundling may simply have been inhibited by a nonspecific, electrostatically based competition between CaM and tubulin for binding the EF-1α homolog. Although the Ca^{2+} binding sites on 9 μM CaM would have been saturated at a 10-fold lower Ca^{2+} concentration, the use of 1 mM free Ca^{2+} further complicated the assay, because Ca^{2+} binds to tubulin and depolymerizes MTs, which in this case, were diluted near the critical concentration (Fosket and Morejohn, 1992). (Bovine brain tubulin and plant tubulin have critical concentrations of 3–4 μM and 0.6–1.3 μM, respectively, in the presence of taxol [Schiff et al., 1979; Kumar, 1981; Bokros et al., 1993].) Because no polymer sedimentation analysis was performed on replicate samples, it is not clear whether the mass of MTs remained constant in the presence and absence of Ca^{2+}. Thus, in the presence of 1 mM Ca^{2+}, fewer MTs may have been available to form bundles.

The data of Durso and Cyr (1994) on the carrot EF-1α homolog resemble those published on glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a ubiquitous glycolytic enzyme that binds to MTs and bundles MTs in vitro (e.g., Kumagai and Sakai, 1983). Interestingly, GAPDH binds to MTs with the unusual stoichiometry of 1 mol GAPDH per mol tubulin (Bramblett et al., 1989). GAPDH binding to MTs in vitro is probably an artifact, however, because GAPDH is not associated with MTs in cells (Bramblett et al., 1989; Balaban and Goldman, 1990). Thus, it remains to be demonstrated that homologs of protein synthesis factors are utilized as MAPs in plant cells.

Interestingly, after the initial submission of this letter, it was reported that concentrations of animal EF-1α substoichiometric to polymerized tubulin rapidly sever MTs in vitro and that MAPs inhibit this severing (Shiina et al., 1994). Also, human EF-1α microinjected into rat fibroblasts causes MT destruction (Shiina et al., 1994). Durso and Cyr (1994) reported no MT severing, the effect of which is antithetical to MT bundling. Thus, definitive tests of the potential regulation of MTs by plant EF-1α will require in vivo experimentation with endogenous EF-1α. In any case, animal EF-1α and proteins with similar

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activities have been designated "MT-severing proteins" rather than MAPs (Vale, 1991; Shiina et al., 1992, 1994; McNally and Vale, 1993).

In summary, the increased pace and growing complexity of research on the plant MT cytoskeleton creates the possibility for confusion. To better understand MT function in plant cells it will be important for workers to carefully characterize the in vitro interactions of MTs with putative MAPs. It is anticipated that in most cases MT binding proteins may be designated as MAPs after satisfying the conservative criterion of their transient or stable colocalization with MTs in cells. This distinction is important, because the claim of the discovery of a MAP implies a considerably more rigorous test of a protein's function, and, thus, its biological significance.

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Reply: A MAP by Any Other Name Would Still Bind to Microtubules

Recently, we published data to indicate that a homolog of elongation factor-1α (EF-1α) interacts with microtubules (MTs), causing them to bundle in vitro (Durso and Cyr, 1994a). Furthermore, this in vitro interaction could be modulated by the addition of Ca^{2+} plus calmodulin (Ca^{2+}/CaM). In his letter, Morejohn raises a number of questions about this work, as well as two general points that warrant a more general discussion. First, what defines a microtubule-associated protein (MAP)? Second, how should we experimentally examine and critically evaluate data regarding the interaction between soluble proteins and the cytoskeleton?

Numerous laboratories have been working on identifying microtubule-associated proteins (MAPs) in plants with the aim of understanding how these proteins affect the behavior of cellular MTs. Morejohn presents one opinion of how to define a MAP, namely as "proteins confirmed to be associated with MTs in cells fixed prior to their extraction." This definition does not, however, represent an invariant standard used by all workers in the field. Moreover, the papers cited as substantiating this definition do not actually do so. Sherline and Schiavone (1977) and Sheterline (1978) do, in fact, utilize an immunocytochemical approach to localize MAPs to MTs, but they make no claim for this method being the definitive technique for MAP identification.

Defining "MAP" is problematic because it is a descriptive term which has evolved over the years. A recent review by Cleveland (1993) emphasizes the constant evolution of the definition and points out some of the historical pitfalls that have occurred as a consequence of adopting too narrow a definition for a MAP. Moreover, one of the most commonly cited review articles (Olmsted, 1986) on MAPs states that "MAPs [are] a collection of varied molecules that have been *defined* on the basis of their binding and/or *putative* interaction with microtubules" (our emphases). The dogmatic application of only one set of criteria to define a MAP runs the risk of arbitrarily ranking proteins in importance. Currently, it is not uncommon to use the term MAP to describe any protein for which evidence exists that it associates with MTs. The evidence may be biochemical, immunocytochemical, or genetic. Of course, corroborative data using two or more approaches (like biochemistry and immunocytochemistry) provide more compelling evidence that a given protein functions in the cell to affect MT activity.

Although a large number of proteins have been classified as MAPs there are only two, kinesin and dynein, for which it is known with any degree of certainty how their presence affects the functioning of MTs in vivo. As an object lesson in the dangers of adopting an inflexible definition for a MAP, Cleveland (1993) points out that "kinesin . . . among the most interesting microtubule related components, would fail to qualify under [the] early defi-