Progress Report
FY 92

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Role of Ca^{++} /calmodulin in the regulation of microtubules in
higher plants
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In FY92 we continued to define the role of Ca^{++} /calmodulin in regulating cortical microtubules (Mts) in higher plants. Enclosed is a manuscript that has recently been submitted to Plant Physiology which describes our immunocytochemical localization of calmodulin to MTs. This work is significant because it demonstrates that calmodulin can bind Mts in a calcium independent fashion. This, coupled to our earlier work (J. Cell Sci. 100:311, 1991) indicates calmodulin can interact in both a calcium dependent, as well as independent fashion with cortical Mts. These two findings are exciting because they raise the possibility that calmodulin may have differing effects upon MTs depending upon the concentration of calcium. Previous to this finding we had focused upon calcium dependent effects of calmodulin upon MTs, however in the future calcium independent effects will also need to be considered. Progress has also been made in three other areas in the last year.

A major thrust of the grant is to define the effects of calcium upon microtubules in vivo. In our initial report, using lysed protoplasts (which is closer to an in vitro observation), we noted Mts to be destabilized by calcium/calmodulin. As we had discussed, this effect could be the result of gross

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depolymerization induced by Ca^{++} /calmodulin, or by an increase in the dynamic flux rate. Joe Sliman, an undergraduate researcher working in the lab is now able to estimate intracellular calcium levels in cultured cells. He has confirmed that elevation of extracellular calcium levels results in an increase in intracellular calcium levels in carrot protoplasts. Debra Fisher, a research assistant in the lab, has found that protoplast possessing elevated intracellular levels of calcium contain highly fragmented cortical Mts. Interestingly, the mitotic and phragmoplast Mts are relatively unaffected. These results indicate that calcium, in conjunction with calmodulin functions to destabilize sensitive microtubules. Further experiments are planned in the upcoming year to further document this work not only in protoplasts, but also in intact cells of both carrot and tobacco.

The second area of interest is to identify the Mt proteins that are affected by Ca^{++} /calmodulin. With this goal in mind Neil Durso, a graduate student in the lab, has isolated Mt-binding proteins using tubulin affinity chromatography. These proteins were then adsorbed to a second column containing calmodulin covalently bound to the matrix. Using this technique, proteins can be identified that absorb in either a calcium dependent, or independent fashion (see attached figure). The MT/calmodulin binding proteins were then assayed for their ability to affect Mt function using in vitro assays. The calcium dependent calmodulin-binding proteins contained a great deal of Mt-bundling activity. This activity was sensitive to the

presence of Ca^{++} /calmodulin which was found to reversibly abolish the bundling activity. This finding is relevant because cortical microtubules (which we have shown to be calcium sensitive) are bundled. It will be important to know at the molecular level how Ca^{++} /calmodulin regulates MT-bundling. In the upcoming year we hope to learn more about how this complex interacts with MT-bundling proteins.

The third area of pursuit entails defining the proteins involved in mediating the attachment of microtubules to the plasma membrane. Our initial attempts at working with these proteins were hampered by the inability to procure sufficient quantities of proteins with which to work. In the last year alternative methodologies have been developed to obtain greater yields. Attached is a figure which summarizes our current approach. In the upcoming year we will work towards the production of antibodies as well as to define the effect Ca^{++} /calmodulin has on the ability of these proteins to interact with Mts.

In this second year we have continued to make headway into understanding how Ca^{++} /calmodulin affects plant microtubules and we are confident that in the upcoming year we will continue to further define the role that Ca^{++} /calmodulin plays in mediating microtubule organization in plant cells.

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Tubulin/CaM binding proteins

- 1-Mt-binding proteins before CaM column
2. Unbound proteins
3. Calcium-independent CaM binding proteins
4. Calcium-dependent CaM binding proteins

Soluble carrot proteins were absorbed to a column in which tubulin was covalently bound. The adsorbed proteins were eluted with 0.3M NaCl (lane 1). These proteins also bind to taxol stabilized Mts. After dialysis in the absence of calcium the proteins were allowed to absorb to a second column in which calmodulin was covalently bound. Adsorbed proteins were eluted with 1 mM Ca^{++} (lane 3). The proteins that did not absorb were reapplied to the column in the presence of calcium. Bound proteins were then eluted with 1 mM EGTA (lane 4).

Microsomal/MT-binding proteins

- 1-Microsome fraction
- 2-unbound fraction
- 3-0.3M eluate
- 4-Supernatant after MT-binding
- 5-MT pellet

A crude microsomal fraction was prepared from BY-2 tobacco suspension cultured cells. The proteins were solubilized with 10 mM CHAPs detergent (lane 1) and absorbed onto a column to which tubulin was covalently bound. The absorbed proteins were eluted with 0.3M NaCl (lane 3) and dialyzed. The eluted and dialyzed proteins were absorbed to taxol stabilized MTs and sedimented through a sucrose cushion. The supernatant (lane 4) and the pellet (lane 5) were collected and analyzed using SDS-PAGE.

Research Summary

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The cytoskeleton, and in particular its microtubule (Mt) component, participates in several processes that directly affect growth and development in higher plants. Normal cytoskeletal function requires the precise and orderly arrangement of Mts into several cell cycle and developmentally specific arrays. One of these, the cortical array, is notable for its role in somehow directing the deposition of cellulose, the most prominent polymer in the biosphere. Unfortunately, little molecular information is available regarding the formation of these arrays, or the cellular signals to which they respond. It is therefore important to acquire information regarding the molecules which regulate Mts within the different arrays. Experimental data has been obtained to suggest that plant cells use calcium, in the form of a Ca^{++} /calmodulin complex, to affect the dynamics of Mts within the cortical array. Owing to the importance of Ca^{++} as a regulatory ion in higher plants we are probing for a putative Ca^{++} /Mt transduction pathway which may serve to integrate Mt activities within the growing and developing plant cell. To aid

in our investigations we are using a lysed cell model in conjunction with immunocytochemical and biochemical methodologies to dissect how Ca^{++} /calmodulin interacts with Mts to affect their function. The information gained in these studies will be useful in understanding how developmentally important signals are transduced into morphogenic events during plant growth and development.

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