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Organization of the actin cytoskeleton is crucial for a diverse set of cell functions including cell division, intracellular signaling, cell shape, and motility. Cancerous cells demonstrate changes in cell adhesion and growth consistent with altered regulation of the actin cytoskeleton. Our research has focused on the regulation of protein phosphorylation, specifically signals mediated by protein phosphatase 1 (PP1), a serine/threonine phosphatase, in these processes. Current work is focused on a class of regulatory subunits, neurabin I and neurabin II, that direct PP1 to the actin cytoskeleton.

Neurabin I and neurabin II were cloned from rat tissue either as PP1 or F-actin binding proteins and contain multiple domains. We have cloned homologs of these proteins from *Xenopus laevis* and *C. elegans*. The high degree of structural homology from worms to mammals in specific domains, such as the PP1 binding domain and PDZ domain, begin to highlight regions most likely to be important for the *in vivo* function of these proteins in these diverse species.

GFP-tagged constructs of full length neurabin I transfected into HEK-293 cells show that the full-length protein localizes to the actin cytoskeleton. A C-terminal truncation of the coiled-coil and PDZ domains also localizes neurabin I to the actin cytoskeleton and causes cells to form filopodia-like extensions. However, an N-terminal deletion, which deletes the actin binding domain, localizes the protein to the cytosol. These experiments indicate that mislocalization of neurabin can cause rearrangement of the actin cytoskeleton.

We have also begun to study the *in vivo* phosphorylation of neurabin I. Sp-cAMPS, a PKA agonist, increased phosphorylation of the full length and N-terminal truncated proteins. The C-terminal truncated protein failed to get phosphorylated in cells suggesting a link between its covalent modification and subcellular localization.

Future study will define the molecular basis of neurabin localization, phosphorylation, and regulation of the actin cytoskeleton, as well as, the potential role of the neurabin-PP1 complex in cytoskeletal dynamics.

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Introduction

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Annual Summary

The role of protein phosphorylation in many cell processes is well established. Much of the focus of this research, however, has been on protein kinases, while the importance of protein phosphatases has not been addressed to the same extent. There is growing evidence that protein dephosphorylation is as important as protein phosphorylation in several cellular events such as proliferation, migration, and cell shape. My work over the last year has focused on neurabins, a family of cytoskeletal proteins, which we identified in a protein expression screen of a Xenopus cDNA library using protein phosphatase 1 (PP1) as a probe.

PP1 is a serine/threonine phosphatase which has been implicated in various cellular events, many of which involve hormonal and second messenger signals. However, PP1 itself is not directly modified in response to these signals. A major question in the field has been, how then is PP1 activity controlled by the cell? It now appears that the catalytic subunit of PP1 is controlled by its interaction with various regulatory subunits. Over 40 regulatory/targeting subunits have now been identified and these proteins affect the localization and possibly substrate specificity of the PP1 catalytic subunit. Unlike the catalytic subunit, many of these targeting subunits are modulated by second messenger signaling.

The neurabin family of proteins, neurabin I(NrbI) and neurabin II/spinophilin(NrbII), were first identified as F-actin binding proteins (1,2) and soon after found to interact with PP1 as well (3). NrbI is localized exclusively to brain while NrbII is ubiquitously expressed. Both of these proteins contain a PDZ domain and a coiled-coil domain, which are thought to mediate protein-protein interactions. They also contain a consensus PP1 binding domain, RVXF, which is found in many PP1 interacting proteins (4). The high degree of homology between NrbI and NrbII among several different

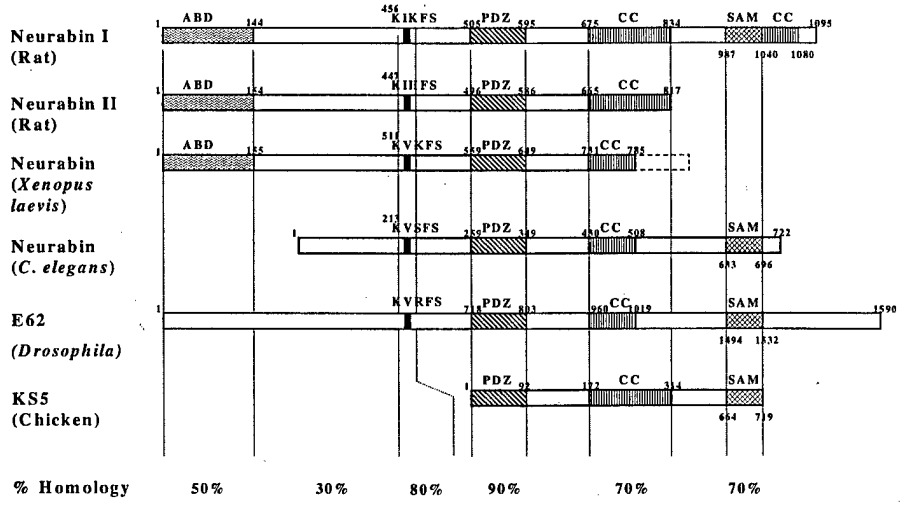

Figure 1 - Homology among different domains of neurabin family members. ABD - actin binding domain, PDZ - PSD95/DLG/ZO-1, CC - Coiled-Coil domain, SAM - sterile alpha motif.

species points to domains which may be important for neurabins' physiological role (Fig. 1).

To determine the *in vivo* relevance of these domains, we made several mutations of the NrbI protein containing an N-terminal GFP tag (Fig. 2). We then transfected these

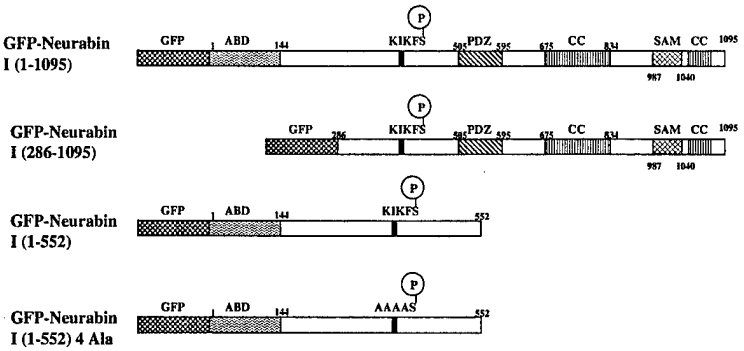

Figure 2 - GFP-NrbI constructs

constructs into HEK-293 cells which contain no endogenous NrbI. Confocal microscopy was then used to determine the localization of these GFP-tagged constructs. As can be seen in Figure 3, the full-length protein localizes exclusively to the membrane, whereas deletion of the first 286 amino acids, which includes the actin-binding domain, localizes

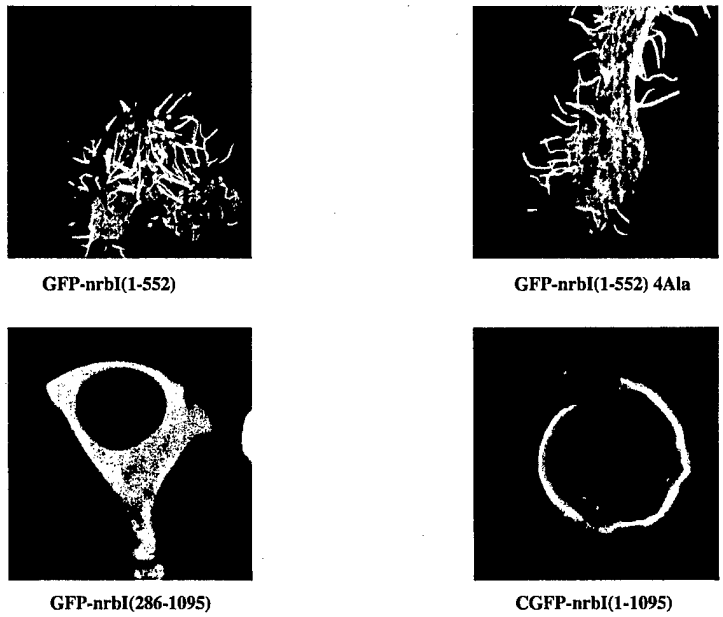

Figure 3 - Confocal sections of HEK-293 cells transiently transfected with various GFP-tagged NrbI constructs.

the protein predominantly to the cytoplasm. Interestingly, if you delete the PDZ and coiled-coil domains, the protein remains bound to the membrane, but also localizes to filopodial extensions and may induce the formation of these filopodia. When the PP1 binding domain is mutated in the context of GFP-NrbI (1-552), the morphology of the cells looks very similar to that of GFP-NrbI (1-552) containing cells. A recent study has

shown that mice lacking the NrbII gene contain neurons with an increased number of filopodial-like extensions (5). This suggests that the wild-type neurabins may be important for either retraction of filopodia or inhibition of filopodial formation. To confirm that the localization seen in the confocal microscope was due to neurabin binding to the actin cytoskeleton, we performed two experiments. First we lysed cells transfected with the various GFP-NrbI constructs using 0.75% Triton X-100, to keep the actin cytoskeleton intact, and spun the lysates at 100,000xg. Proteins which bind the actin cytoskeleton will pellet at this speed and proteins in the cytosol will remain in the

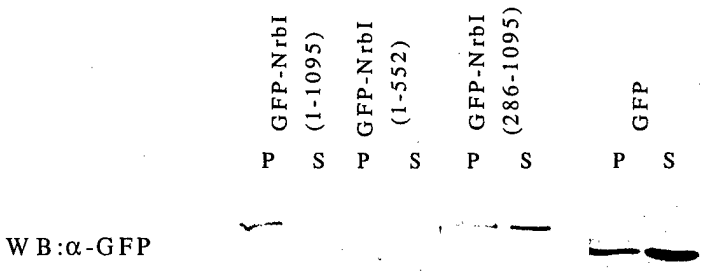

Figure 4 - HEK-293 cells were transiently transfected with various GFP tagged Neurabin I (GFP-NrbI) constructs and cells were lysed in 0.75% Triton-X-100. Lysates were then spun at 100,000 x g through a 6% sucrose cushion.

supernatant. Both GFP-NrbI (1-1095) and GFP-NrbI (1-552) remained in the pellet fraction while GFP-NrbI (286-1095) stayed in the supernatant (Figure 4). Second, we incubated cells which had been transfected with the GFP-NrbI constructs with

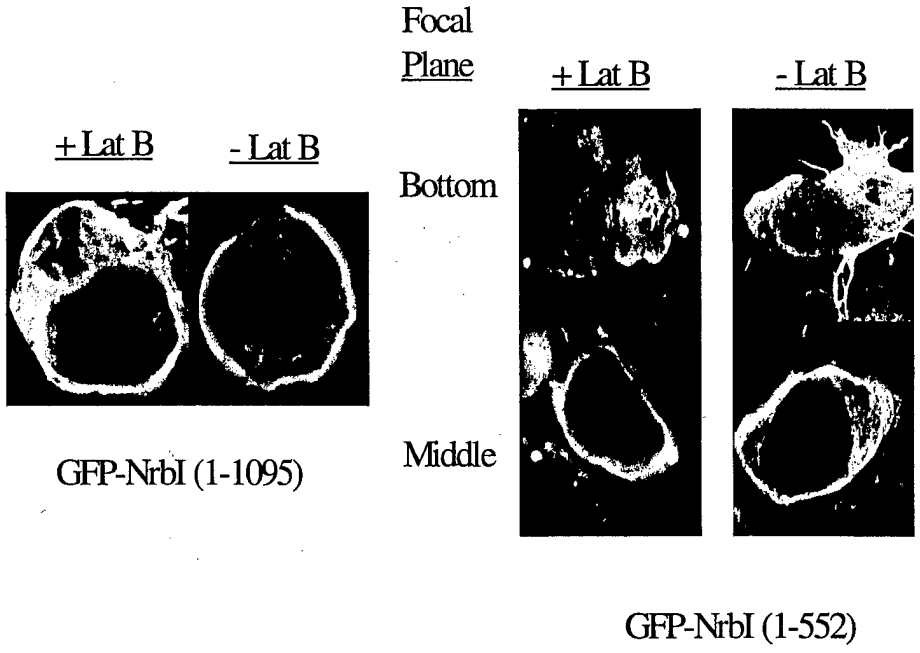

Figure 5 - HEK-293 cells were transiently transfected with various GFP tagged Neurabin constructs and treated with Latrunculin B for 30 minutes to disrupt actin structures. Cells were then fixed and visualized using confocal microscopy.

Latrunculin B, an actin depolymerizing agent. As can be seen in figure 5, Latrunculin B relocalized GFP-NrbI (1-1095) and GFP-NrbI (1-552) to the cytoplasm, as well as, eliminating the filopodia normally seen in the GFP-NrbI (1-552) containing cells. These two experiments together with *in vitro* data showing that amino acids 1-103 are absolutely required for neurabin I to co-sediment with F-actin (data not shown) prove that neurabin I is an F-actin binding protein *in vivo* and that this binding is involved in filopodial localization of the GFP-NrbI (1-552) mutant.

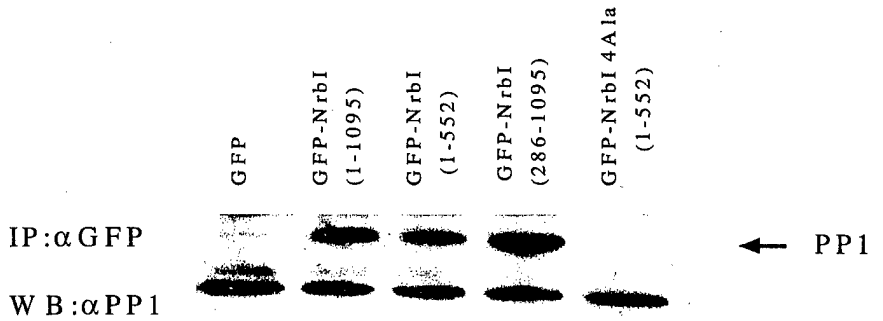

Figure 6 - HEK-293 cells were transiently transfected with various GFP tagged Neurabin constructs, lysed in RIPA buffer and immunoprecipitated with α-GFP antibody

Next we wanted to see if any of the binding partners of the full-length NrbI protein had altered binding to the 1-552 mutant which might explain its altered localization and the altered morphology of cells which contain the protein. The first protein we looked at was PP1 which bound equally well to all of the NrbI proteins except the one which had the mutated PP1 binding site (Figure 6). Future experiments will look at binding of these proteins to p70S6K and neurabin II.

Another possible explanation for the altered localization of the NrbI mutants is their phosphorylation status. It has been shown that Neurabin I can be phosphorylated *in vitro* by PKA, most likely on Serine 461 which is directly C-terminal to the PP1 binding domain (6). We decided to look *in vivo* to see if our NrbI constructs could be phosphorylated in response to Sp-cAMPS, a PKA agonist. Figure 7 shows that GFP-NrbI (1-1095) and GFP-NrbI (228-1095) are phosphorylated in response to Sp-cAMPS, but not Rp-cAMPS, a PKA antagonist. In contrast, GFP-NrbI (1-552) is not phosphorylated in response to Sp-cAMPS. Future studies will attempt to determine if this mutant is not phosphorylated because it localizes to filopodia, or vice versa, the protein localizes to filopodia because it cannot be phosphorylated.

Future experiments will further concentrate on the physiological effects of the various GFP-NrbI mutations. Our ultimate goal is to determine why the GFP-NrbI (1-552) protein causes filopodial extensions to form, as we believe this will lead us to a better understanding of the physiologically relevant role of this protein.

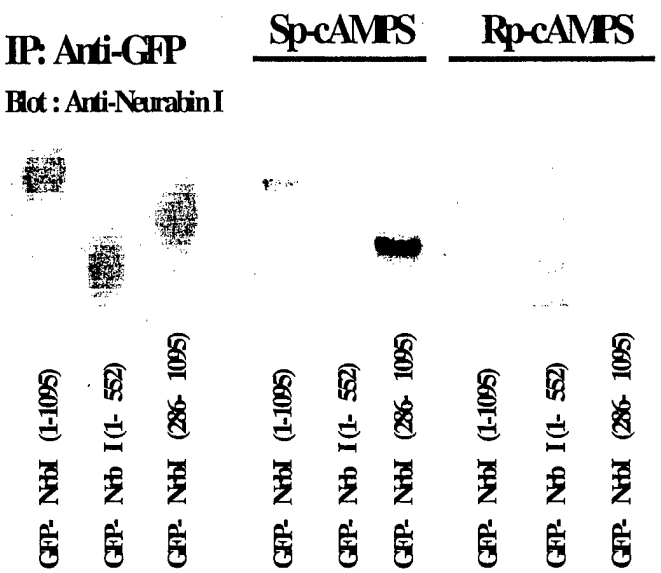

Figure 7 - HEK-293 cells were transiently transfected with various GFP tagged Neurabin constructs and labeled with ^{32}P-orthophosphate. Cells were induced with 100μM Sp- or Rp-cAMPS for 15 minutes, lysed in RIPA buffer, and immunoprecipitated with α-GFP antibody

Key Research Accomplishments

- Screened a *Xenopus* ovary phage cDNA library for PP1 binding proteins and found a neurabin family member.

- Constructed several GFP-tagged NrbI cDNAs, GFP-NrbI(1-1095), GFP-NrbI(1-552), GFP-NrbI(228-1095), and GFP-NrbI(1-552)4Ala.

- Expressed GFP-NrbI protein in HEK-293 cells and showed that the full-length protein localizes to the actin cytoskeleton by immunofluorescence and cell fractionation.

- Showed that GFP-NrbI(228-1095) localizes to the cytoplasm in HEK-293 cells by immunofluorescence and cell fractionation.

- Showed that GFP-NrbI(1-552) causes HEK-293 cells to form filopodial extensions to which the protein localizes and that this is not dependent on PP1 binding.

- Showed that neurabin I can be phosphorylated by PKA *in vivo* and that GFP-NrbI(1-552) is not phosphorylated by PKA *in vivo*.

Reportable Outcomes

Oliver, C.J., Li, S., Colbran, R.J., and Shenolikar, S. Protein phosphatase-1 - targeting the actin cytoskeleton. (2000) FASEB Summer Research Conference on Protein Phosphatases.

Oliver, C.J. and Shenolikar, S. Protein phosphatase-1 and cytoskeletal organization. (2000) Department of Defense Breast Cancer Program Era of Hope Meeting.

Oliver, C.J., Swenson, K., and Shenolikar, S. Targeting as a mechanism for protein phosphatase-1 regulation. (1999) Biological Sciences Graduate Student Symposium: Duke University Medical Center.

Oliver, C.J., Swenson, K., and Shenolikar, S. Essential role of protein phosphatase-1 in maturation of *Xenopus laevis* oocytes (1998). Biological Sciences Graduate Student Symposium: Duke University Medical Center.

Conclusions

In summary, we have begun to look at the physiological relevance of one member of the neurabin family of proteins, neurabin I. By expressing various GFP-tagged proteins in a heterologous cell line, HEK-293 which do not contain endogenous neurabin I, we have been able to determine several properties of this protein *in vivo*. One, the actin binding domain contained within the first 150 amino acids is absolutely required for interaction of this protein with the actin cytoskeleton. Two, deletion of the PDZ and coiled-coil domains leads to an increase in filopodial formation and localization of the protein to filopodia, neither of which require PP1 association. Three, the RVXF motif of neurabin I is absolutely required for PP1 binding *in vivo*, since mutation of these residues to alanine abrogates PP1 binding. Four, the full-length protein can be phosphorylated in response to PKA activation, whereas GFP-NrbI(1-552) is not phosphorylated in response to PKA activation. Future studies will examine various neurabin I binding partners, such as p70 S6K, that may be involved in the morphological phenotype seen in the GFP-NrbI(1-552) expressing cells. We will also examine the movement of these cells to determine what the effect of increased filopodial formation is on cell motility. In addition, the movement of neurabin I inside the cell will be examined using Fluorescence Recovery After Photobleaching or FRAP on live cells expressing the GFP-tagged proteins.

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Appendix 1

PROTEIN PHOSPHATASE-1 - TARGETING THE ACTIN CYTOSKELETON

Carey J. Oliver, Shi Li, Roger J. Colbran* and Shirish Shenolikar
Department of Pharmacology and Cancer Biology, Duke University Medical Ctr, Durham, NC
**Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN*

Organization of the actin cytoskeleton is crucial for a diverse set of cell functions including cell division, intracellular signaling, cell shape, and motility. Our research has focused on the signals mediated by protein phosphatase-1(PP1), a serine/threonine phosphatase, in cytoskeletal signalling. PP1 is a multisubunit enzyme containing a catalytic subunit and one or more regulatory subunits. Current work is focused on a class of regulatory subunits, neurabin I and neurabin II, that may direct PP1 to the actin cytoskeleton.

Neurabin I and neurabin II were cloned from rat tissue either as PP1 or F-actin binding proteins and contain multiple domains. We have also cloned homologs of these proteins from *Xenopus laevis* and *C. elegans*. The high degree of structural homology from worms to mammals in specific domains, such as the PP1 binding domain and PDZ domain, begin to highlight regions most likely to be important for the *in vivo* function of these proteins in these diverse species.

GFP-tagged constructs of full length neurabin I transfected into HEK-293 cells show that the full-length protein localizes to the actin cytoskeleton. An N-terminal deletion of the actin binding domain localizes neurabin I to the cytosol, implying that this domain is crucial for targeting the protein to the membrane/cytoskeleton. However, a C-terminal truncation, which deletes the PDZ and coiled-coil domains, causes cells to form filopodia-like extensions to which the mutant neurabin localizes. Yet other studies indicate similar morphologies in transfected cultured neurons. This suggests that mislocalization of neurabin can cause rearrangement of the actin cytoskeleton.

Analysis of the *in vivo* phosphorylation of neurabin I, indicates Sp-cAMPS, a PKA agonist, increased phosphorylation of the full length and N-terminal truncated neurabin proteins. In contrast, the C-terminal truncated protein failed to get phosphorylated in cells suggesting subcellular localization determines the covalent modification of neurabin.

Future study will define the potential role of the neurabin-PP1 complex in cytoskeletal dynamics, specifically, the molecular basis of neurabin localization, phosphorylation, and regulation of the actin cytoskeleton.

Appendix 2

PROTEIN PHOSPHATASE-1 AND CYTOSKELETAL ORGANIZATION

Carey J. Oliver and Shirish Shenolikar
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Organization of the actin cytoskeleton is crucial for a diverse set of cell functions including cell division, intracellular signaling, cell shape, and motility. Cancerous cells demonstrate changes in cell adhesion and growth consistent with altered regulation of the actin cytoskeleton. Our research has focused on the regulation of protein phosphorylation, specifically signals mediated by protein phosphatase-1(PP1), a serine/threonine phosphatase, in these processes. PP1 is a multisubunit enzyme containing a catalytic subunit and one or more regulatory subunits. Current work is focused on a class of regulatory subunits, neurabin I and neurabin II, that direct PP1 to the actin cytoskeleton.

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GFP-tagged constructs of full length neurabin I transfected into HEK-293 cells show that the full-length protein localizes to the actin cytoskeleton. An N-terminal deletion of the actin binding domain still localizes neurabin I to the actin cytoskeleton, implying that other domains also target the protein to the membrane/cytoskeleton. However, a C-terminal truncation, which deletes the PDZ and coiled-coil domains, localizes the protein to the cytosol and causes cells to form filopodia-like extensions. This indicates that mislocalization of neurabin can cause rearrangement of the actin cytoskeleton.

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Future study will define the molecular basis of neurabin localization, phosphorylation, and regulation of the actin cytoskeleton, as well as, the potential role of the neurabin-PP1 complex in cytoskeletal dynamics.

The U.S. Army Medical Research and Materiel Command under DAMD17-98-1-8075 supported this work.

Appendix 3

Targeting as a Mechanism For Protein Phosphatase 1 Regulation

Carey J. Oliver, Katherine Swenson, and Shirish Shenolikar
Department of Pharmacology and Cancer Biology

Protein Phosphatase 1 (PP1) is a ser/thr phosphatase which is involved in many different biological processes including, growth control, glycogen metabolism, mitosis, and LTD/LTP. *In Vitro*, PP1 has a broad range of substrates and this has led to the hypothesis that *in vivo* PP1 is a nonspecific phosphatase with very little regulation. However, there is now growing evidence that in fact PP1 is a multisubunit enzyme. It has a catalytic subunit which by itself has little substrate specificity, but it also has regulatory subunits which target PP1 to a specific location within the cell and/or increase its specificity towards one or more substrates. To find regulatory subunits of PP1 which might be involved in growth control, I performed an expression library screen from a *Xenopus laevis* ovary cDNA library to look for PP1 binding proteins. Two proteins which may potentially be involved in growth control were identified, PKC Zeta and spinophilin. Further studies on the interaction of PP1 with these two proteins will be discussed.

Appendix 4

Essential role of protein phosphatase-1 in maturation of *Xenopus laevis* oocytes

Carey J. Oliver, Katherine Swenson*, and Shirish Shenolikar
Program in Cell and Molecular Biology, Department of Pharmacology, and *Department of Molecular Cancer Biology

Protein phosphatase-1 (PP1) acts at multiple points in the eukaryotic cell cycle to control growth.. Genetic studies in yeast, fungi, and flies have highlighted a crucial role for PP1 in mitosis. *Xenopus* oocytes represent an excellent model system to study some of the events in mitosis. Following progesterone stimulation, there is a decrease in cAMP levels in the oocytes which leads to an increase in the translation of the mos proto-oncogene and enhancement of its kinase activity. Mos activation leads to subsequent stimulation of MAP kinase and the cdc2 protein kinase. Together the activation of these kinases promotes oocyte maturation. As a means to study the role of PP1 in mitosis, we have begun to study the effects of PP1 and its endogenous regulator, inhibitor-1, on *Xenopus* oocyte maturation.

Earlier studies showed that microinjection of several PP1 inhibitors blocked progesterone induced oocyte maturation, highlighting PP1's role in this process. To identify PP1 target(s) in this pathway, we have performed *in vitro* kinase assays using *Xenopus* oocyte extracts incubated with either Mos alone, Mos + thiophosphorylated (i.e. active) I-1, or Mos + PP1. The extracts are then incubated with P-32 ATP and a MAP kinase substrate to determine the efficiency of Mos signaling to MAP kinase. Our results showed that thiophosphorylated I-1 completely blocked Mos dependent activation of MAP kinase. Conversely, PP1 addition enhanced this signaling pathway.

These data suggest that PP1 activity is essential for signaling by the Mos proto-oncogene. Future studies will investigate whether Mos itself or components downstream are the targets of regulation by PP1.