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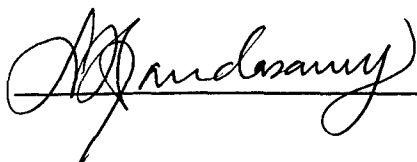
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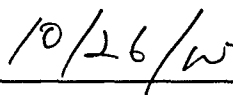
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13. ABSTRACT (Maximum 200 words)

There is growing evidence that protein dephosphorylation is as important as protein phosphorylation in cell proliferation. I am currently studying the role of protein phosphatase 1 (PP1), a serine/threonine phosphatase, in mitogenic signaling and breast cancer progression. To accomplish this goal, the following objectives are being addressed: (1) Determination of the changes in expression and function of MAP kinase and PP1 isoforms in a diversity of breast cancer cells. (2) Using *Xenopus* oocytes, we are delineating the role of PP1 in the activation of the MAP kinase pathway. (3) Identification of regulatory proteins capable of binding to PP1 and modulating its function in mitogenic signaling by screening *Xenopus* and MCF-7 expression libraries. (4) Determination of the effect of PP1 regulatory proteins on its function in mitogenic signaling and growth regulation. We have begun work on objectives one and two and made significant progress on objective three. We have identified a number of *Xenopus* proteins which bind to PP1 and are focusing on two proteins, spinophilin and PKC Zeta. We have begun *in vivo* studies of the interactions between PP1 and these two proteins and shown that they can each be co-immunoprecipitated with PP1. Future work will further delineate the biological consequences of PP1's interaction with these two proteins.

14. SUBJECT TERMS

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Annual Summary

The role of protein phosphorylation in mitogenic signaling is well established. Much of the focus of this research 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 cell proliferation. I am currently studying the role of protein phosphatase 1 (PP1), a serine/threonine phosphatase, in mitogenic signaling and breast cancer progression. To accomplish this goal, the following objectives are being addressed: (1) Determination of the changes in expression and function of MAP kinase and PP1 isoforms in a diversity of breast cancer cells. (2) Using *Xenopus* oocytes, a model system for the study of cell cycle regulation and the MAP kinase pathway, we are delineating the role of PP1 in the activation of the MAP kinase pathway. (3) Screening *Xenopus* and MCF-7 expression libraries to identify regulatory proteins capable of binding to PP1 and modulating its function in mitogenic signaling. (4) Determination of the effect of PP1 regulatory proteins on its function in mitogenic signaling and growth regulation, particularly in MCF-7 breast cancer cells. The completion of this study will define the role of PP1 and its regulators in mitogenic signaling and their potential contribution to breast cancer progression.

Objective one – Levels of the four different PP1 isoforms in MCF-7 cells were established by western blot using isoform specific antibodies obtained from Dr. Brian Wadzinski. MCF-7 cells predominately contain the α isoform of PP1, with lesser amounts of the β and $\gamma 1$ isoforms, and no appreciable amount of the $\gamma 2$ isoform (data not shown). Next, the total levels of PP1 were checked in several different breast cancer cell lines by western blot using a non-isoform specific PP1 antibody. It was determined that the total level of PP1 was similar in SKBR3, MD468, MCF-7, HBL100, TH7d, ZR75-1, and a control, noncancerous breast epithelial cell line, NC26 (data not shown). However, it is possible that one or more isoforms of PP1 has varied expression levels in breast cancer cells. Future work will determine whether the levels and activity of PP1 isoforms and MAPK change in the various breast cancer cell lines when compared to control cell lines.

Objective two – The initial observation that Inhibitor-1, a specific endogenous inhibitor of PP1, prevents Mos activation of the MAP kinase cascade in *Xenopus* oocyte extracts has been difficult to repeat because of the extreme variability in *Xenopus* oocyte extracts. Therefore, we have temporarily put aside these experiments and concentrated on objective three, which has produced very interesting results. Work on objective two will be continued in the near future.

Objective three – We have performed an expression screen of a *Xenopus* ovary phage cDNA library, kindly provided by Dr. Sally Kornbluth, using digoxigenin labeled PP1. Briefly, bacteria were infected with the phage library and grown on agar plates until plaques formation began. Nitrocellulose filters were soaked in IPTG and placed on the agar plates to induce protein expression in the phage-infected bacteria. The filters were then incubated with digoxigenin-labeled PP1 and subsequently with an I^{125} labeled anti-digoxigenin antibody. Then the filters were visualized using autoradiography and positive plaques were cored and amplified. After performing two sets of three rounds of

screening, we have identified 37 positive clones which encode 23 different proteins (See Table 1).

Clone # isolates			Identity			Clone # isolates			Identity		
XPBP1	2		putative glucoamylase (Gm?)			XPBP13	1		Novel		
XPBP2	3		putative ATP binding protein			XPBP14	1		Novel		
XPBP3	3		PKC Zeta			XPBP15	2		Ral A		
XPBP4	2		Novel			XPBP16	1		Putative Translational Release Factor		
XPBP5	7		Neurabin II/Spinophilin			XPBP17	1		Novel		
XPBP6	1		Zona pellucida B			XPBP18	1		No Insert		
XPBP7	1		Novel			XPBP19	1		Putative 60S ribosomal protein L18A		
XPBP8	1		putative adipose differentiation related protein			XPBP20	2		Threonyl-tRNA Synthetase		
XPBP9	1		Putative BTG family protein			XPBP21	1		Glutamine Synthetase		
XPBP10	1		Putative ASMTL			XPBP22	1		Putative Tropomyosin		
XPBP11	1		Putative Nucleosome Assembly Protein			XPBP23	1		Putative Caspase		
XPBP12	1		RLF								

Table 1 – Clones isolated from expression screen of *Xenopus* ovary phage cDNA library.

Once the 5' ends of the clones were sequenced, we wanted to confirm that these proteins were in fact PP1 binding proteins. First, we attempted to bacterially express the clones directly from the library vector. Several attempts were unsuccessful at producing any protein. We then produced S³⁵ labeled protein using an *in vitro* transcription/translation reaction to use in *in vitro* binding assays. Unfortunately, the background in these assays was unacceptably high. Therefore, we decided to concentrate on two known proteins, clone 3 and clone 5.

Clone 3 encodes for amino acids 49-510 of the atypical protein kinase C isoform zeta which had been previously cloned in *Xenopus*. The atypical PKCs, unlike the classical PKC isoforms, are not activated by calcium or diacylglycerol, but by lipids. Interestingly, it has already been shown that PKC Zeta can activate the MAP kinase pathway in *Xenopus* oocytes and cause maturation in the absence of hormonal stimulus (1). Thus, it is exciting to hypothesize that the mechanism of action of PP1 in oocyte maturation is to activate PKC Zeta and subsequently the MAP kinase pathway. However, it is unknown whether or not PKC Zeta is in the same pathway as Mos. Our future research will address this question. To further delineate the PKC Zeta/PP1 interaction we have obtained several myc-tagged PKC Zeta constructs from Dr. Peter Parker and expressed them in HEK-293 cells to look at the *in vivo* interaction with PP1. There are two sites on PKC Zeta which are especially important for its activation, the pseudosubstrate domain and Threonine-410. Threonine-410 is in the activation loop of

the kinase domain and the protein is activated when phosphorylated on this residue. This phosphorylation releases the pseudosubstrate domain from the catalytic site allowing PKC Zeta to phosphorylate its substrates. We have shown that endogenous PP1 can co-immunoprecipitate wild-type and constitutively active mutants of PKC Zeta (A119E or Δ PSS), but not kinase dead PKC Zeta mutants(T410A) (Figure 1).

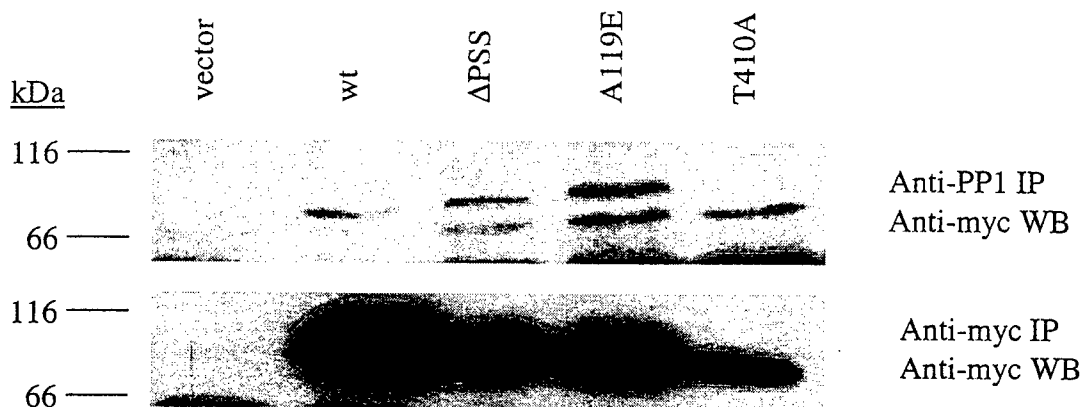


Figure 1 – Co-immunoprecipitation of PP1 and myc-PKC Zeta from HEK-293 cells. Briefly, various myc-tagged PKC Zeta constructs were transiently overexpressed in HEK-293 cells, the cells were lysed, and immunoprecipitated with either PP1 or myc antibodies. Samples were run on SDS-PAGE, transferred to nitrocellulose, and blotted with anti-myc antibody. The higher molecular weight band is PKC Zeta.

In addition, PKC Zeta can not be isolated from HEK-293 cell lysates using microcystin sepharose, a small molecular weight marine toxin which binds to the catalytic site of PP1 (data not shown). This suggests that PKC Zeta may be a substrate of PP1. In support of this idea, a recent paper has shown that okadaic acid, a PP1 and PP2A inhibitor, can activate PKC Zeta in 3T3 L1 adipocytes (2). Future research will further study the nature of this interaction and the biological consequences of it.

Clone 5 encodes for amino acids 1-719 of spinophilin/neurabin II, a previously identified PP1 binding protein which is ubiquitously expressed in mammalian tissues. Spinophilin contains an N-terminal actin-binding domain followed by a PP1 binding motif. It also has a PDZ domain and a C-terminal coiled-coil motif. This protein has not previously been cloned from *Xenopus*. Thus, we have learned from a sequence alignment of the rat and frog proteins that the PP1 and PDZ domains are highly homologous, indicating that these regions are very important for spinophilin function. In addition, we have been able to co-immunoprecipitate endogenous PP1 with endogenous spinophilin. We have also been able to purify spinophilin using microcystin sepharose indicating that it is not a substrate for PP1 (data not shown). We have also shown that spinophilin is a phosphoprotein *in vivo* and a PKA substrate *in vitro* (data not shown). How this phosphorylation affects PP1 binding and spinophilin function inside a cell will be a subject of future study. In addition, we will attempt to determine spinophilin's effect on oocyte maturation by microinjection of antisense oligonucleotides or peptides which disrupt PP1 binding.

Objective Four – work has not yet begun on objective four.

References

1. Berra,E., Diaz-Meco,M.T., Dominguez,I., Municio,M.M., Sanz,L., Lorzano,J., Chapkin,R.S. and Morcat,J. (1993). *Cell* 74, 555-63.
2. Standaert,M.L., Bandyopadhyay,G., Sajan,M.P., Cong,L., Qwon,M.J. and Farese,R.V. (1999). *J. Biol. Chem.* 274, 14074-78.

Key Research Accomplishments

- Determined levels of different PP1 isoforms in MCF-7 cells
- Determined total levels of PP1 in several different breast cancer cell lines
- Screened a *Xenopus* ovary phage cDNA library using digoxigenin-labeled PP1 as a probe. Focused attention on 2 clones for further research
- Showed association of PP1 and PKC Zeta, as well as, PP1 and spinophilin in HEK-293 cells by co-immunoprecipitation
- Showed that spinophilin could be isolated using microcystin sepharose and PKC Zeta could not be isolated using microcystin sepharose
- Established that spinophilin is a phosphoprotein *in vivo* and a PKA substrate *in vitro*



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