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14. ABSTRACT The ongoing purpose of this work is to examine the effects of altered cyclophilin A (CypA) levels and activity on the prolactin receptor (PRLr) associated signaling and its role in breast cancer development and/or progression. To this end, we have further demonstrated that siRNA-mediated CypA knockdown or the use of CypA inhibitors such as cyclosporine A (CsA) analogs-CsD and CsH significantly abrogated the PRLr signaling and breast cancer cell growth <i>in vitro</i> . In addition, by using a novel antibody phage display technology, we have developed several synthetic antibodies, which exhibit nanomolar affinities for the CypA. Experiments are currently underway to test these potential synthetic antibodies <i>in vitro</i> and <i>in vivo</i> .					
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INTRODUCTION

Breast cancer is the most prevalent cancer among women evidenced by nearly 2254,650 new cases diagnosed and approximately 40,170 deaths in the United States in 2009. A variety of potential risk factors are involved in its etiology, such as genetic, environmental, dietary and hormonal factors. Earlier diagnosis and therapeutic molecular targets that influence cancer growth and progression are urgently needed in order to improve outcomes in patients with breast cancer. Recently, there is increasing evidence that the hormone prolactin (PRL) and its receptor (PRLr) are involved in the development of human breast cancer [1]. Thus, the PRL/PRLr complex and its signal transduction networks are appropriate targets for the development of anti-breast cancer drugs [2].

The major objective of this proposal is to understand how cyclophilin A (CypA) regulates the PRLr signaling and to determine the effects of altered CypA expressions and activity on the PRLr signaling and breast cancer phenotypes. We aim to accomplish this through a multidisciplinary approach that combined cellular biological, biophysical structural and animal studies. There are two major hypotheses in this proposal: (1). CypA regulates PRLr signaling function by altering structure of the PRLr complex via its intrinsic PPIase activity; (2). CypA levels and activity substantively contribute to the biology of human breast cancer through its regulation of cell surface signaling, including that of the PRLr. We believe that the knowledge obtained from this work will contribute to a greater understanding of the mechanism of proximal PRL transduction following ligand binding. Given prolyl isomerases are also associated with other cell surface receptors such as transforming growth factor beta receptor (TGF β r) and epidermal growth factor receptor (EGFr) [3; 4], this study may provide a general structural model for the study of the functional interactions between prolyl isomerases and cell surface receptors.

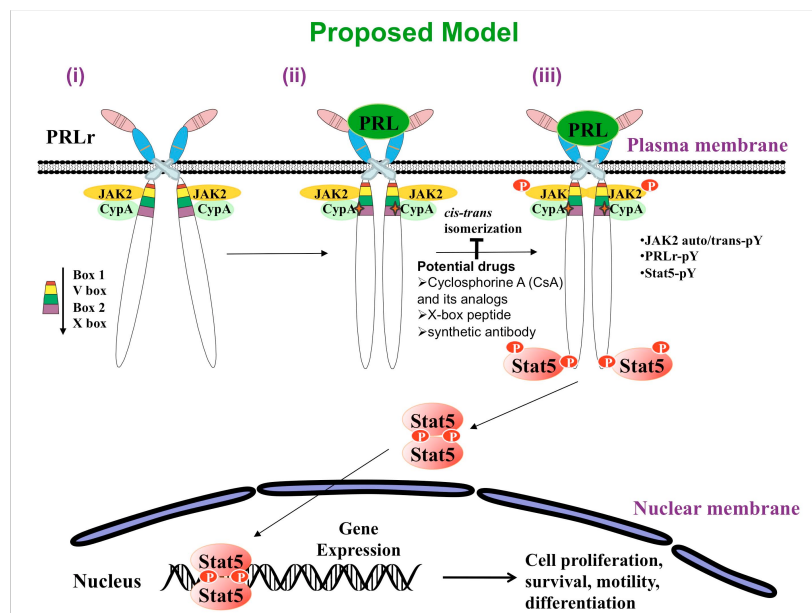


Figure 1. Regulation of the prolactin receptor/Janus kinase 2 (PRLr/Jak2) complex by PRLr-associated cyclophilin A (CypA). (i) CypA is constitutively associated with PRLr and Jak2 during unstimulated conditions. (ii) Upon binding of PRL to PRLr, CypA positively regulates Jak2 activity by exerting its isomerase activity, (iii) presumably through its switching function of a cis-trans peptidyl prolyl isomerase. Ablation of CypA PPI activity by cyclosporine A (CsA) and other approaches inhibits PRL signaling and might be a novel therapeutic strategy in the treatment of human breast cancer. The red arrow in (ii) denotes cis-trans interconversion of proline 334 in the X-box motif of the PRLr; the red circle containing P indicates phosphorylation of JAK2 kinase, PRLr, and signal transducer and activator of transcription 5a (Stat5a).

Our 2009 annual report highlighted that CypA, serving as a molecular switch via its peptidyl-prolyl isomerase (PPI) activity, binds to and regulates the function of the PRLr through the X-box motif. From a translation perspective, we also demonstrated that the X-box peptide significantly blocks PRLr signaling and inhibits PRL-induced gene expression. These studies suggested that the inhibition of CypA levels and activity maybe a novel therapeutic strategy in the treatment of breast cancer (illustrated in Figure 1) [5; 6].

In the past year, in Task 1 we have made a set of Jak2 mutants, and experiments are currently underway to map of the Jak2-CypA interaction domain. In Task 2, we have purified recombinant CypA and PRLr-ICD proteins. The major challenge we encountered is partial degradation of recombinant GST-PRLr-ICD protein. In Task 3, we have conducted several proof-of-concept studies that may lead to novel anti-breast cancer agents. First, we have demonstrated that siRNA-mediated CypA knockdown or the use of CypA inhibitors (CsA analogs such as CsD and CsH) significantly abrogated PRLr signaling and breast cancer cell growth *in vitro*. In addition, by using a novel antibody phage display technology, several synthetic antibodies targeted to the CypA have been developed.

BODY

Three specific aims were proposed in this multidisciplinary postdoctoral award.

Specific Aim #1: To map the domains involved in the CypA-PRLr-Jak2 interactions.

Specific Aim #2: To determine the functional structure of the CypA-PRLr-Jak2 complex.

Specific Aim #3: To evaluate the functional effects of manipulating CypA levels and PPlase activity on PRLr signaling and breast cancer phenotype *in vitro* and *in vivo*.

Statement of Work

The tasks to achieve the specific aims listed above are covered in the statement of work as outlined below.

Task 1: *In vitro* mapping of the CypA-PRLr-Jak2 interaction domains (Months 0-6).

- a. Synthesis of Jak2 mutants and PRLr mutants by PCR-directed mutagenesis (Months 0-3).
- b. Identification of the region of the PRLr and Jak2 which bind to CypA by co-immunoprecipitation studies using a 293 cell-based transfection *in vivo* model system (Months 3-6).

Task 2: Determination of the functional structure of the CypA-PRLr-Jak2 complex (Months 6-36).

- a. Co-crystallization of the CypA/PRLr/Jak2 interaction domains by the chaperone-assisted crystallography “CDC” technology (Months 6-36).
- b. Determination of the magnitude and nature of the conformational changes that are induced in the ICD of the PRLr and Jak2 by EPR spectroscopy analysis (Months 12-36).

- c. Functional characterization of the putative proline residues that are the target of CypA activity by site-directed mutagenesis, co-immunoprecipitation, and in vitro cell culture models (Months 12-36).

Task 3: Assessment of the effects of manipulating CypA levels and PPlase activity on PRLr signaling and breast cancer phenotype *in vitro* and *in vivo* (Months 12-36).

- a. Design and synthesis of interaction-defective CypA mutants and constructs (Months 12-18).
- b. Generation of stable transfectants that overexpress interaction-defective CypA mutant via a lentivirus-based delivery system, and that express the reduced level of CypA via a retrovirus-based siRNA knockdown system (Months 12-18).
- c. *In vitro* testing of the effects of manipulating CypA levels and activity on PRLr signaling. (Months 12-36).
- d. *In vivo* testing of the effects of manipulating CypA levels and activity on the biology of breast cancer using xenografted murine model. (Months 12-36).

Task 1. *In vitro* mapping of the CypA-PRLr-Jak2 interaction domains (Months 0-6).

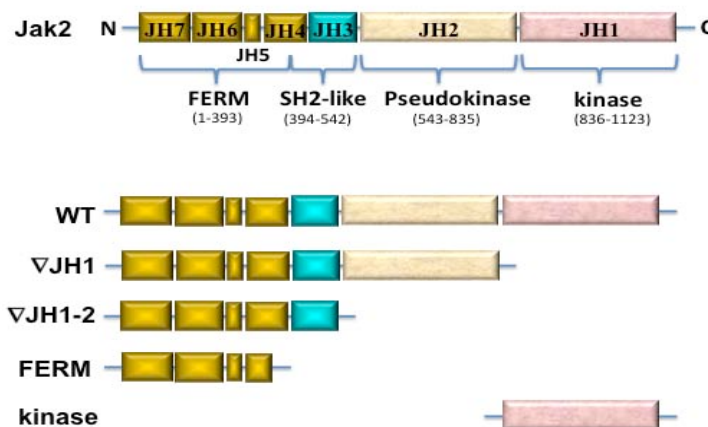


Figure 2. Schematic diagrams of the Jak2 structure and its mutants. Jak2 shares seven significant Janus homology domains (JH1-7) (from the C- to the N-terminus) to other members of the Jak kinase family as described below: (1) JH1 at the C-terminus is a tyrosine kinase domain crucial for kinase activity; (2) Adjacent to JH1 is a catalytically inactive pseudo-kinase or kinase-like domain (JH2) important for autoinhibition of the catalytic activity of JH1 in the absence of ligand stimulation; (3) The first half of JH4 and the whole of JH3 resemble an Src-homology-2 (SH2) domain, termed as SH2-like domain; (4) The N-terminal portion spanning the middle of JH4 to JH7 is referred to as the FERM domain, which is necessary for the association with cell surface receptors and other regulatory proteins. A set of Jak2 C-terminal deletion mutants (WT, ΔJH1, ΔJH1-2 and the FERM domain) and the kinase domain were amplified by PCR and cloned into the eukaryotic expression pTracer vector.

In the first year, we have completed the determination of the CypA-PRLr interaction domains. These data suggested that the PRLr is associated with CypA through its X-box motif, and that a proline residue at 334 within this motif is a potential target of the PPlase activity of CypA [5; 6]. During the second year of the award period, we have followed a similar research strategy to map the interaction domain of Jak2 with CypA. To clone human Jak2, we obtained Jak2 cDNA from Open Biosystems. Using

this cDNA as a template, a set of its C-terminal deletion mutants and the kinase domain were amplified by PCR and cloned into the eukaryotic expression pTracer vector using standard methods. The resulting constructs express the wild type Jak2 (WT) spanning residues 1-1123, ∇ JH1 spanning residues 1-835, ∇ JH1-2 spanning residues 1-542, the FERM domain spanning residues 1-393, and the kinase domain spanning residues 836-1123 (Figure 2). A V5-tag was fused to the 3'-end of all deleted constructs. Experiments are currently underway to determine physical interaction or lack of these mutants with CypA in a 293 cell-based transfection system.

Hence, a part of this task will be delayed and continue into Year 3. Once CypA-interacting domain of Jak2 is determined, DNA fragment encoding this domain will be amplified with PCR and cloned into a suitable prokaryotic expression vector. The recombinant CypA-interacting domain of Jak2 will be produced and purified for crystallization trials as described in Task 2.

Task 2: Determination of the functional structure of the CypA-PRLr-Jak2 complex (Months 6-36).

This task a, b and c focus on the elucidation of the structural basis for the CypA-PRLr-Jak2 complex using several biophysical techniques including x-ray crystallization, EPR analysis, and other biophysical approaches. In the first year of the award period, a part of Task 2c was completed. Our data demonstrated that the X-box motif is an essential mediator for the interaction of the PRLr with CypA, and a replacement mutation of the PRLr (Pro to Ala) caused this receptor to lose the interaction with CypA and to inhibit the activation of wt-PRLr associated signaling. During the second year of the award period, we are working on the production of recombinant proteins using the prokaryotic expression system (*E.coli*) for crystallization and EPR experiments as proposed in Task 2a and 2b. Given this protein complex plays an important role in breast tumor formation and progression, the determination and functional analysis of the three-dimensional structure of this complex will help us to unravel the molecular mechanisms of how CypA modulates PRLr signaling.

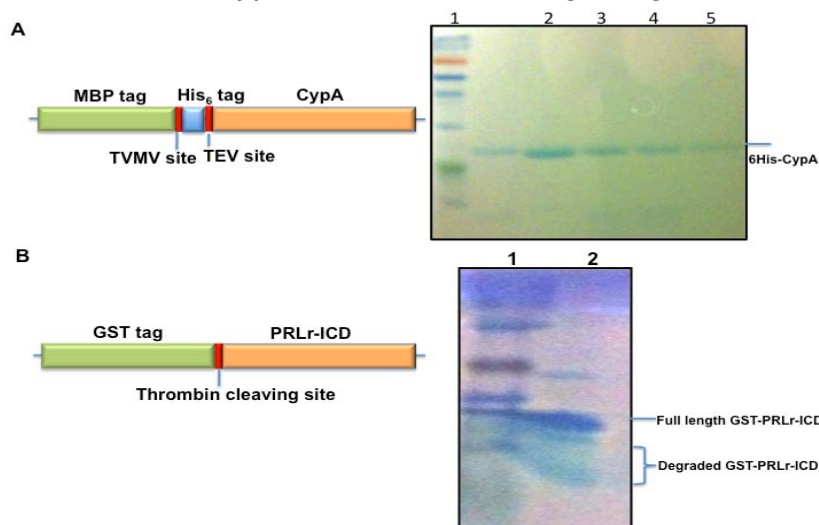


Figure 3. Expression and purification of human CypA and PRLr-ICD. (A) Expression of human CypA protein. Left panel shown is a schematic diagram of recombinant dual-tag CypA fusion protein produced in *E. coli*. The MBP tag was cleaved in *E. coli* BL21 (DE3), which expresses a TVMV protease. Right panel shown is purified recombinant his-tagged CypA protein. Lane1: protein marker; lanes 2-5: a dilution series of recombinant CypA protein. The position of recombinant CypA protein is indicated with an arrow. (B) Expression of human PRLr-ICD domain (259-349). Left panel shown is a schematic diagram of recombinant GST-tagged PRLr-ICD fusion protein. Right panel shown is purified recombinant GST-tagged PRLr-ICD protein. Lane1: protein marker; lanes 2: purified GST-tagged PRLr-ICD protein. The position of the recombinant GST-tagged PRLr-ICD protein and its partial protein degradation are indicated with symbols.

For the expression of recombinant human CypA, PCR-amplified CypA DNA fragment was cloned into pMCSG19C prokaryotic vector using ligation-independent cloning method as previously described [7]. The cloned gene in pMCSG19C was expressed as a fusion protein with a N-terminal dual tag, which is ordered by maltose-binding protein (MBP), a TVMV protease cleave site, poly-histidine, and a TEV protease cleave site (left panel in Figure 3A). This prokaryotic expression system has several advantages over others. For example, fusion to MBP significantly enhances protein solubility in most cases. In addition, the MBP tag could be cleaved in cells co-expressing TVMV protease, such as *E.coli* BL21(DE3) (Novagen). In our experience, this system usually expresses foreign protein at a high level, and the his₆-tag could also be removed by TEV protease *in vitro*. In brief, *E.coli* BL21(DE3) was transformed with the resulting construct DNA and the cells were grown in the LB medium supplemented with ampicillin (100 ug/ml). Expression of recombinant CypA protein was initiated by the addition of IPTG (0.1 mM) at OD₆₀₀ of around 0.6-0.7. After overnight induction at 25°C, highly soluble 6His-tag recombinant CypA protein was purified on Ni-NTA resin under native conditions. In general, using this expression approach we were able to obtain up to 20 mg of recombinant protein (>95% pure as estimated by SDS-PAGE analysis, about 18 kDa) per liter of bacterial culture (right panel in Figure 3A). The purified protein was frozen at -80°C before they were used for experiments.

For the expression of recombinant PRLr-ICD protein, DNA fragment encoding amino acids 259-349 of the intermediate form of the PRLr was amplified by PCR and cloned into pGEX vector (GST Gene Fusion System, GE Healthcare life Sciences) [8]. The resulting construct were verified by DNA sequencing and used to transform *E. coli* BL21 (DE3) cells. Cultures were grown in LB medium with ampicillin (100 ug/ml) at 37°C until OD₆₀₀ reach about 0.6-0.7. Expression of recombinant PRLr-ICD was induced by IPTG (0.1 mM) overnight at 18°C. *E. coli* cells collected by centrifugation were resuspended in 50 mM Tris HCL buffer (pH7.5) containing 200 mM NaCl, 10 mM MgSO₄, 50 µg/ml Dnase and 1x protease inhibitor cocktail, and lysed by French press. Recombinant GST-PRLr-ICD protein was recovered from cell extracts on glutathione-superflow resin (Clontech). In order to mimic the predimerized PRLr in cells, we kept GST-PRLr-ICD fusion protein intact since GST or GST fusion proteins are know to dimerize. Using this expression system we were able to obtain up to 1 mg of recombinant protein (>80% pure as estimated by SDS-PAGE analysis, about 40 kDa) per liter of bacterial culture (right panel in Figure 3B). The purified protein was frozen at -80°C before they were used for experiments. The major challenge we encountered in the purification of recombinant GST-PRLr-ICD protein was partial protein degradation due to unknown reasons as indicated in the right panel of Figure 3B. Currently we are in the process of optimizing this protein purification conditions in order to obtain a highly pure protein sample. Once having purified these proteins (CypA, PRLr-ICD and Jak2) to near homogeneity at a larger scale, we will proceed with crystallization and EPR experiments in Year 3.

Task 3: Assessment of the effects of manipulating CypA levels and PPIase activity on PRLr signaling and breast cancer phenotype *in vitro* and *in vivo* (Months 12-36).

In the first year of the award period, we have identified the X-box motif of the PRLr as an essential mediator for the interaction of the PRLr with CypA. As consequence of this finding, a peptide inhibitor was developed to specifically block the activation of the PRLr signaling and downstream signaling responses. Given the vision

of DOD-BCRP is to cure breast cancer, the successful development of such specific and potent peptide inhibitor of PRLr signaling and CypA activity will open a novel avenue for the development of novel anti-breast cancer agents, and ultimately benefit breast cancer patients. During the second year of the award period, we have further conducted several proof-of-concept studies that may lead to the development of novel anti-breast cancer drugs as described in Task 3 a-d. The major approaches we used to manipulate CypA levels and activity are CypA inhibitors (CsA analogs such as CsD and CsH), siRNA-mediated suppression of CypA protein expression, and generation of synthetic antibodies that neutralize CypA activity.

CypA inhibitors	anchorage-dependent growth	anchorage-independent growth	Cell motility and invasion in vitro	PRL signaling	PRL gene expression	Tumor growth in vivo
CsA	+	+	+	+	+	+
CsD	+	+	ND	+	ND	ND
CsH	+	+	ND	+	ND	ND

+: significantly inhibit; ND: not determined

Table 1. The inhibition of CypA activity by CypA inhibitors (CsA, CsD and CsH) is associated with abrogation of PRLr signaling and tumor growth *in vitro* and *in vivo*. While CsA is a well-known FDA-approved immunosuppressive drug, CsD and CsH are its non-immunosuppressive chemically modified derivatives. Using several representative breast cancer cell *in vitro* models (ER+: T47D and MCF7; ER-: MDA231) and/or xenografted breast cancer nude murine *in vivo* models, we have demonstrated that CsA and its analogs have anti-breast cancer efficacy. In cell culture-based models, CsA and its analogs (CsD and CsH) inhibit PRL signaling (e.g. Jak2, Stat5, Erk and Akt) and PRL-induced gene expression (e.g. CISH and Cyclin D1), suppress anchorage-dependent and independent cell growth, block motility and invasion of human breast cancer cells. In xenografted nude models, we have further demonstrated that CsA significantly enhances central necrosis of primary tumors and blocks tumor metastasis.

First, using CypA inhibitors, we have demonstrated that CsA as we previously published [5] and its non-immunosuppressive analog (CsD and CsH) treatment of breast cancer cells significantly abrogated PRLr signaling and PRL-induced gene expression, inhibited cell growth, motility and invasion *in vitro*, and blocked tumor growth *in vivo* (summarized in Table 1). Thus, the development of novel CypA inhibitors represents a useful therapeutic strategy for treating breast cancer patients.

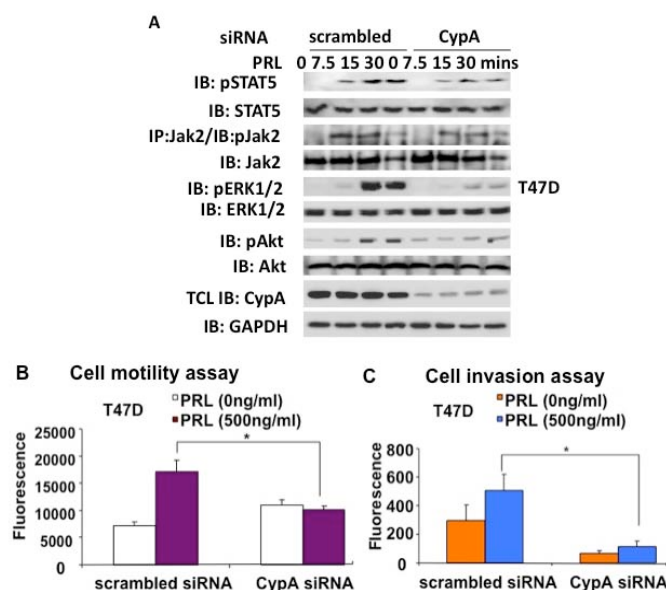
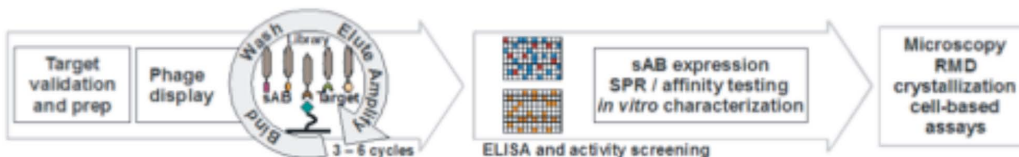


Figure 3. siRNA-mediated knockdown of CypA inhibits PRL-induced signaling and PRL-induced gene expression, and blocks motility and invasion of human breast cancer cells. (A) Stable depletion of CypA inhibits PRLr signaling. Stable pools of T47D transfectants expressing scrambled or CypA siRNA were stimulated with PRL (100 ng/ml) at indicated times, harvested, and lysates were subject to immunoblot analysis as indicated. (B-C) Stable depletion of CypA blocks motility and invasion of breast cancer cells. Stable transfectants of T47D cells with scrambled siRNA and CypA siRNA were allowed to migrate or invade toward 3% fetal bovine serum for 24 hours. The number of cells migrating or invading to the lower surface of the membrane was quantified by CyQuant (Invitrogen).

Second, we provided evidence that elimination of endogenous CypA protein by using RNA interference technology resulted in a significant inhibition of Jak2, Stat5, ERK1/2, and Akt activation in breast cancer cells (Figure 3A). More importantly, siRNA-mediated knockdown of CypA can block motility and invasion of breast cancer cells *in vitro* (Figure 3B-C). Thus, suppression of CypA expression using small interfering RNA may hold a potential as a therapeutic strategy for breast cancer. The concept of the use of RNA interference to suppress expression of oncogenes in breast cancer is encouraged by a recent study that nanoparticle-delivered siRNA successfully turns off expression of ribonucleotide reductase (RRM2) gene in human tumors [9].

A antibody phage display selection and screening



B Sequences of selected FABs

FABs	CDR-H1	CDR-H2	CDR-H3
1	F S S S Y I	Y I S S S S G Y T Y	G P Y F Y Y P E E M E W W Q A M
2	I S Y S S I	S I Y S Y S G Y T S	Y I G G E Y Y Y G T A M
3	F S Y Y Y I	S I Y P Y S G Y T S	Q Y Y P F Y Y S S K A M
4	V Y S S S I	S I S S Y Y G Y T Y	E W F G G M
5	F S Y Y Y I	S I Y P Y S G Y T S	Q Y Y P F Y Y S S K A M
6	I S Y S S I	S I Y S Y S G Y T S	Y I G G E Y Y Y G T A M
7	F S Y Y Y I	S I Y P Y S G Y T S	G P Y F Y Y P E E M E W W Q A M
8	F S Y S S I	S I Y S S S G Y T Y	F G L S G Y G A A M
9	F Y S S S I	S I Y P S Y S G T Y	Y Y Y G V P G L
10	F S S S Y I	Y I S S S S G Y T Y	Y M
11	F Y S S S I	S I Y P S Y G S T Y	Y Y Y G V P G L
12	I Y Y S S I	S I Y S S S G Y T Y	G V G W S A L
13	F Y Y S S I	S I Y P Y S S S T S	N W W Y D W A M
14	I S S S S I	Y I Y P S S G S T S	K W W Y H S Y P P G W W S A L
15	I Y Y S S I	S I S S Y Y G Y T Y	L G W G Y G S A A L
16	V Y S Y S I	S I Y S Y S G Y T S	M Q W S A L
17	V S S S Y I	S I Y P S Y G S T Y	S K Y W H M G L
18	F S Y S S I	S I Y S Y S G Y T S	Y I G G E Y Y Y G T A M

Color code: Y tyrosine S serine G glycine

C Expression and purification of selected FABs

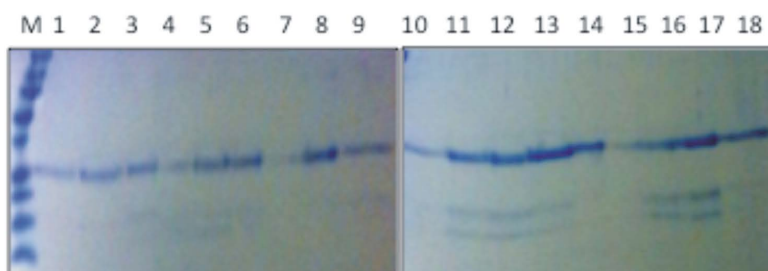


Figure 4. Generation of synthetic antibodies against CypA. (A) A schematic diagram of synthetic antibody production, which involves target protein immobilization, phage display library screening, and structural and functional analysis of selected FABs. (B) Sequences of selected FABs. Tyrosine (Y), Serine (S) and Glycine (G) are randomized residues within complementarity determining region (CDR) H1, H2 and H3. In order to show their abundance in the sequence, these residues are highlighted by yellow, red and green, respectively. (C) Expression and purification of selected FABs. Through the introduction of a stop codon between the FAB heavy chain and the phage p3 coat protein using Kunkel mutagenesis, a soluble form of FABs was obtained. The resulting phagemid DNAs were used to transform *E. coli* 55244 and the FAB proteins were expressed under the PhoA alkaline phosphatase promoter in the CRAP medium at 30 °C for 24 hours. The FAB proteins were purified on protein A resin. Lane 1-18: purified FAB proteins (#1-18) (>90 pure as estimated by coomassie blue-stained SDS-PAGE gel).

Third, using an antibody phage display technology, we have successfully generated 18 synthetic antibodies targeted to the CypA. Antibody-based therapies are highly advantageous because they provide specificity, thus minimizing toxicity. By using the most appropriate delivery techniques, we can deliver these synthetic antibodies into cancer cells, and stop cancer cell growth. The production of functional synthetic antibodies involves immobilization of target proteins, phage display library screening, and functional analysis of selected antigen-binding fragments (FABs) (Figure 4A). In brief, recombinant his-tag CypA proteins were produced and purified as described in Task 2. Target proteins were then biotinylated through EZ-Link NHS-SS-PEG4-Biotin kit (Pierce), and the resulting proteins were immobilized on streptavidin-coated paramagnetic beads. The immobilized target proteins were mixed with the YSG FAB phage library (Genentech, Inc.) to capture target-specific phage particles. After four rounds of library sorting and amplification, individual bound phages were randomly selected for competitive phage enzyme-linked immunosorbent assay (ELISA) in the presence or absence of competitors (soluble native CypA proteins or CsA). Positive clones were sequenced and the FABs with unique CDRs were identified. As shown in Figure 4B, phage display library screening results in the generation of total 18 CypA-specific FABs including CsA sensitive (#1-11) and non CsA-sensitive (#12-18). A free protein form of FABs other than FAB-p3 fusion protein was achieved by the introduction of a stop codon between the FAB heavy chain and the phage p3 coat protein using Kunkel mutagenesis. The 55244 *E. coli* strain (Genentech, Inc) was transformed with the phagemid DNA, and cultures were grown in the CRAP medium supplemented with ampicillin (100 µg/ml) for 24 hours at 30°C. The FAB proteins were purified with protein A resin and subjected to binding affinity analysis. Based on our preliminary surface plasmon resonance (SPR) analysis, several FABs have exhibited binding affinity within a low nanomolar range, and are good candidates for further studies (Table 2). Experiments are currently underway to test these potential synthetic antibodies *in vitro* and *in vivo*. In order to establish the binding model, we will determine the three dimensional structure of the FAB/CypA complex using x-ray crystallography in Year 3.

FABs	3	4	5	8	10	14	16	18
Kd (nM)	3	2	7	12	2	12	4	0.9

Table 2. Preliminary SPR analysis of binding affinity of selected FABs. Purified FABs were injected over Sensor Chip NTA where 6His-tag CypA was immobilized on the surface. The equilibrium constant Kd was used to measure the binding affinity of two components.

KEY RESEARCH ACCOMPLISHMENTS

- Cloning of human Jak2 gene and its deletion mutants into the eukaryotic expression vector.
- Cloning of human CypA and PRLr-ICD into prokaryotic expression vectors.
- Expression of human CypA and PRLr-ICD proteins in *E.coli* BL21(DE3) cells and purification of recombinant proteins using chromatographic methods.
- Treatment of CsA analogs (CsD and CsH) on breast cancer cells that blocks PRL signaling and inhibits multiple breast cancer phenotypes.
- siRNA-mediated stable depletion of endogenous CypA protein in breast cancer cells that inhibits PRLr signaling and PRL-induced gene expression.
- Generation of synthetic antibodies against CypA using antibody phage display technology.

REPORTABLE OUTCOMES

Conference Abstracts and Posters

Zheng J., A. Paduch M., Harrington K., Kossiakoff A., and Clevenger C.
“Multidisciplinary Analysis of Cyclophilin A/Prolactin Receptor Complex Function in Human Breast Cancer”. DOD The Leading Innovation and Knowledge Sharing (LINKS) Meeting, Vienna, VA, February 16 – 17, 2010.

CONCLUSIONS

During the second year of the award period, in Task 1 we have made a set of Jak2 mutants, and mapping of the Jak2-CypA interaction domain is currently underway. In Task 2 we have purified recombinant CypA and PRLr-ICD proteins. The major challenge we encountered was partial protein degradation of recombinant GST-PRLr-ICD protein. Currently we are in the process of optimizing this protein purification conditions in order to obtain a highly pure protein sample. Once having purified these proteins (CypA, PRLr-ICD and Jak2) to near homogeneity at a larger scale, we will proceed with crystallization experiments in Year 3. In Task 3, we have conducted several proof-of-concept studies that may lead to novel anti-breast cancer agents. First, using CypA inhibitors, we have demonstrated that CsA analogs such as CsD and CsH significantly abrogated PRLr signaling and tumor growth. Second, using RNA interference approach, stable depletion of endogenous CypA protein inhibited PRL signaling and blocked motility and invasion of breast cancer cells. Third, we have developed several synthetic antibodies against CypA. Antibody-based therapies are highly advantageous because they provide specificity, thus minimizing toxicity. Our ultimate goal is that, by using the most appropriate delivery techniques, we can deliver synthetic antibodies, siRNA molecules or other novel CypA inhibitors into cancer cells, and stop cancer cell growth.

In the final year of the award, we will proceed with the completion of remaining experiments as follows:

1. To map the Jak2-CypA interacting domains (Task 1)
2. To determine the 3D structure/function of the CypA/PRLr-ICD/Jak2 complex (plus to establish a binding model of CypA with its FAB) (Task 2)
3. To determine the *in vivo* effects of manipulating CypA levels and activity in breast cancer (Task 3)

The completed research and training grant will support the PI's planned career in breast cancer research, by enabling him to translate structure/function relationships of receptor action into pre-clinical models of this disease.

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APPENDICES

A copy of abstract from 2010 DOD's The Leading Innovation and Knowledge Sharing (LINKS) Meeting

2010 DOD's The Leading Innovation and Knowledge Sharing (LINKS) Meeting

Multidisciplinary Analysis of Cyclophilin A/Prolactin Receptor Complex Function in Human Breast Cancer

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The growth and progression of human breast cancer is regulated by several cell surface receptors, including the prolactin receptor (PRLr). These receptor-triggered signals directly contribute to prolactin (PRL) induced proliferation, survival, and motility of human breast cancer. The major objective of this proposal is to understand how cyclophilin A (CypA) regulates the PRLr signaling and to determine the effects of altered CypA expression and activity on the PRLr signaling and breast cancer phenotypes. We aim to accomplish this through a multidisciplinary approach that combined biological, biophysical structural and animal studies. Our data suggest that CypA, serving as a molecular switch via its peptidyl-prolyl isomerase (PPI) activity, binds to and regulates the function of the PRLr through its X-box motif. We have also conducted several proof-of-concept studies that may lead to novel anti-breast cancer agents. First, using CypA inhibitors, we have demonstrated that CsA and its analogs significantly abrogated PRLr signaling and tumor growth. Second, using RNA interference approach, stable depletion of endogenous CypA protein inhibited PRL signaling and blocked motility and invasion of breast cancer cells. Third, by mapping of the PRLr-CypA interaction, we have identified and developed a peptide inhibitor, which specifically blocked the activation of

the PRLr signaling and downstream signaling responses. Lastly, we have developed several synthetic antibodies against CypA. Antibody-based therapies are highly advantageous because they provide specificity, thus minimizing toxicity. Our ultimate goal is that, by using the most appropriate delivery techniques, we can deliver these synthetic antibodies, peptide inhibitors or other CypA inhibitors into cancer cells, and stop cancer cell growth. The knowledge we obtained from this study will contribute to a greater understanding of the mechanism of PRL action. Given that the PRLr-triggered signals directly contribute to PRL-induced proliferation, survival, and motility of human breast cancer, the proposed study in detail on the effect of CypA on PRLr structure and function will have a significant impact on human breast cancer.