

AD _____

Award Number: W81XWH-08-1-0169

TITLE: Multidisciplinary Analysis of Cyclophilin A Function in Human Breast Cancer

PRINCIPAL INVESTIGATOR: Jiamao Zheng

CONTRACTING ORGANIZATION: Northwestern University
Chicago, IL 60611

REPORT DATE: March 2009

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE 1 March 2009		2. REPORT TYPE Annual Summary		3. DATES COVERED 1 Mar 2008 – 28 Feb 2009	
4. TITLE AND SUBTITLE Multidisciplinary Analysis of Cyclophilin A Function in Human Breast Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-08-1-0169	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Jiamao Zheng E-Mail: jiamaoz@yahoo.com				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Northwestern University Chicago, IL 60611				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The major goal of the proposed project is to understand how cyclophilin A (CypA) modulates the prolactin (PRL) receptor (PRLr)-associated signaling and to determine the effects of altered CypA levels and activity on PRL signaling and breast cancer phenotype by combining biophysical structural investigations with studies in cell and animal models. The knowledge we obtained from this study will contribute to a greater understanding of the mechanism of PRL action. Our recent 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. From a translation perspective, we have also shown that the X-box peptide significantly blocks PRLr signaling. 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.					
15. SUBJECT TERMS Cyclophilin A, prolactin, prolactin receptor, Jak2, breast cancer					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 48	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

Introduction.....	4
Body.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusion.....	7
References.....	8
Appendices.....	8

INTRODUCTION

We have previously implicated the PPI activity of CypA in the function of the prolactin/prolactin receptor (PRL/PRLr) complex during the pathogenesis of human breast cancer (1). In this project, we will study how CypA modulates PRLr signaling. We aim to accomplish this through a multidisciplinary approach that combined 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 level and activity substantively contributes 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 PRLr 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) (2, 3), this study may provide a general structural model for the study of the functional interactions between prolyl isomerases and cell surface receptors.

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 PPIase 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. Items that had to be completed in the first year of the award period are indicated in bold.

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 PPIase 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).

In the first year, one of the tasks to be completed was determination of the CypA-PRLr-Jak2 interaction domains. For this purpose, we utilized biochemical techniques to determine the domain of the PRLr that interacts with CypA. On the basis of the primary structure of the PRLr, a set of its C-terminal deletion constructs were assembled into the pTracer vector using PCR-directed mutagenesis, including the wild-type long form of the PRLr, the short form of the PRLr, and truncations of ICD between conserved domains of the PRLr at residues 326, 314, 276, and 264. A V5-tag was fused to the 3'-end of all deleted constructs. Physical interaction or lack of these mutants with CypA thereof was tested by co-immunoprecipitation (anti-CypA immunoprecipitation followed by anti-V5 immunoblot) in a 293 cell-based transfection system. Our published data (Figure 4) suggested that truncations of the PRLr membrane-proximal to the X-box motif were incapable of interacting with CypA, revealing a contribution of the X-box motif to the engagement of CypA (4). Taken together, our data suggests that the PRLr is associated with CypA through its X-box motif. Note that the results of these studies will be used to determine the structure/function relationships of this protein complex when analyzed with data from crystallography and EPR spectroscopy as proposed in Aim #2.

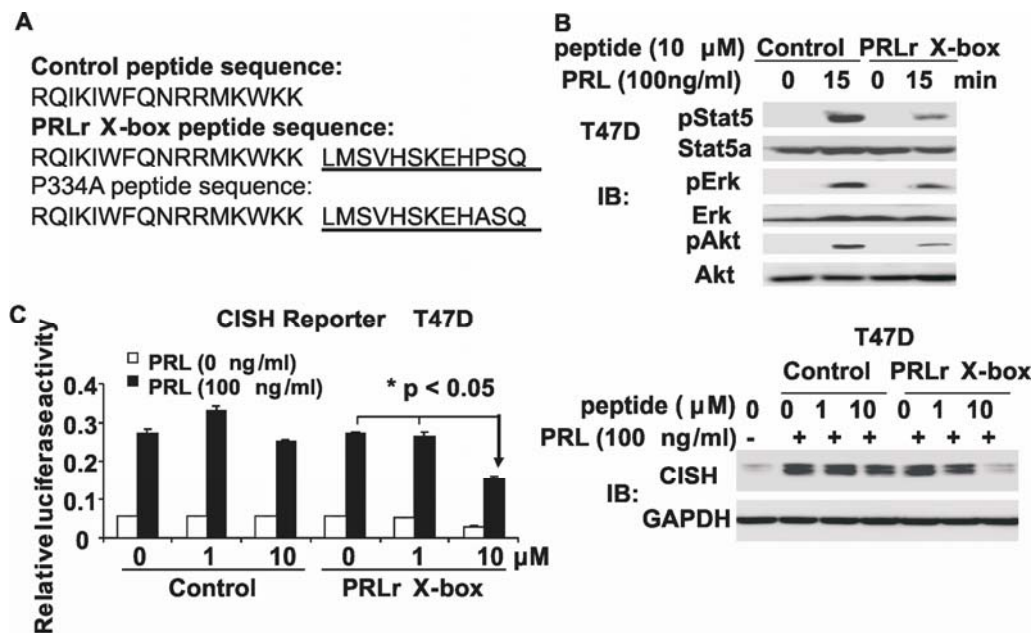
Task 2: Determination of the functional structure of the CypA-PRLr-Jak2 complex (Months 6-36).

- 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).**

According to schedule, the plans for functional characterization of the putative proline residues within the PRLr that are targets of CypA activity, will be pursued after the determination of the functional structure of the PRLr-CypA complex. Our data, however, have revealed that the X-box motif is an essential mediator for the interaction of the PRLr with CypA. Furthermore, analysis of the X-box motif revealed a proline at residue 334 of the PRLr that is highly conserved across species. Therefore, we hypothesized that P334 in the X-box may contribute to the engagement of CypA. To test this hypothesis a proline-to-alanine replacement mutation of the PRLr was generated (P334A), and the ability of this mutant PRLr to interact with CypA and mediate PRL-induced signaling was tested (see more detail in Figure 4) (4). These studies revealed that when expressed in T47D cells, the mutant PRLr P334A demonstrated a significantly reduced association with CypA. Furthermore, the ability of the P334A mutant to induce Jak2 and Stat5 phosphorylation (in comparison to wild type PRLr transfected control)

was also significantly diminished. Further, overexpression of the PRLr P334A mutant in T47D cells resulted in a dose-dependent reduction in the PRL-induced expression of both co-transfected LHRE and CISH luciferase reporters and endogenous CISH protein. These findings are notable, in that T47D cells are known to express high levels of endogenous PRLr, indicating that the P334A mutant, most probably through heterodimerization with the wild type PRLr, functions as a dominant negative receptor. When assessed with the data presented above, these findings would indicate that the direct action of the CypA on the PRLr is required for effective PRL-induced signaling, a process that is inhibited by the PRLr P334A mutant. These findings were further confirmed by control experiments which demonstrated that the PRLr/Jak2 interaction was not affected by the P334A mutation.

From a translation perspective, we synthesized the X-box peptide and examined its effects on PRL signaling in T47D breast cancer cells using cell-permeable peptide as a delivery system.



The X-box peptide blocks PRL signaling. (A), Sequences of control and the PRLr X box peptides. Control peptide is the Antennapedia homeodomain, a cell-permeable peptide; The X-box peptide is composed of the Antennapedia homeodomain and the X-box motif (underlined sequence). (B), The X-box peptide blocks the activation of the PRLr-associated signaling. T47D cells were starved overnight and pre-treated with 10 μ M of control or PRLr X-box peptide for 1 hour as indicated. Cells were stimulated with PRL (100 ng/ml) for 15 minutes. Blots were probed with the indicated antibodies. (C), The X-box peptide inhibits PRL-induced gene expression from CISH luciferase reporter and endogenous CISH. T47D cells were transfected with CISH luciferase reporter. Cells were starved overnight and pre-treated with 0, 1, and 10 μ M of control or the X-box peptide for 1 hour as indicated. After 24 hours PRL (100 ng/ml) treatment, cell lysates were analyzed for luciferase activity and endogenous CISH protein level. The results are representative of two independent experiments.

Our data demonstrated that the X-box peptide but not control peptides (either cell-permeable peptide or P334A peptide) was able to significantly block PRL signaling and reduce PRL-induced gene expressions from CISH luciferase reporters and endogenous CISH. Taken together, these data suggest that the X-box peptide appears to be a specific and potent inhibitor of PRL signaling.

Multidisciplinary Training Program:

The proposed studies are multidisciplinary in nature as they combine the two distinct fields of cell biology and biophysics. For training in tumor biology, the principal investigator continued attending breast cancer progression seminar series at the Northwestern University. In order to be able to carry out the proposed functional structure analysis of the PRLr/Jak2/CypA complex, the principal investigator acquired the necessary training in structure biology, X-ray crystallography and NMR spectroscopy by auditing the relevant course at the University of Chicago.

KEY RESEARCH ACCOMPLISHMENTS

- Identifying that the PRLr X-box motif interacts with CypA
- Developing a peptide inhibitor which blocks PRL signaling
- Course training of the principal investigator in X-ray crystallography and NMR spectroscopy

REPORTABLE OUTCOMES

Journal Papers

Zheng, J., J. E. Koblinski, L. V. Dutson, Y. B. Feeney, and C. V. Clevenger. 2008. Prolyl isomerase cyclophilin A regulation of Janus-activated kinase 2 and the progression of human breast cancer. *Cancer Research* 68:7769-7778.

Clevenger, C.V., S.L. Gadd, and J. Zheng. 2009. New mechanisms for PRLr action in breast cancer. *Trends in Endocrinology and Metabolism*. In press.

Conference Abstracts and Posters

Zheng J., A. Kossiakoff, and C. Clevenger. "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 9 – 10, 2009.

CONCLUSIONS

In the first year of the award period, the specific aims we accomplished were mapping of the PRLr/CypA interacting-domain, development of a X-box derived peptide inhibitor which blocks PRL signaling, and training of the principal investigator in breast cancer and structure biology. During this period, we haven't encountered any major challenges. Next steps in the study will be:

- Determining the three-dimensional structures of CypA complexed with the X-box peptide from the PRLr by standard X-ray crystallography.
- Determining the three-dimensional structures of Jak2 FERM domain complexed with the PRLr ICD domain by Chaperone-Assisted Crystallography "CAC" technology.
- Mapping the Jak2/CypA interacting domains and determining their structures by standard X-ray crystallography.

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.

REFERENCES

1. F. Syed, M. A. Rycyzyn, L. Westgate, C. V. Clevenger, *Endocrine* 20, 83 (Feb-Mar, 2003).
2. Y. G. Chen, F. Liu, J. Massague, *Embo J* 16, 3866 (Jul 1, 1997).
3. M. Lopez-Illasaca *et al.*, *J Biol Chem* 273, 9430 (Apr 17, 1998).
4. J. Zheng, J. E. Koblinski, L. V. Dutson, Y. B. Feeney, C. V. Clevenger, *Cancer Res* 68, 7769 (Oct 1, 2008).

APPENDICES

A copy of published paper: Zheng, J., J. E. Koblinski, L. V. Dutson, Y. B. Feeney, and C. V. Clevenger. 2008. Prolyl isomerase cyclophilin A regulation of Janus-activated kinase 2 and the progression of human breast cancer. *Cancer Research* 68:7769-7778.

A copy of manuscript: Clevenger, C.V., S.L. Gadd, and J. Zheng. 2009. New mechanisms for PRLr action in breast cancer. *Trends in Endocrinology and Metabolism*. In press.

A copy of abstract from 2009 Dod's The Leading Innovation and Knowledge Sharing (LINKS) Meeting

Prolyl Isomerase Cyclophilin A Regulation of Janus-Activated Kinase 2 and the Progression of Human Breast Cancer

Jiamao Zheng, Jennifer E. Koblinski, Laura V. Dutson, Yvonne B. Feeney, and Charles V. Clevenger

Breast Cancer Program, Robert H. Lurie Comprehensive Cancer Center and Department of Pathology, Northwestern University, Chicago, Illinois

Abstract

The activation of the Janus-activated kinase 2 (Jak2) tyrosine kinase following ligand binding has remained incompletely characterized at the mechanistic level. We report that the peptidyl-prolyl isomerase (PPI) cyclophilin A (CypA), which is implicated in the regulation of protein conformation, is necessary for the prolactin (PRL)-induced activation of Jak2 and the progression of human breast cancer. A direct correlation was observed between the levels or activity of CypA and the extent of PRL-induced signaling and gene expression. Loss of PRLr-CypA binding, following treatment with the PPI inhibitor cyclosporine A (CsA), or overexpression of a dominant-negative PRLr mutant (P334A) resulted in a loss of PRLr/Jak2-mediated signaling. *In vitro*, CsA treatment of breast cancer cells inhibited their growth, motility, invasion, and soft agar colony formation. *In vivo*, CsA treatment of nude mice xenografted with breast cancer cells induced tumor necrosis and completely inhibited metastasis. These studies reveal that a CypA-mediated conformational change within the PRLr/Jak2 complex is required for PRL-induced transduction and function and indicate that the inhibition of prolyl isomerases may be a novel therapeutic strategy in the treatment of human breast cancer. [Cancer Res 2008;68(19):7769–78]

Introduction

Peptidyl-prolyl isomerases (PPI) are a family of enzymes that catalyze the *cis-trans* interconversion of imide bonds of proline residues (1). One member of this family, cyclophilin A (CypA), was initially identified as the primary cytosolic receptor of the immunosuppressive drug cyclosporine A (CsA; ref. 2). The immunosuppressive activity of CsA is thought to be mediated by the engagement of calcineurin by the CsA-CypA complex (3), an observation supported by the finding that CypA knockout mice are resistant to immunosuppression by CsA (4). As a PPI, CypA has been found to assist in protein folding *in vivo* (5). Several lines of research, however, have revealed that CypA and other PPIs may function as molecular signaling “switches” (6). Indeed, it has been shown that PPI activity promotes viral replication and infection of HIV (7), modulates Itk and Crk signaling (8, 9), regulates the pore opening of a neurotransmitter-gated ion channel (10), functions

as a molecular timer for phage infection (11), and controls transcription factor activity (12).

Additional data also suggest that CypA may contribute to the pathology of human malignancy. CypA is overexpressed in a large number of lung (13), pancreatic (14), and oral squamous cancer cells and tissues (15). Significantly, in a case cohort of 25,000 women who had received CsA as therapy for renal and cardiac allografts, a reduction in the incidence of breast cancer of up to 50% in the CsA-treated group was noted during a 10-year follow-up period. Indeed, in their first year of therapy, the CsA-treated cohort showed a 90% reduction in the incidence of breast cancer (16). Taken together, these data suggest that CypA may play an important role in tumorigenesis and serve as a target for PPI inhibitors in the oncologic setting.

The important role of the hormone prolactin (PRL) in the development and progression of breast cancer has been supported by epidemiologic studies and transgenic model studies (17–19). The effects of PRL on normal and malignant breast tissues are mediated by the prolactin receptor (PRLr), a member of the cytokine receptor superfamily and relative of the growth hormone receptor. Both the growth hormone receptor and the PRLr are predimerized; following ligand binding, it has been reported that the growth hormone receptor and PRLr may undergo conformational change (20, 21), enabling the rapid activation of the Janus-activated kinase 2 (Jak2) tyrosine kinase, which results in PRLr/growth hormone receptor phosphorylation and the activation of several signaling cascades including the Jak2/signal transducer and activator of transcription-5 (Stat5), Ras/mitogen-activated protein kinase, and Nek3/Vav2/Rac1 pathways (17, 22). The triggering of these signaling pathways summates in the activation of multiple gene loci, including cyclin D1 (23) and the cytokine inducible SH2-containing protein (CISH; ref. 24). Like other cytokine receptors that associate with Jak2, the activation of many of these downstream signaling pathways is dependent on the initial activation of Jak2. However, the molecular mechanism following ligand binding that triggers a conformational change in the Jak2-receptor complex enabling Jak2 activation has remained elusive. Given the important role that the PRLr, growth hormone receptor, and other cytokine receptors play in normal and pathologic physiology, an understanding of this phenomenon is highly relevant.

Our laboratory has previously reported on a role for cyclophilin family members in the regulation of the PRLr-mediated signaling. Triggered by PRL, cyclophilin B potentiated Stat5 function by inducing the release of the Stat repressor, PIAS3 (12, 25). Here, we report that the PPI activity of CypA contributes to the proximal activation of Jak2 and other PRLr-associated signaling pathways following binding of ligand to the PRLr as a receptor-associated conformational switch. Finally, our findings reveal that ablation of CypA activity by several approaches, including the use of the Food

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Requests for reprints: Charles V. Clevenger, Department of Pathology, Northwestern University, Lurie 4-107, 303 East Superior Street, Chicago, IL 60611. Phone: 312-503-5750; Fax: 312-503-0095; E-mail: clevenger@northwestern.edu.

©2008 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-08-0639

and Drug Administration–approved drug CsA, inhibits the *in vitro* and *in vivo* outgrowth of human breast cancer.

Materials and Methods

Cell culture and reagents. The human breast cancer cell lines T47D, MCF7, and MDA-231 were maintained in recommended conditions (26). The mouse pre-adipocyte cell line 3T3F442A, kindly provided by Dr. Stuart J. Frank (Department of Medicine, University of Alabama, Birmingham, AL), was maintained in DMEM supplemented with 10% calf serum. CsA (Gengraf) was purchased from Abbott Laboratories. Recombinant human PRL was obtained from Dr. Michael Hodsdon (Department of Pathology, Yale University, New Haven, CT), and recombinant human growth hormone was from Dr. Stuart J. Frank. CyQuant (Invitrogen) and QuickChange (Stratagene) were used as directed. Antibodies used in these studies were obtained from Santa Cruz (cyclophilin A and CISH), Invitrogen (Stat5a, pStat5a, glyceraldehyde-3-phosphate dehydrogenase, V5, cyclin D1, and PRLr-ECD), and Cell Signaling [Jak2, pJak2, extracellular signal–regulated kinase (Erk)-1/2, pErk1/2, Akt, and pAkt].

Immunoblotting and immunoprecipitation. Immunoblotting and immunoprecipitation were done as previously described (26). Target proteins were visualized by enhanced chemiluminescence (GE Healthcare), and images were captured using Fujifilm LAS-3000 system. The band intensities were quantified by densitometry using ImageQuant and normalized to those of their respective control bands. Data were expressed as fold changes compared with an appropriate control.

Plasmids, transfection, and retrovirus production. The PRLr, CypA constructs, Stat5-responsive luteinizing hormone response element (LHRE), and CISH reporters were previously described (26, 27). A set of COOH-terminal deletion constructs of the PRLr were assembled into the pTracer vector. A V5-tag was fused to the 3' end of all deleted constructs. All point mutations were generated using the QuickChange kit. The pSilencer 5.1 carrying a DNA insert encoding a CypA siRNA or a scrambled control siRNA was purchased from Ambion.

The PRL-induced, luciferase-linked gene reporters used here contained either a synthetic LHRE reporter or the endogenous promoters from the *CISH* gene. For luciferase assay, cells were transiently transfected with the LHRE and CISH reporters, in addition to either the empty vector (control)

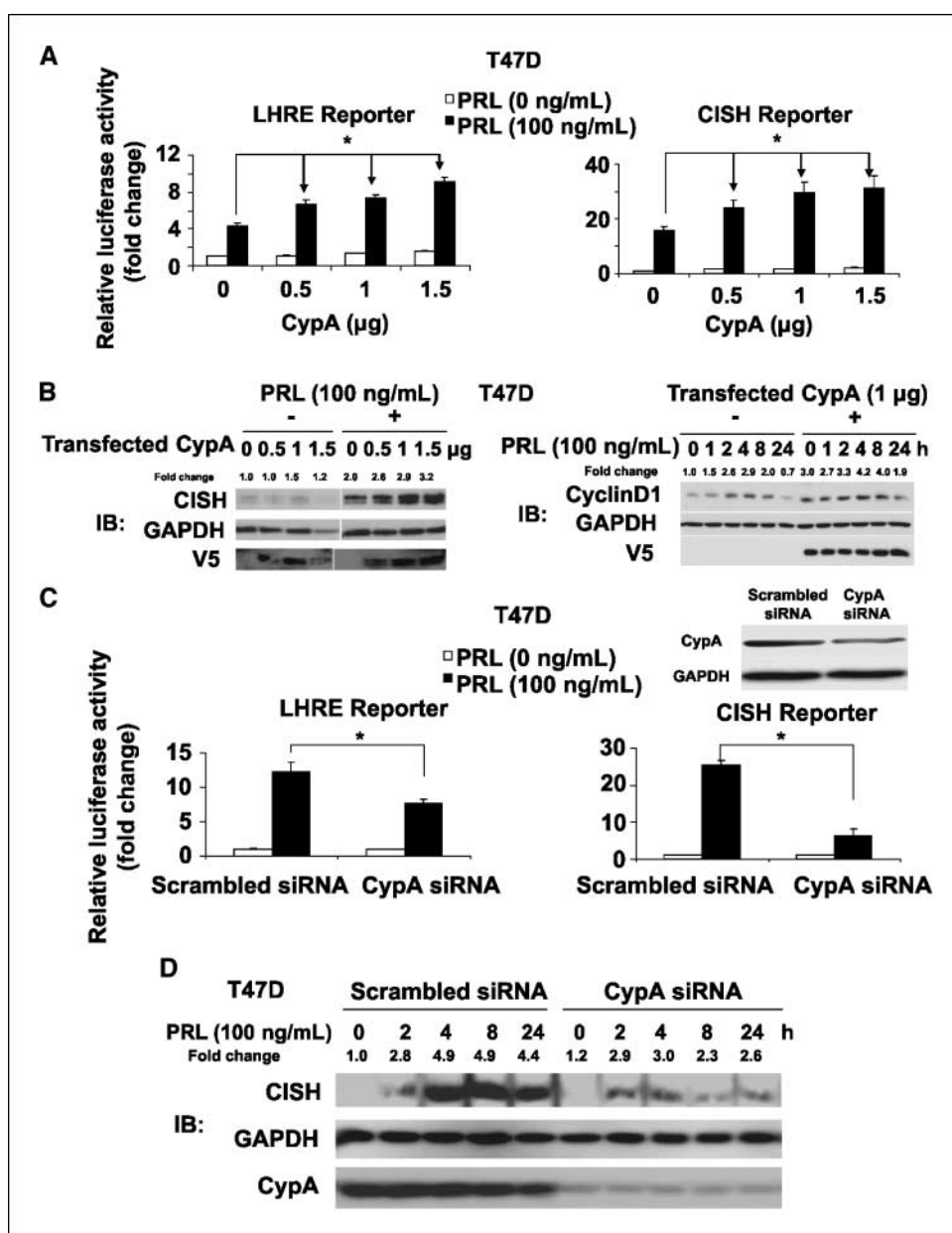


Figure 1. Modulation of CypA levels regulates PRL-induced gene reporter and endogenous gene expression. **A**, CypA overexpression enhances PRL-induced gene expression from luciferase reporters. T47D cells were cotransfected with LHRE or CISH reporters with increasing amount of CypA overexpression construct as indicated. Cells were treated with PRL (100 ng/mL) in defined medium for 24 h. Columns, mean of at least two independent experiments; bars, SE. *, $P < 0.05$ (*t* test). **B**, CypA overexpression enhances PRL-induced endogenous gene expression of CISH and cyclin D1 proteins in T47D cells. The expression of transfected CypA was verified by Western blot analysis of total cell lysates with an anti-V5 antibody. **C**, siRNA-mediated knockdown of CypA results in reduced PRL-induced reporter gene expression. T47D cells were transfected with CypA siRNA or scrambled control siRNA and the indicated luciferase reporters. Following 24-h stimulation with PRL (100 ng/mL), transfectants were lysed and assayed for luciferase activity. Knockdown of CypA by siRNA was confirmed by Western blot analysis (top right). Columns, mean of at least two independent experiments; bars, SE. *, $P < 0.05$ (*t* test). **D**, stable depletion of CypA represses endogenous *CISH* gene expression in T47D cells. Stable transfectants of T47D cells expressing either scrambled siRNA or CypA siRNA were stimulated with PRL (100 ng/mL) for 24 h. Suppression of endogenous, PRL-induced CISH expression by CypA knockdown was detected by Western blot analysis.

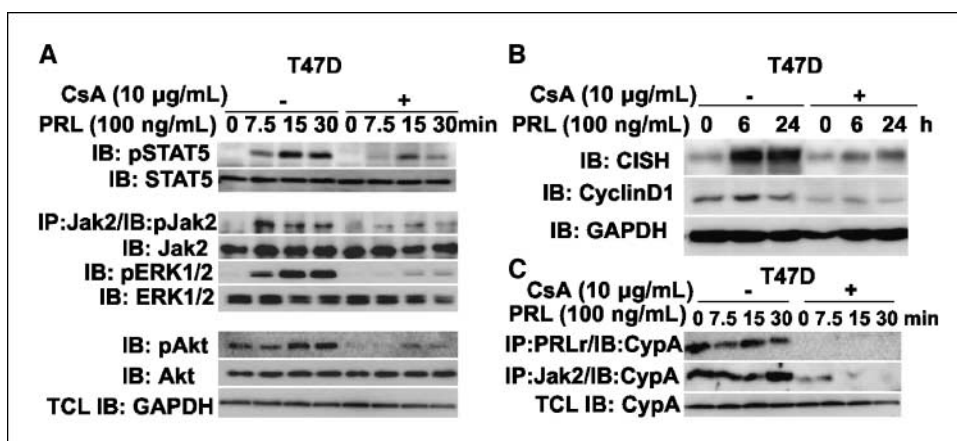


Figure 2. Ablation of CypA activity by CsA suppresses the PRL-associated signaling, reduces PRL-induced gene expression, and inhibits the association of CypA with the PRLr and Jak2. **A**, CsA inhibits PRLr signaling. T47D cells in defined medium were pretreated with CsA for 4 to 6 h. Cells were stimulated with PRL (100 ng/mL) at the indicated times. Blots were probed with the indicated antibodies. **TCL**, total cell lysates; **IP**, immunoprecipitation. **B**, CsA treatment of T47D cells results in reduced PRL-induced gene expression. T47D cells treated without or with CsA for 6 or 24 h were assessed for CISH and cyclin D1 protein expression by Western blot analyses. **C**, CsA inhibits the association of CypA with the PRLr and Jak2. T47D cells in defined medium were treated as described above. Cell lysates were immunoprecipitated with anti-PRLr-ECD or anti-Jak2 antibody and immunoblotted with anti-CypA antibody. Representative of three independent experiments.

or the indicated plasmid. Cells were arrested for 24 h posttransfection and then unstimulated or treated with the indicated amount of PRL. Twenty-four hours after PRL stimulation, cells were lysed and extracts were assayed using Victor3 Multilabel Counter (Perkin-Elmer).

The association of PRLr P334A or PRLr deletion mutants with wild-type CypA, the association of Cyp-PPI mutants with wild-type PRLr, and the Stat5 activation by P334A mutant were determined in 293FT cells transfected with the indicated plasmids. The effects of wild-type CypA, Cyp-PPI, and PRLr P334A overexpression on endogenous gene expression were assessed in T47D cells transfected with the increased amounts of plasmids as indicated. In all transfection experiments, the total amount of transfected plasmid was equalized with empty vector.

For production of recombinant retroviruses, PT67 cells were transfected with pSilencer 5.1 Retro-CypA siRNA or pSilencer 5.1 Retro-scrambled siRNA, and viruses were harvested 48 h posttransfection. To generate stable knockdown cells, T47D cells were infected with the retroviral supernatants, and stable colonies were selected with 2 µg/mL puromycin.

Cell growth assay. Cells were plated the day before CsA treatment in 24-well plates at 1×10^4 per well. Cells were incubated in complete medium with various concentrations of CsA as indicated for an additional 6 d, and viable cells were counted with a hemocytometer every other day by the trypan blue exclusion method.

Soft agar colony formation assay. Cells (5×10^3) were seeded in 1 mL of 0.3% agar in complete medium in the presence of various doses of CsA over a 1-mL base layer of 0.6% agar in complete medium in six-well plates. Growth medium containing various doses of CsA was added and replaced every 2 or 3 d. Cells were incubated at 37°C for 3 to 4 wk. Colonies were photographed with a phase-contrast microscope. Colonies (≥ 50 µm) were counted with Image J software.

Cell motility and invasion assay. For cell invasion assay, cells were placed in a Matrigel-coated transwell chamber with 8-µm-pore positron emission tomography membranes in the presence of various doses of CsA and were allowed to migrate toward 3% fetal bovine serum for 24 h. The number of cells invading to the lower surface of the membrane was quantified by CyQuant (Invitrogen). Cell motility was assessed by two distinct assays. Transwell migration assays were carried out as described above for Matrigel invasion assays except that the chambers were not coated with Matrigel (26). For wound healing assay, the confluent cell monolayer was wounded with a p200 pipette tip and cultured in serum-free medium in the presence of various doses of CsA. Representative images of a wound closure assay were acquired with a phase-contrast microscope at the indicated times. The wound areas were measured using Image J and the percentage of the wound closed was calculated.

Xenograft model. MCF7 or MDA231 cells (1×10^6) were suspended in Matrigel and injected into the teat of the fourth abdominal mammary gland of nude (*nu/nu*) mice as described (28). In brief, following injection, animals were randomized and treated with control carrier (apple juice) or CsA (100 mg/kg/d, standard dose; doses from 5 to 300 mg/kg/d were also examined) 3 d postinjection for 12 wk by twice-daily gavage. The standard dose of CsA given the mice exceeded that received by human patients (human dose range, 0.2–10 mg/kg/d) given the rapidity at which CsA is excreted by the murine kidney. At no point in these experiments did mice receiving the standard dose (or less) show any signs of neurologic toxicity or weight loss. Tumor growth was measured by weekly caliper measurement. Mice xenografted with MCF7 cells received a subcutaneous estrogen pellet (Innovative Research) implant before injection to ensure the growth of this estrogen receptor (ER)-positive cell line. All animal experiments were conducted in accordance with a protocol approved by Northwestern University Institutional Animal Care and Use Committee. After 12 wk, the mice were euthanized by CO₂ followed by cervical dislocation, and the entire primary mammary tumors were removed and weighed. In addition, all visceral organs, brain, and superficial lymph nodes were harvested for microscopic examination for metastasis. One half of each tumor was immediately frozen in liquid nitrogen and then stored at –80°C. The remaining tumor and other organs (lung, lymph node, liver, etc.) removed from sacrificed animals were fixed in 10% formalin for subsequent H&E staining or immunohistochemistry, which was done by the Northwestern University Pathology Core Facility. The necrotic area of primary tumor was quantified by morphometric determination of the proportion of total tumor area that was necrotic in H&E-staining section as described above. Organ metastasis was detected by gross and histologic examination of H&E-staining section of organ tissues from the control mice and CsA-treated mice. The number of tumor nuclei labeling positive for cyclin D1 was quantified in four preassigned high-power fields per tumor by microscopic examination of anti-cyclin D1 immunohistochemistry of primary tumor tissues from mice treated with CsA or control vehicle.

Statistical analysis. Data were reported as the means $\pm$ SE and analyzed using the appropriated statistical methods as indicated. $P < 0.05$ was considered significant.

Results

PRL-induced gene expression is modulated by CypA levels and CsA treatment. To test whether CypA could serve as a molecular toggle for the PRLr, the effects of manipulating CypA levels and activity on both PRL-responsive gene reporters and

endogenous genes were examined in breast cancer cells (Fig. 1). When overexpressed in T47D transfectants, CypA dose-dependently enhanced the PRL-induced expression from the Stat5-responsive LHRE and CISH luciferase reporters. These findings were paralleled by corresponding increases in the endogenous expression of CISH and cyclin D1 protein (Fig. 1A and B). Conversely, the suppression of endogenous CypA using a siRNA-mediated approach resulted in a decrease of PRL-induced gene expression as measured by LHRE and CISH luciferase reporters and endogenous CISH protein in T47D cells (Fig. 1C and D). Taken together, these findings show that alterations in CypA levels directly correlate with PRL-induced gene expression.

To examine whether the inhibitory effects of CypA knockdown were related to a reduction in the PPIase activity within the cell, T47D cells were treated with the PPI inhibitor CsA, followed by an analysis of PRL-induced signaling and gene expression evaluated (Fig. 2). As noted with the siRNA-mediated reduction of CypA, CsA treatment of T47D cells suppressed both basal and PRL-induced expression of endogenous CISH and cyclin D1 (Fig. 2B). To further delineate a mechanism for the reduction in PRL-induced gene expression mediated by CsA treatment, analysis of PRL-induced signaling was done. These studies (Fig. 2A) revealed a marked inhibition of PRL-stimulated phosphorylation of Jak2, Stat5, Erk1/2, and Akt. PRL-induced activation of these signaling molecules was also significantly inhibited at lower doses (i.e., 1 μ g/mL; data not

shown). Because many of the functions of the PRLr require and are initially triggered by Jak2 (29, 30), these findings would suggest that PPI activity contributes to proximal PRLr signaling.

To confirm this hypothesis, several follow-up and control experiments were done. If CsA was inhibiting the action of CypA at the PRLr, then one could anticipate that CsA treatment could interfere with CypA-PRLr interaction. Indeed, this effect was noted (Fig. 2C), as minimal CypA could be detected in the anti-PRLr or anti-Jak2 immunoprecipitates from CsA-treated T47D cells. In contrast, CsA had no effect on the PRLr-Jak2 interaction (Supplementary Fig. S1). The effects of CsA on receptor-mediated signaling were specific, as parallel experiments examining the activation of Erk1/2 and Akt CsA-treated T47D cells showed no significant diminution of insulin- or epidermal growth factor-triggered signaling (Supplementary Fig. S2). In addition, CsA had no effect on PRL binding to the PRLr (Supplementary Fig. S3). Thus, taken as a whole, these data support the notion that PPI activity, such as that found in CypA, significantly contributes to PRLr-induced signal transduction and gene expression. Interestingly, CsA also inhibits growth hormone-induced Akt and Erk signaling (Supplementary Fig. S2). Given that the growth hormone and PRL receptors are highly related members of the cytokine receptor superfamily that use similar signaling pathways, this observation is not surprising and is an area of ongoing research.

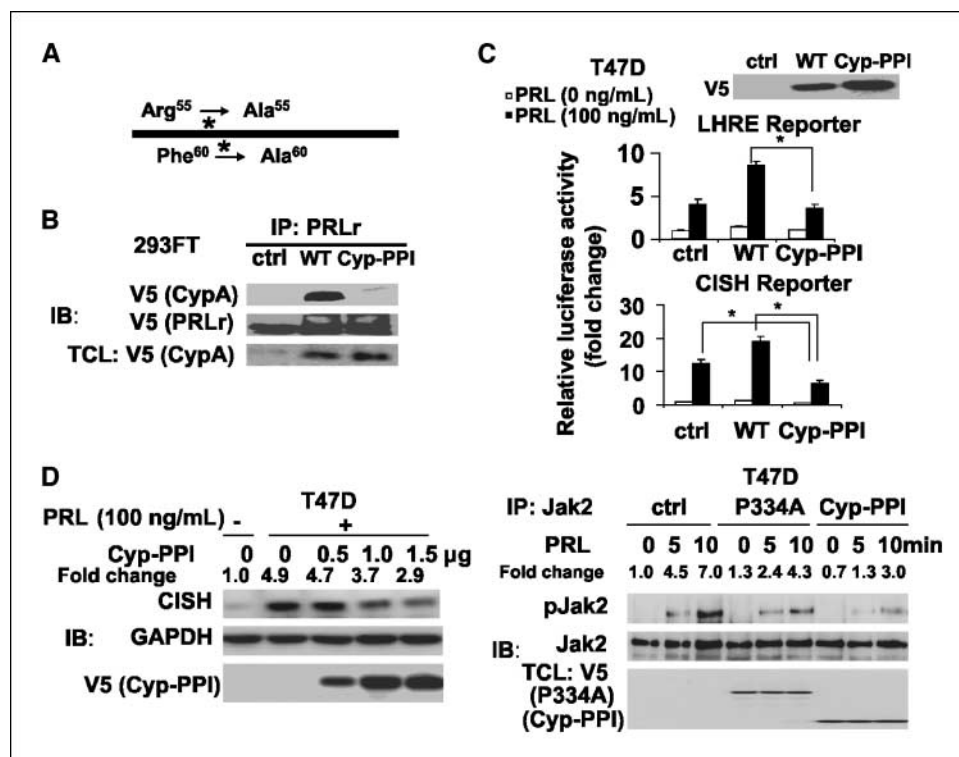
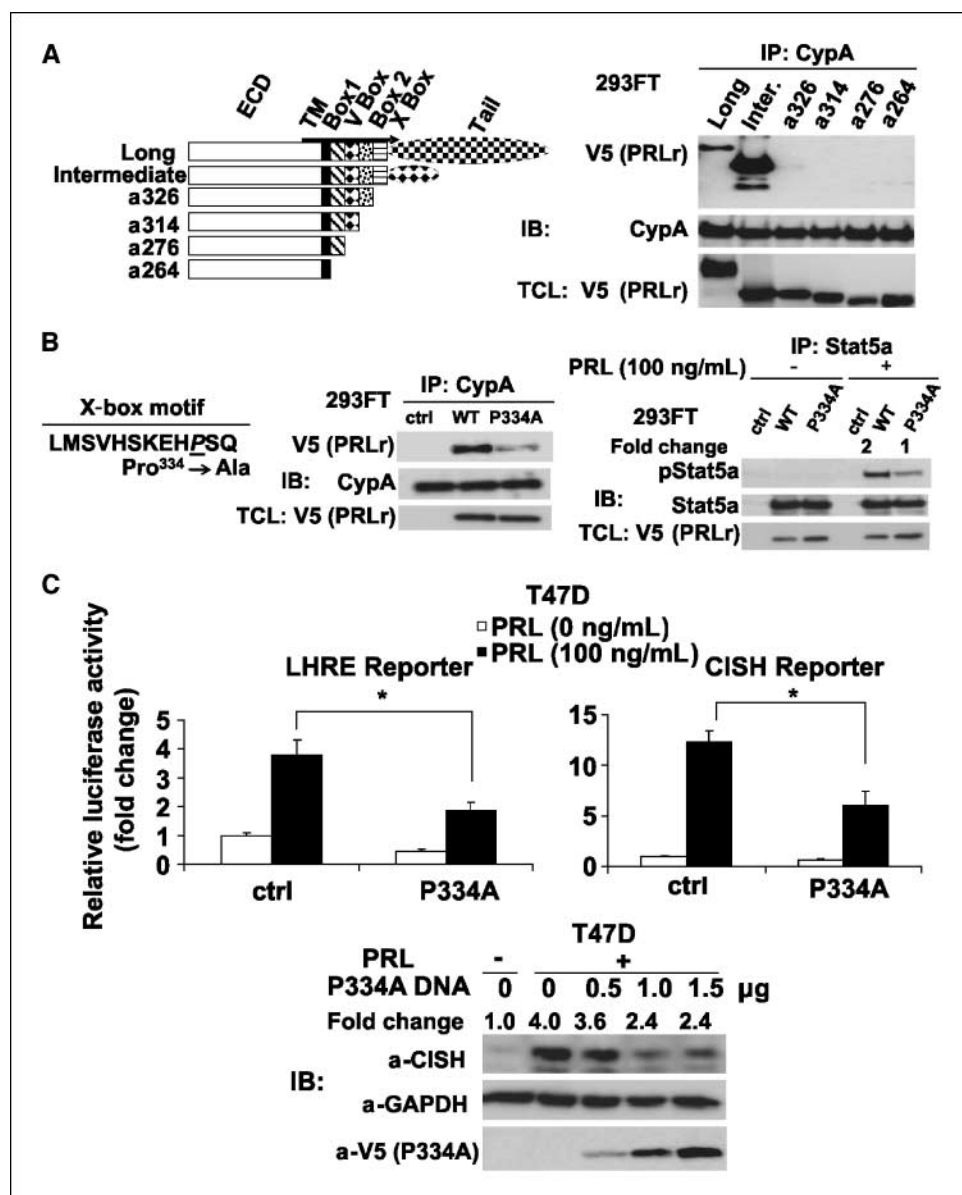


Figure 3. Overexpression of PPI-defective mutant of CypA is unable to associate with the PRLr and potentiate PRL-induced gene expression. **A**, generation of a PPI-defective CypA mutant (Cyp-PPI). Two residues (Arg⁵⁵ and Phe⁶⁰) were mutated to Ala using site-directed mutagenesis. **B**, Cyp-PPI is unable to bind to the PRLr. Wild-type (WT) CypA, its mutant (Cyp-PPI), or empty vector control (ctrl) was coexpressed with the PRLr in 293FT transfectants and immunoprecipitated by using an antibody against PRLr-ECD. Cell lysates and immunoprecipitates were probed in immunoblots with the indicated antibodies. **C**, Cyp-PPI fails to potentiate PRL-induced gene expression. T47D cells were cotransfected with LHRE or CISH luciferase reporter and wild-type CypA, Cyp-PPI, or empty vector expression construct. Following 24-h stimulation with PRL (100 ng/mL), transfectants were lysed and assayed for luciferase activity. Columns, mean of two independent experiments; bars, SE. *, $P < 0.05$ (t test). Western blot analysis (top) of total cell lysates with an anti-V5 antibody was done to verify the expression of transfected CypA. **D**, Cyp-PPI inhibits PRL-induced endogenous CISH protein expression (left), and Cyp-PPI and PRLr P334A inhibit Jak2 phosphorylation (right). Left, suppression of endogenous, PRL-induced CISH protein expression by the overexpression of Cyp-PPI was detected by Western blot analysis. The cells were treated with PRL (100 ng/mL) for 24 h. Right, immunoprecipitation and immunoblots of T47D cells overexpressing either Cyp-PPI or PRLr P334A (or empty vector control) were done as indicated.

Figure 4. Mapping of the CypA-binding site on the PRLr results in the generation of an interaction-defective point mutant PRLr that functions as a dominant negative receptor. **A**, CypA binding requires the PRLr X-box. A set of the PRLr COOH-terminal truncation mutants were prepared using PCR-based mutagenesis. 293FT transfectant lysates were immunoprecipitated with an anti-CypA antibody and sequentially immunoblotted with an anti-V5 antibody. **B**, *left*, analysis of the PRLr X-Box reveals a conserved proline residue. PCR mutagenesis was used to generate a receptor termed PRLr P334A. *Middle*, the association of CypA with the PRLr P334A is markedly decreased. 293 transfectants overexpressing wild-type PRLr and the PRLr P334A mutants were immunoprecipitated with an anti-CypA antibody and analyzed by immunoblot analyses as indicated. *Right*, Stat5 activation by PRLr P334A is significantly impaired. Wild-type PRLr and its PRLr P334A were coexpressed with rabbit Stat5a in 293FT transfectants. The cells were stimulated with PRL (100 ng/mL) for 10 min and immunoprecipitated with an antibody against Stat5. Total cell lysates and immunoprecipitates were probed with the indicated antibodies and subjected to Western blot analyses as indicated. **C**, PRLr P334A inhibits PRL-induced gene expression in T47D cells in a dominant negative manner. *Top*, T47D cells were transfected with LHRE or CISH luciferase reporters and a PRLr P334A expression construct (or empty vector control). Following 24-h stimulation with PRL (100 ng/mL), transfectants were lysed and assayed for luciferase activity. *Columns*, mean of two independent experiments; *bars*, SE. *, $P < 0.05$ (t test). *Bottom*, PRLr P334A inhibits PRL-induced endogenous CISH protein expression. Suppression of endogenous, PRL-induced CISH protein levels by PRLr P334A transfectants was detected by Western blot analysis.



The PPIase activity of CypA is required for potentiation of PRL-induced gene expression and association of CypA with the PRLr. To delineate the molecular basis for interaction between the PRLr and CypA and correlate this to function, selective mutagenesis was done. Many of the interactions of cyclophilins are mediated by the PPI active site (31), and the inhibition of PRLr-CypA binding by CsA further suggested that the PPI pocket was involved in this event. Point mutagenesis to residues Arg⁵⁵ and Phe⁶⁰ of the PPI pocket of CypA (Fig. 3A) resulted in a mutant CypA lacking 99% of PPI activity found in wild-type CypA (32). Subsequent analysis then queried whether this PPI-defective form of CypA was capable of interacting with the PRLr and potentiating its signal, as observed with wild-type CypA. When coexpressed with the PRLr in 293FT cells, unlike wild-type CypA, Cyp-PPI interacted poorly with the PRLr (Fig. 3B). Furthermore, in contrast to wild-type CypA, which potentiated both the PRL-induced LHRE and CISH luciferase reporters when overexpressed in T47D cells, overexpressed Cyp-PPI failed to potentiate PRL-induced gene

expression of the LHRE (Fig. 3C). Indeed, Cyp-PPI overexpression significantly repressed gene expression from CISH luciferase reporter (Fig. 3C) and blocked Jak2 phosphorylation and the endogenous expression of CISH protein (Fig. 3D). These data show that the PPIase active site of CypA is involved in the PRLr-CypA interaction and that this PPIase activity is required for the potentiation of PRL-induced gene expression.

A conserved proline residue in the PRLr X-box motif contributes to CypA binding and is required for PRLr transduction. The studies presented above reveal a proximal contribution of the PPI activity of CypA to PRLr/Jak2 signaling. However, the possibility remained that the action of CypA could be indirect, acting at a site other than the PRLr to effect its potentiation of Jak2/Stat5 signaling. To address this question, the binding site of CypA on the PRLr was mapped. As an initial step, COOH-terminal PRLr truncation mutants were generated and tested for their ability when expressed to interact with CypA (Fig. 4A). These studies revealed that truncations of the PRLr membrane proximal

to the X-box motif were incapable of interacting with CypA, revealing a contribution of the X-box to the engagement of CypA. Analysis of the X-box motif (Fig. 4B) revealed a proline at residue 334 of the PRLr that was highly conserved across species. Given the affinity of cyclophilins for proline residues, it was hypothesized that P334 in the X-box could contribute to the engagement of CypA. To test this hypothesis, a proline-to-alanine replacement mutation of the PRLr was generated (P334A), and the ability of this mutant PRLr to interact with CypA and mediate PRL-induced

signaling was tested. These studies revealed that when expressed in T47D cells, the mutant PRLr P334A showed a significantly reduced association with CypA (Fig. 4B). Furthermore, the ability of the P334A mutant to induce Jak2 and Stat5 phosphorylation (in comparison with wild-type PRLr transfected control) was also significantly diminished (Figs. 3D and 4B). Overexpression of the PRLr P334A mutant in T47D cells resulted in a dose-dependent reduction in the PRL-induced expression of both cotransfected LHRE and CISH luciferase reporters and endogenous CISH protein

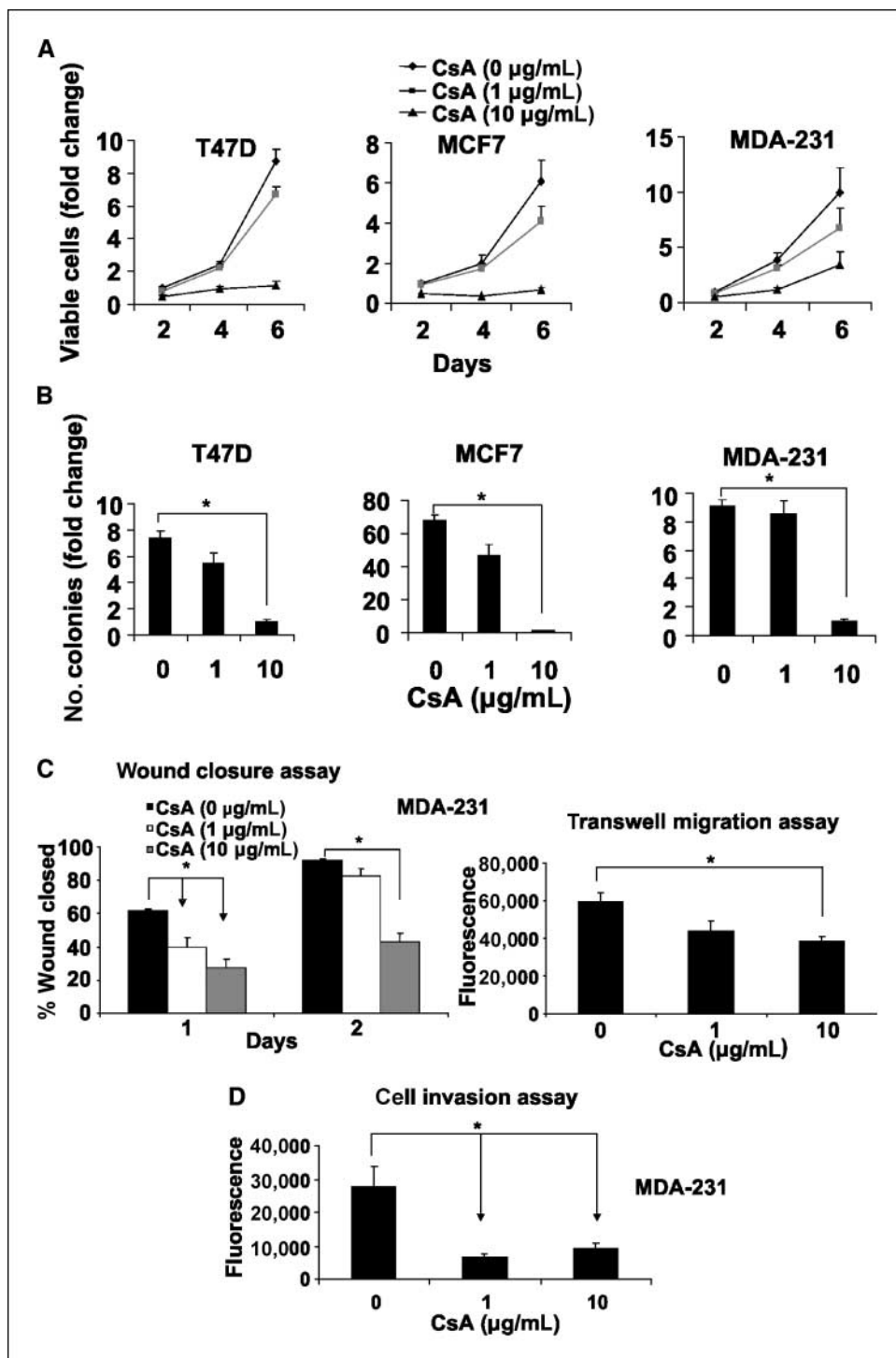
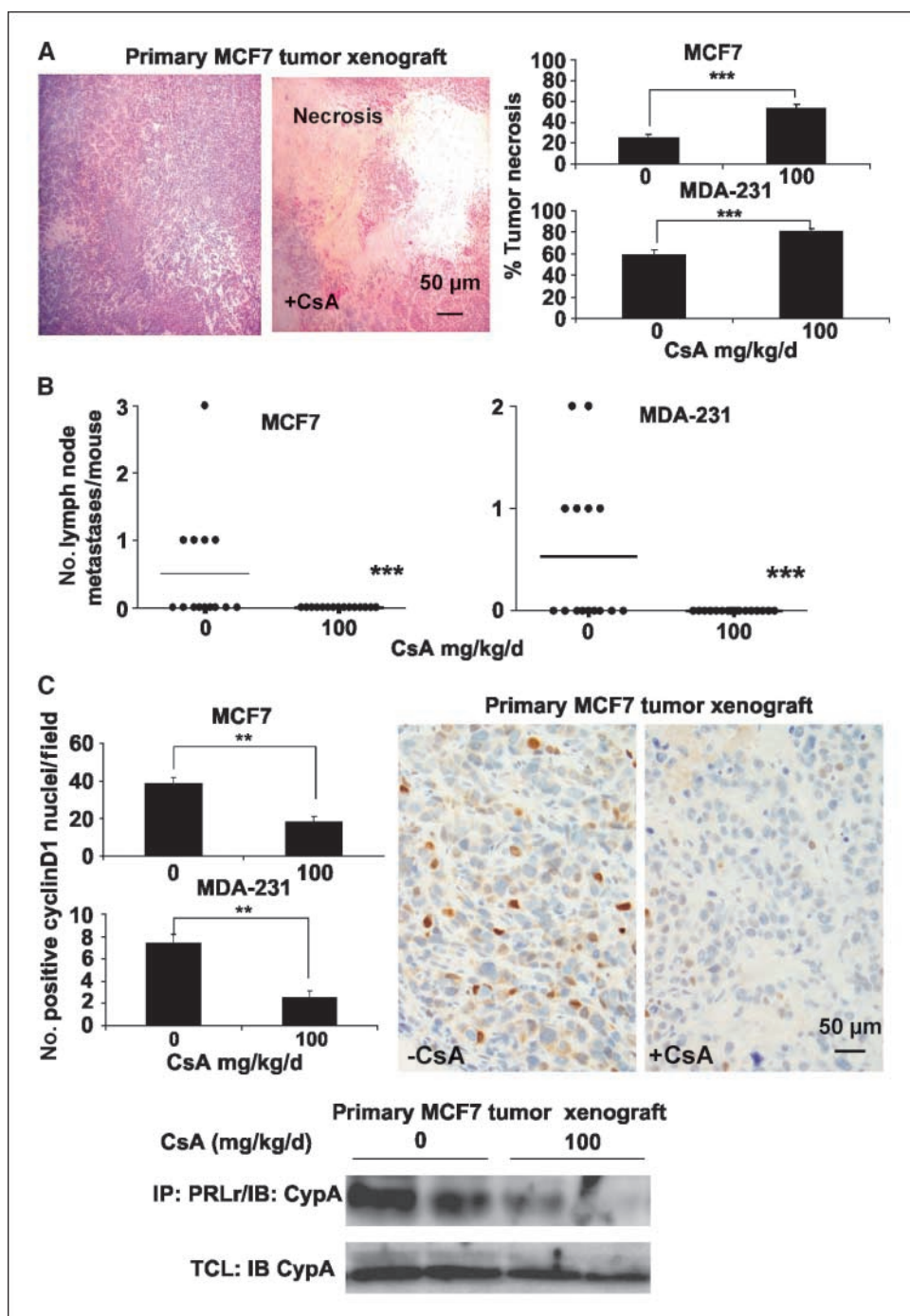


Figure 5. CsA inhibits anchorage-dependent and anchorage-independent growth and motility and invasion of breast cancer cells *in vitro*. **A**, growth inhibition by CsA of breast cancer cells. T47D, MCF7, and MDA-231 cells were incubated with various doses of CsA, and viable cells were quantified every other day by trypan blue exclusion. Points, mean of two independent experiments; bars, SE. **B**, colony formation inhibition by CsA. Colony enumeration used Image J software. Columns, mean of two independent experiments; bars, SE. *, $P < 0.05$ (t test). **C**, inhibition of motility by CsA as measured by wound healing assay (left) and Boyden chamber assay (right). Left, a confluent cell monolayer of MDA-231 was wounded with a pipette tip and cultured in serum-free medium in the presence of various doses of CsA. Representative images of a wound closure assay were acquired with a phase-contrast microscope and the percentage of the wound closed was quantified. Representative of three independent experiments. *, $P < 0.05$ (two-way ANOVA). Right, the number of cells migrating through pores was quantified by CyQuant labeling. Representative of three independent experiments. *, $P < 0.05$ (one-way ANOVA). **D**, inhibition of invasion by CsA. The number of MDA-231 cells invading through Matrigel-occluded pores was quantified by CyQuant labeling. Representative of three independent experiments. *, $P < 0.05$ (one-way ANOVA).

Figure 6. CsA induces central primary tumor necrosis and the metastasis of ER⁺ and ER⁻ human breast cancer xenografts, inhibits primary tumor cyclin D1 expression, and blocks CypA-PRLr association. **A**, induction of primary tumor necrosis by CsA. Mice xenografted with MCF7 and MDA231 cells were treated with CsA (100 mg/kg/d; *n* = 18) or control carrier (apple juice; *n* = 18) for 12 wk by twice-daily gavage. *Left*, representative H&E-stained sections (20 $\times$; bar, 50 μ m). *Right*, the necrotic area of primary tumor (*right*) was quantified by determination of the proportion of total tumor area that was necrotic in H&E-stained section of primary MCF7 tumor xenograft. ***, *P* < 0.001 (*t* test). **B**, inhibition of metastasis by CsA. Lymph node metastases were detected by histologic examination of H&E-stained section of lymph nodes harvested from mice treated for 12 wk with either CsA (100 mg/kg/d; *n* = 18) or control carrier (apple juice; *n* = 18). ***, *P* < 0.001 (Fisher's exact test). **C**, inhibition of primary tumor cyclin D1 expression (*top*) and CypA-PRLr association (*bottom*) by CsA. *Top*, the number of positive cyclin D1 nuclei was quantified by microscopic examination of anti-cyclin D1 immunohistochemical labeling of primary tumor tissue from mice treated for 12 wk with either CsA (100 mg/kg/d; *n* = 5) or control carrier (apple juice; *n* = 5). **, *P* < 0.01 (*t* test). Representative sections (bar, 50 μ m) of anti-cyclin D1 immunohistochemical labeling of primary MCF7 tumor xenograft. *Columns*, mean; *bars*, SE. *Bottom*, CsA blocks the association of CypA with the PRLr in primary MCF7 tumor xenograft. The inhibition of CypA-PRLr association was detected by coimmunoprecipitation analysis.



(Fig. 4C). These findings are notable in that T47D cells are known to express high levels of endogenous PRLr, indicating that the P334A mutant, most probably through heterodimerization with the wild-type PRLr (33), functions as a dominant negative receptor. When assessed with the data presented above, these findings would indicate that the direct action of the CypA on the PRLr is required for effective PRL-induced signaling, a process that is inhibited by the PRLr P334A mutant. These findings were further confirmed by control experiments, which showed that the PRLr/Jak2 interaction was not affected by the P334A mutation (data not shown).

CsA treatment blocks multiple cellular functions in breast cancer cells *in vitro*. The data presented above have revealed that the function of the PRLr and its associated downstream signaling are regulated by the PPIase activity of CypA. Thus, given the function of cyclophilins, not only at the level of the PRLr but at other signaling loci that contribute to the pathogenesis of breast cancer (i.e., such as NFAT ref. 34 and Stat 5 ref. 12, 25) as well, it was reasoned that the use of PPI inhibitors such as CsA might effectively inhibit the malignant phenotype of breast cancer. To assess the *in vitro* effects of CsA on the biology of human breast cancer, analysis of the effects of CsA on the cell

growth, soft agar colony formation, cell motility, and invasion was done. When cultured in monolayer in the presence of CsA, both the ER⁺ lines T47D and MCF7 and the ER⁻ line MDA-231 showed a dose-dependent inhibition of viable cell growth (Fig. 5A). At concentrations ≤ 10 $\mu\text{g/mL}$, this antiproliferative effect was reversible following washing and medium change; at concentrations of ≥ 30 $\mu\text{g/mL}$, these effects were not reversible and cell death ensued (data not shown). To assess the effects of CsA on anchorage-independent growth, soft agar colony formation assays were done. As shown in Fig. 5B, CsA treatment resulted in a marked suppression of the anchorage-independent growth of human breast cancer cells in a dose-dependent manner. When introduced into either cell migration or invasion assays using the highly metastatic ER⁻ MDA-231, CsA dose-dependently inhibited both cell motility [as assessed by wound healing assay (Fig. 5C, left; Supplementary Fig. S4) or Boyden chamber assay (Fig. 5C, right)] and cell invasion through Matrigel (Fig. 5D). Taken together, these findings indicate that CsA inhibited many of the malignant properties of breast cancer cells *in vitro*, suggesting a potential *in vivo* role for PPI inhibition in the treatment of breast cancer.

Inhibition of PPI activity *in vivo* by CsA induces primary tumor necrosis and blocks metastasis of breast cancer xenografts. Given the *in vitro* effects of CsA on breast cancer cell biology, it was hypothesized that CsA could inhibit the *in vivo* outgrowth of human breast cancer. To test this hypothesis, both the ER⁺ MCF7 and the ER⁻ MDA-231 human breast cancer lines were xenografted into nude mice, using a novel approach that dramatically facilitates the metastatic spread of breast cancer xenografts (28), and subsequently randomized into CsA therapy or control groups 3 days postinjection. Whereas all CsA-treated tumors showed a trend toward a decrease in size and weight over time, these parameters did not achieve statistical significance (data not shown). However, two parameters were found to be significantly changed as a function of CsA therapy, notably central tumor necrosis and metastasis. As seen in Fig. 6A, central tumor necrosis of both ER⁺ and ER⁻ was significantly increased, with a somewhat more pronounced effect in ER⁺ MCF7 tumors. Most interestingly, not a single metastasis to lymph nodes or organs was noted in CsA-treated mice (Fig. 6B) from either ER⁺ or ER⁻ xenografts. Indeed, in this entire line of experimentation with 94 mice receiving varied doses of CsA (some as low as 10 mg/kg/d), not a single CsA-treated mouse xenografted with either MCF7 or MDA-231 cells was noted to have a metastasis, whereas between 30% and 50% of the control mice showed metastasis. To address whether the *in vivo* use of CsA resulted in inhibition of Stat5-driven gene expression, immunohistochemistry of the primary tumors with an anti-cyclin D1 antibody was done and the number of labeled nuclei was quantified. As shown in Fig. 6C (top), cyclin D1 gene expression in both ER⁺ and ER⁻ primary tumors from CsA-treated mice was significantly inhibited when compared with tumors from control mice. To further support the *in vitro* observation that the association of CypA with the PRLr was blocked by CsA, the *in vivo* status of PRLr-CypA binding in CsA-treated tumor tissues was also examined. As shown in Fig. 6C (bottom), the CypA-PRLr association in CsA-treated tumor tissues compared with control vehicle-treated tumor tissues was inhibited. Thus, the *in vivo* actions of CsA, while reducing cyclin D1 expression and blocking CypA-PRLr association, also promoted central tumor necrosis and inhibited metastasis of breast cancer xenografts, irrespective of ER status.

Discussion

The proximate mechanisms that enable receptor-mediated signal transduction following ligand binding remain poorly characterized. Following ligand binding to the type I cytokine receptors, such as the PRLr and growth hormone receptor, members of the Jak family of tyrosine kinases are activated within 30 to 60 seconds by an autophosphorylation-based mechanism (35). Recent analysis of the growth hormone receptor using mutagenesis and fluorescence resonance energy transfer approaches has suggested that a conformational change may occur within the receptor following ligand binding approximating the growth hormone receptor-associated Jak2 kinases, thereby enabling their autophosphorylation and activation (20). Nuclear magnetic resonance spectroscopy of the conserved Box 1 motif of the PRLr, a hydrophobic and proline-rich intracellular domain adjacent to the transmembrane region of this receptor, also has suggested that a conformational change within the PRLr is also feasible (21). However, a mechanism that would enable such a conformational change to occur within either the growth hormone receptor or PRLr has not been elaborated.

The data presented here indicate that the PPI activity of CypA significantly contributes to proximate receptor activation, enabling transduction through the PRLr/Jak2 complex. The PRL-induced expression of both Stat5-responsive reporter constructs and endogenous genes was directly correlated to CypA levels by both overexpression and knockdown approaches. Loss of PPI activity in CypA (CypA-PPI) following mutagenesis resulted in reduced PRLr binding, Jak2 phosphorylation, and PRL-induced gene expression. Similarly, replacement by mutation of a conserved proline residue (PRLr-P334A) also resulted in reduced PRLr-CypA interaction, decreased Jak2 and Stat5 phosphorylation, and decreased Stat5-responsive reporter and endogenous gene expression. Indeed, when transfected into the T47D breast cancer line, which expresses high levels of PRLr, the PRLr-P334A functioned in a dominant negative manner. Taken together, these results would argue that the potentiation of PRLr-induced signaling by CypA is a consequence of the direct actions of the PPI of CypA on the PRLr/Jak2 complex, and not an epiphenomenon of CypA function at another site.

As a member of the cytokine receptor superfamily, the membrane-proximal portion of the intracellular domain of the PRLr contains the conserved Box 1/Variable Box/Box 2/X-box motifs. Whereas the functions of the Variable Box, Box 2, and X-Box are largely uncharacterized, the proline-rich Box 1 motif has been implicated in the binding and ligand-induced activation of Jak2. Deletion of this motif from the PRLr or nonconservative replacement of its COOH-terminal proline residue with leucine results in a loss of Jak2 binding via its NH₂-terminal FERM domain (36) and downstream PRL-induced gene expression (37). However, although necessary for Jak2 engagement, the Box 1 motif alone is not sufficient for lactogenic signaling (33, 37), clearly indicating that other determinants in the PRLr contribute to Jak2 activation. The identified contribution of the proline residue at position 334 for both CypA-PRLr association and PRL-induced Jak2 activation would indicate that the PPI activity of CypA at this site or its immediate environs (such as the Box 1 motif) immediately affects Jak2-driven signaling.

CsA has been a widely used immunosuppressive drug in organ transplantation and in the treatment of autoimmune diseases (38, 39). As an immunosuppressant, CsA has been classically thought to form a complex with cyclophilin that binds with high

affinity to the phosphatase calcineurin. As a consequence of this event, interleukin-2 transcription is inhibited by a loss of calcineurin-mediated dephosphorylation of the transcription factor NFAT activation, resulting in a loss of clonal T-cell proliferation following antigen stimulation (40). However, our data would indicate that, at least in human breast cancer cells, CsA also targets the proximal Jak2 kinase, presumably through its inhibition of the PPI activity of CypA. The loss of Jak2 activity readily explains the loss of PRL-induced activation of downstream signaling factors such as Stat5, Akt, and Erk, and the concomitant loss of PRL-induced, Stat5-mediated gene expression and the inhibition of outgrowth of CsA-treated breast cancer cells. Was the inhibition of breast cancer cell outgrowth secondary to the effects of CsA on Stat5 or NFAT? *In vitro* evidence does exist showing that NFAT activity regulated by the Akt kinase stimulates breast cancer motility and invasion (34, 41). However, the precise role of this transcription factor in either normal breast biology or breast cancer pathobiology *in vivo* remains uncertain. In contrast, *in vivo* studies using conditional Jak2^{-/-} knockout mice, and cells derived thereof, have revealed that Jak2 is required for normal mammary alveogenesis and lactation at the tissue level and the activation of Stat5 and expression of cyclin D1 at the cellular level (29, 30). Furthermore, when transgenic WAP-T antigen mice, a genetic model of mammary cancer, were mated to heterozygous Stat5^{+/-} mice, a significant reduction in mammary tumor size, incidence, and progression was noted (42). Most importantly, our knock-down and overexpression studies presented here (using both wild-type and mutant CypA and PRLr) showed the effects on signaling and gene expression comparable to those observed following CsA treatment. These complementary, non-CsA-based approaches, which should not result in the chelation of calcineurin or the inhibition of other cyclophilin family members, would argue the importance of the Jak2/Stat5 axis in breast cancer pathobiology and suggest that the inhibition of this pathway by CsA may, in part, explain the actions of this drug both *in vitro* and *in vivo*. However, it is important to note that the inhibitory effects of CsA may be unique to breast malignancies, as CsA is recognized to increase or enhance the incidence and progression of epidermal, lymphoid, and gastrointestinal malignancies in the laboratory and the clinic (43–45).

Our *in vitro* studies showed that CsA markedly inhibited the growth, motility, and invasion of both ER⁺ and ER⁻ human breast cancer cells in a dose-dependent manner. Interestingly, *in vivo* CsA therapy did not have a statistically significant effect on overall tumor size (although CsA-treated tumors did trend toward being smaller). However, a statistically significant increase in central primary tumor necrosis and a complete absence of metastasis were noted in CsA-treated mice xenografted with either ER⁺ or ER⁻ breast cancer cells. Significantly, as noted *in vitro*, CsA-treated

xenografts showed a significant reduction in the expression of the Stat5-responsive cyclin D1. There are many potential mechanisms through which these CsA-mediated effects on xenograft progression may have been mediated. In this context, it is interesting to note that the inhibition of cyclin D1 expression is angiostatic (46). In addition, epidemiologic studies have implicated a role for PRL in the metastatic progression of human breast cancer (47, 48). It is tempting to speculate, therefore, that some of the progression-inhibitory effects of CsA may result from its blockade of the PRL/Jak2/Stat5 pathway, the activity of which has been implicated in the proliferation, survival, motility, and invasion of human breast cancer (17). It is also important to note that the significant reduction in breast cancer observed in female patients undergoing CsA therapy following allograft transplant (16) was at that time interpreted to be secondary to the immunosuppressive effects of CsA. Our *in vitro* and *in vivo* data in models lacking functional immune systems, however, would argue that some, if not many, of the effects of CsA are due to its direct action on breast cancer cells, and not due to its secondary immunosuppressive actions.

In many tumor types, including breast cancer, CsA has been found to bind to and inhibit the efflux functions of multidrug resistance proteins including P-glycoprotein, breast cancer resistance protein, and multidrug resistance protein (49, 50). First documented in Chinese hamster ovary cells (51) and extended into human leukemias (52), these studies have served as the basis for the first phase II clinical trials with cyclosporine in patients with advanced breast cancer. In theory, by reducing drug efflux, thereby enhancing the efficacy of chemotherapy, the combination of Paclitaxel and CsA was found to be safe and effective in patients with advanced disease (53) when administered simultaneously on a once-weekly basis. Our findings, however, would argue that although CsA may also function as a drug efflux inhibitor, this PPI inhibitor has direct actions on breast cancer signaling that should be exploited in patients with this disease, by establishing continuous serum levels of CsA that would inhibit Jak2/Stat5 transduction. Indeed, as alternative PPI-inhibitors continue to be developed, novel and more specific pharmacotherapies against breast cancer should be expected.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 2/21/2008; revised 6/30/2008; accepted 7/7/2008.

Grant support: Susan G. Komen Foundation grant BCTR05-03790 and NIH grants CA69294 and CA102682. Additional support was received from the Zell Scholar's Fund, the Lynn Sage Foundation, and the Avon Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

- Gothel SF, Marahiel MA. Peptidyl-prolyl *cis-trans* isomerases, a superfamily of ubiquitous folding catalysts. *Cell Mol Life Sci* 1999;55:423–36.
- Handschumacher RE, Harding MW, Rice J, Drugge RJ, Speicher DW. Cyclophilin: a specific cytosolic binding protein for cyclosporin A. *Science* 1984;226:544–7.
- Bram RJ, Hung DT, Martin PK, Schreiber SL, Crabtree GR. Identification of the immunophilins capable of mediating inhibition of signal transduction by cyclosporin A and FK506: roles of calcineurin binding and cellular location. *Mol Cell Biol* 1993;13:4760–9.
- Colgan J, Asmal M, Yu B, Luban J. Cyclophilin A-deficient mice are resistant to immunosuppression by cyclosporine. *J Immunol* 2005;174:6030–8.
- Harrison RK, Stein RL. Substrate specificities of the peptidyl prolyl *cis-trans* isomerase activities of cyclophilin and FK-506 binding protein: evidence for the existence of a family of distinct enzymes. *Biochemistry* 1990;29:3813–6.
- Lu KP, Finn G, Lee TH, Nicholson LK. Prolyl *cis-trans* isomerization as a molecular timer. *Nat Chem Biol* 2007;3:619–29.
- Bosco DA, Eisenmesser EZ, Pochapsky S, Sundquist WI, Kern D. Catalysis of *cis/trans* isomerization in native HIV-1 capsid by human cyclophilin A. *Proc Natl Acad Sci U S A* 2002;99:5247–52.
- Colgan J, Asmal M, Neagu M, et al. Cyclophilin A regulates TCR signal strength in CD4⁺ T cells via a proline-directed conformational switch in Itk. *Immunity* 2004;21:189–201.

9. Nicholson LK, Lu KP. Prolyl *cis-trans* isomerization as a molecular timer in Crk signaling. *Mol Cell* 2007;25:483–5.
10. Lummis SC, Beene DL, Lee LW, Lester HA, Broadhurst RW, Dougherty DA. *Cis-trans* isomerization at a proline opens the pore of a neurotransmitter-gated ion channel. *Nature* 2005;438:248–52.
11. Eckert B, Martin A, Balbach J, Schmid FX. Prolyl isomerization as a molecular timer in phage infection. *Nat Struct Mol Biol* 2005;12:619–23.
12. Ryczyn MA, Clevenger CV. The intranuclear prolactin/cyclophilin B complex as a transcriptional inducer. *Proc Natl Acad Sci U S A* 2002;99:6790–5.
13. Campa MJ, Wang MZ, Howard B, Fitzgerald MC, Patz EF, Jr. Protein expression profiling identifies macrophage migration inhibitory factor and cyclophilin A as potential molecular targets in non-small cell lung cancer. *Cancer Res* 2003;63:1652–6.
14. Shen J, Person MD, Zhu J, Abbruzzese JL, Li D. Protein expression profiles in pancreatic adenocarcinoma compared with normal pancreatic tissue and tissue affected by pancreatitis as detected by two-dimensional gel electrophoresis and mass spectrometry. *Cancer Res* 2004;64:9018–26.
15. Rey O, Baluda MA, Park NH. Differential gene expression in neoplastic and human papillomavirus-immortalized oral keratinocytes. *Oncogene* 1999;18:827–31.
16. Stewart T, Tsai SC, Grayson H, Henderson R, Opelz G. Incidence of de-novo breast cancer in women chronically immunosuppressed after organ transplantation. *Lancet* 1995;346:796–8.
17. Clevenger CV, Furth PA, Hankinson SE, Schuler LA. The role of prolactin in mammary carcinoma. *Endocr Rev* 2003;24:1–27.
18. Hankinson SE, Willett WC, Michaud DS, et al. Plasma prolactin levels and subsequent risk of breast cancer in postmenopausal women. *J Natl Cancer Inst* 1999;91:629–34.
19. Rose-Hellekant TA, Arendt LM, Schroeder MD, Gilchrist K, Sandgren EP, Schuler LA. Prolactin induces ER α -positive and ER α -negative mammary cancer in transgenic mice. *Oncogene* 2003;22:4664–74.
20. Brown RJ, Adams JJ, Pelekanos RA, et al. Model for growth hormone receptor activation based on subunit rotation within a receptor dimer. *Nat Struct Mol Biol* 2005;12:814–21.
21. O'Neal KD, Chari MV, McDonald CH, et al. Multiple *cis-trans* conformers of the prolactin receptor proline-rich motif (PRM) peptide detected by reverse-phase HPLC, CD and NMR spectroscopy. *Biochem J* 1996;315:833–44.
22. Waters MJ, Hoang HN, Fairlie DP, Pelekanos RA, Brown RJ. New insights into growth hormone action. *J Mol Endocrinol* 2006;36:1–7.
23. Brockman JL, Schroeder MD, Schuler LA. PRL activates the cyclin D1 promoter via the Jak2/Stat pathway. *Mol Endocrinol* 2002;16:774–84.
24. Raccurt M, Tam SP, Lau P, et al. Suppressor of cytokine signalling gene expression is elevated in breast carcinoma. *Br J Cancer* 2003;89:524–32.
25. Ryczyn MA, Reilly SC, O'Malley K, Clevenger CV. Role of cyclophilin B in prolactin signal transduction and nuclear retrotranslocation. *Mol Endocrinol* 2000;14:1175–86.
26. Miller SL, DeMaria JE, Freier DO, Riegel AM, Clevenger CV. Novel association of Vav2 and Nek3 modulates signaling through the human prolactin receptor. *Mol Endocrinol* 2005;19:939–49.
27. Syed F, Ryczyn MA, Westgate L, Clevenger CV. A novel and functional interaction between cyclophilin A and prolactin receptor. *Endocrine* 2003;20:83–90.
28. Harrell JC, Dye WW, Allred DC, et al. Estrogen receptor positive breast cancer metastasis: altered hormonal sensitivity and tumor aggressiveness in lymphatic vessels and lymph nodes. *Cancer Res* 2006;66:9308–15.
29. Sakamoto K, Creamer BA, Triplett AA, Wagner KU. The Janus kinase 2 is required for expression and nuclear accumulation of cyclin D1 in proliferating mammary epithelial cells. *Mol Endocrinol* 2007;21:1877–92.
30. Wagner KU, Krempler A, Triplett AA, et al. Impaired alveologenesis and maintenance of secretory mammary epithelial cells in Jak2 conditional knockout mice. *Mol Cell Biol* 2004;24:5510–20.
31. Ke HM, Zydowsky LD, Liu J, Walsh CT. Crystal structure of recombinant human T-cell cyclophilin A at 2.5 Å resolution. *Proc Natl Acad Sci U S A* 1991;88:9483–7.
32. Zydowsky LD, Etzkorn FA, Chang HY, et al. Active site mutants of human cyclophilin A separate peptidyl-prolyl isomerase activity from cyclosporin A binding and calcineurin inhibition. *Protein Sci* 1992;1:1092–9.
33. Chang WP, Clevenger CV. Modulation of growth factor receptor function by isoform heterodimerization. *Proc Natl Acad Sci U S A* 1996;93:5947–52.
34. Jauliac S, Lopez-Rodriguez C, Shaw LM, Brown LF, Rao A, Tokar A. The role of NFAT transcription factors in integrin-mediated carcinoma invasion. *Nat Cell Biol* 2002;4:540–4.
35. Brooks AJ, Wooh JW, Tunny KA, Waters MJ. Growth hormone receptor; mechanism of action. *Int J Biochem Cell Biol*. In press 2008.
36. He K, Wang X, Jiang J, et al. Janus kinase 2 determinants for growth hormone receptor association, surface assembly, and signaling. *Mol Endocrinol* 2003;17:2211–27.
37. Lebrun JJ, Ali S, Ullrich A, Kelly PA. Proline-rich sequence-mediated Jak2 association to the prolactin receptor is required but not sufficient for signal transduction. *J Biol Chem* 1995;270:10664–70.
38. Kahan BD. Cyclosporine. *N Engl J Med* 1989;321:1725–38.
39. Ponticelli C. Cyclosporine: from renal transplantation to autoimmune diseases. *Ann N Y Acad Sci* 2005;1051:551–8.
40. Schreiber SL, Crabtree GR. The mechanism of action of cyclosporin A and FK506. *Immunol Today* 1992;13:136–42.
41. Yoeli-Lerner M, Yiu GK, Rabinovitz I, Erhardt P, Jauliac S, Tokar A. Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. *Mol Cell* 2005;20:539–50.
42. Ren S, Cai HR, Li M, Furth PA. Loss of Stat5a delays mammary cancer progression in a mouse model. *Oncogene* 2002;21:4335–9.
43. Hojo M, Morimoto T, Maluccio M, et al. Cyclosporine induces cancer progression by a cell-autonomous mechanism. *Nature* 1999;397:530–4.
44. Ohsawa I, Murakami T, Uemoto S, Kobayashi E. *In vivo* luminescent imaging of cyclosporin A-mediated cancer progression in rats. *Transplantation* 2006;81:1558–67.
45. Koehl GE, Andrassy J, Guba M, et al. Rapamycin protects allografts from rejection while simultaneously attacking tumors in immunosuppressed mice. *Transplantation* 2004;77:1319–26.
46. Hanai J, Dhanabal M, Karumanchi SA, et al. Endostatin causes G₁ arrest of endothelial cells through inhibition of cyclin D1. *J Biol Chem* 2002;277:16464–9.
47. Wang DY, Stepniowska KA, Allen DS, et al. Serum prolactin levels and their relationship to survival in women with operable breast cancer. *J Clin Epidemiol* 1995;48:959–68.
48. Holtkamp W, Nagel GA, Wander HE, Rauschecker HF, von Heyden D. Hyperprolactinemia is an indicator of progressive disease and poor prognosis in advanced breast cancer. *Int J Cancer* 1984;34:323–8.
49. Gupta A, Dai Y, Vethanayagam RR, et al. Cyclosporin A, tacrolimus and sirolimus are potent inhibitors of the human breast cancer resistance protein (ABCG2) and reverse resistance to mitoxantrone and topotecan. *Cancer Chemother Pharmacol* 2006;58:374–83.
50. Qadir M, O'Loughlin KL, Fricke SM, et al. Cyclosporin A is a broad-spectrum multidrug resistance modulator. *Clin Cancer Res* 2005;11:2320–6.
51. Rauscher FJ III, Beerman TA, Baker RM. Characterization of ouromycin-resistant hamster cell mutants that display a multidrug resistance phenotype. *Mol Pharmacol* 1990;38:198–206.
52. Boesch D, Muller K, Pourtier-Manzanedo A, Loor F. Restoration of daunomycin retention in multidrug-resistant P388 cells by submicromolar concentrations of SDZ PSC 833, a nonimmunosuppressive cyclosporin derivative. *Exp Cell Res* 1991;196:26–32.
53. Helgason HH, Kruijtzter CM, Huitema AD, et al. Phase II and pharmacological study of oral paclitaxel (Paxoral) plus cyclosporin in anthracycline-pretreated metastatic breast cancer. *Br J Cancer* 2006;95:794–800.

New mechanisms for PRLr action in breast cancer

Charles V. Clevenger, Samantha L. Gadd, Jiamao Zheng

Department of Pathology & Robert H. Lurie Comprehensive Cancer Center,
Northwestern University and Children's Memorial Hospital, Chicago, IL 60611

Correspondence should be addressed to: Charles Clevenger, Department of Pathology,
Northwestern University, Lurie 4-107, 303 East Superior Street, Chicago, IL 60611.
Phone: 312-503-5750. Fax: 312-503-0095. Email: clevenger@northwestern.edu

Keywords: Cyclophilin, Jak2, Prolactin receptor, Breast cancer, Cyclosporine A

Running Title: PRLr action in breast cancer

24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40

ABSTRACT

Prolactin (PRL) contributes to the growth of normal and malignant breast tissues. These actions are initiated by the engagement of PRL by its receptor, the PRLr, a transmembrane receptor belonging to the cytokine receptor family. The mechanisms of how the PRL:PRLr interaction triggers the activation of downstream signaling networks however has remained enigmatic. This review examines the physical status of the PRLr before and after ligand binding and the mechanisms through which PRL binding may initiate receptor-associated signaling. Evidence for PRLr pre-dimerization in the absence of ligand will be overviewed, and the actions of the prolyl isomerase cyclophilin A in the ligand-induced activation of the PRLr-associated Jak2 kinase discussed. The therapeutic translation of these observations is considered.

Introduction

The purpose of this review is to provide a background as to the relevance of the prolactin receptor (PRLr) in normal and malignant breast biology; it will then examine the novel proximal mechanisms that have emerged through which ligand binding to the pre-dimerized PRLr results in signaling regulated by the actions of prolyl isomerases.

Role of PRL in the normal and malignant breast

Prolactin (PRL) was originally identified as a neuroendocrine hormone of pituitary origin. While the pleiotropic actions of PRL are legend, its regulation of the growth and differentiation of mammary tissues is most recognized and best understood [3]. These effects are mediated by the interaction of PRL with the prolactin receptor (PRLr), a member of the cytokine receptor superfamily [5]. Synthesis of PRL is not limited to the hypophysis, as numerous extra-pituitary sites of PRL expression including the breast, decidua, and T-lymphocytes have been detected [7]. With respect to the mammary gland, these data have demonstrated that PRL stimulates the proliferation/survival [10], motility [11], and terminal maturation of normal mammary tissues [13,14]. Several lines of evidence have also indicated that PRL acts as both an endocrine and autocrine/paracrine progression factor for mammary carcinoma *in vitro* and *in vivo* in rodents and humans [5]. These data include recent epidemiologic studies that indicate that post-menopausal women with “high-normal” levels of PRL are at increased risk for breast cancer [15]. These findings are bolstered by the observations that transgenic mice overexpressing PRL either at the endocrine or autocrine/paracrine levels, develop

both ER+ and ER-, well-differentiated mammary carcinomas within the first 12-18 months of life [5,16].

PRLr structure and signaling

The effects of PRL in normal and malignant breast tissues are mediated by its cell surface receptor, the PRLr, a member of the cytokine receptor superfamily. The PRLr consists of an extracellular domain (ECD) required for ligand binding, a transmembrane domain (TM), and an intracellular domain (ICD) containing a region of membrane-proximal homology to other cytokine receptors (e.g. the Box1 / Variable Box / Box 2 / X Box, and its unique C-terminal tail) [5]. The hPRLr exists as seven recognized isoforms which have different signaling properties [5].

Insert BOX 1

Ligand binding to the long and presumably the $\Delta S1$ (these two PRLr isoforms being the most functional from a signaling perspective) results in the rapid phosphorylation of the ICD domain of the PRLr, and the activation of PRLr-associated signaling cascades such as the Jak2/Stat5 [17], Grb2-Sos-Ras-Raf-MEK-MAPK [7], and Nek3-Vav2-Rac1 [18,19] pathways. These signaling events induce several PRL-responsive genes such as those involved in cell proliferation (i.e. cyclin D1 and CISH) and in the differentiated mammary phenotype (i.e. β -casein) [20,21]. From garnered kinetic and knockout data [22,23], the Jak2 tyrosine kinase represents the most proximal kinase activated following ligand binding to the PRLr. The PRLr-Jak2 complex

requires the membrane-proximal proline-rich Box1 motif within the PRLr. Deletion of the Box1 motif or replacement of the Box 1's C-terminal proline residue (with leucine) abrogates PRLr function [24]. In addition, the tyrosine residues present within the C-terminal tail of the PRLr are necessary for the engagement of Stat5 as a prerequisite for their phosphorylation by Jak2 [25]. Other structural domains within the PRLr including V Box, Box 2, and X Box are largely uncharacterized and their signaling functions are not currently known.

Jak2 structure, signaling, and function

Jak2 is a member of the larger Jak2 family of tyrosine kinases consisting of four members which demonstrate significant homology, delineated as Jak homology (or "JH") domains. From the C-terminus, this includes a kinase motif (JH1), a regulatory pseudo-kinase domain (JH2), and SH2 motif (JH3) and a FERM protein interaction domain (JH4-7). Precedent studies with the growth hormone receptor (GHR) have revealed that the N-terminal region of the FERM domain of Jak2 is necessary for GHR interaction [26]. Following prolactin stimulation, Jak2 is rapidly activated within 30-60 seconds through trans- and/or auto-phosphorylation of two constitutively-associated Jak2 molecules, revealing an essential proximate role for Jak2 in PRLr-mediated signaling. Indeed, loss of Jak2 activity results in an ablation of PRL-induced Stat5 phosphorylation and gene expression [20]. The activity of Jak2 is necessary for both the in vitro [27] and in vivo [22] growth and differentiation of mammary cells and tissues. Indeed, the actions of Jak2 are not only necessary for both PRLr and GHR action, they may also contribute to cross-talk between these receptors and the erbB2 in breast

tissues [28]. The ligand-induced mechanism that triggers the trans/auto-phosphorylation of Jak2, however, has remained unresolved for not only the PRLr, but for all other cytokine receptors including the GHr, as well.

Ligand-independent dimerization of the PRLr

The accepted view has been that the initial event in PRL signaling is the binding of one PRL molecule to two cell surface PRLr monomers, inducing the dimerization and subsequent activation of the receptors. This model was based on studies with the GHr and other homologous receptors. Early studies determined the nature of the in vitro interaction between the GHr extracellular domain, referred to as growth hormone binding protein (GHBP), and GH, using the bacterially expressed extracellular domain of the GHr [29]. These classic studies, however, were limited in that they utilized high concentrations of only the ECD of the GHr, and did not initially examine the in vivo structural biology.

Other subsequent studies suggested that full length receptor predimerization did occur in vivo in the absence of ligand [30,31]. Indeed, Gent *et al.* [32] confirmed pre-dimerization of GHr, demonstrating that this process occurred in the endoplasmic reticulum (ER) via the transmembrane domain, a process which is facilitated by the ECD. Recent studies employing fluorescence resonance energy transfer (FRET), however, suggest that ECD facilitation of pre-dimerization may not be a prerequisite [33].

Pre-dimerization of the receptor, however, is insufficient to trigger signaling in the absence of ligand, suggesting that the function of pre-dimerization is to hold the

receptor in a “ready” position until addition of ligand induces the necessary conformational changes for activation of Jak2 kinase and subsequent signaling. Conformational changes in the ECD can be transduced into activation of the ICD, as evidenced by a study showing that a monoclonal antibody against the GHr ECD can induce GH-independent signaling [34]. More detailed analysis has suggested that GH activates a pre-formed dimer by transmission of rotational torque through the TM resulting in rotation of the GHr ICDs [33]. However, this study relied exclusively upon transfected, mutant GHrs, and various studies investigating rotational torque to induce activation were not consistent in the degree of rotation required for activation.

Similar data has been reported for the erythropoietin (EPO) receptor. Further studies indicate that the transmembrane domain plays a major role in EPOr ligand-independent dimerization [35]. The proposed mechanism of EPOr activation upon ligand-binding is via a scissor-like movement of the ICD [36], which is slightly different than the rotational torque mechanism proposed for liganded GHr [33]. This indicates that although these homologous receptors pre-dimerize in the absence of ligand, different mechanisms may exist for their ligand-induced activation.

Few studies have looked at ligand-independent dimerization of the PRLr. One report demonstrated that recombinant soluble PRLr-ECD (PRLBP) does not pre-dimerize but rather forms an unstable, transient homodimer upon PRL binding [37]. Furthermore, full length (ie TM domain-containing PRLr) failed to show FRET in the absence of PRL [38]. Other studies have been unable to detect a FRET signal between PRLr and GHr heterodimers in the absence of ligand [38], while another study demonstrated a lack of bioluminescence resonance energy transfer (BRET) signal

between homo and heterodimers of long and the two short PRLr isoforms in the absence of ligand [39]. However, a recent article has demonstrated ligand-independent hetero- and homo- dimerization of the long and 2 short PRLr isoforms using co-immunoprecipitation and bioluminescence resonance energy transfer (BRET) [40]. As the discordant Qazi *et al.* [40] and Tan *et al.* [39] studies both investigated the homo- and hetero- dimerization of the long and 2 short isoforms using BRET analysis in the same cell line, the non-concordant results may be due to differences in the nature of the constructs used by these two labs and/or the relatively insensitive plate-based FRET/BRET approaches used by some of these labs.

Recently our lab has demonstrated ligand-independent dimerization of transfected and, for the first time, endogenous PRLr [41], by biochemical approaches. The co-immunoprecipitation of endogenous PRLr isoforms showed that dimerization of the hPRLr occurs at physiologic concentrations and is not an artifact of transfection. In addition, transfected epitope-tagged isoforms of the PRLr demonstrated that long, intermediate and $\Delta S1$ PRLrs formed ligand-independent homo- and heterodimers. Using PRLr deletion constructs, it was shown that the TM domain has a significant role in ligand-independent PRLr dimerization. Although interactions between the ECD and ICD in the dimeric pair do not appear necessary for this process, interactions between these domains do appear to strengthen the dimerization. Therefore, these findings indicate that the TM domain is sufficient for ligand-independent dimerization and that this interaction is strengthened by the ECD and ICD. These data are consistent with a recent study demonstrating that a mutation of a single cysteine residue in the TM domain inhibited ligand-independent dimerization by approximately 30% [40].

Taken together, these data make a case for ligand-independent dimerization of cytokine receptors, including the PRLr. Rather than induce dimerization of monomeric receptors, the addition of ligand appears to bind a preformed dimer and initiate conformational changes that lead to activation of the receptor ICDs and associated kinases (see Figure 1). Given the rapid activation of Jak2 kinases within 30 seconds after ligand stimulation, pre-dimerization of the cytokine receptors makes teleological sense; the delay monomeric receptors would encounter secondary to lateral diffusion through the cell membrane, could result in a significant delay in ligand stimulated receptor dimerization and signaling. With the dimerization status of the PRLr and GHr within the cell membrane largely resolved, however, the conundrum that now presents itself is what enables the receptor to alter its conformation following ligand binding, such that its associated kinases become activated?

Function of peptidyl prolyl isomerases in signal transduction

Peptidyl-prolyl isomerases (PPI) are a superfamily of enzymes that catalyze the *cis-trans* interconversion of imide bonds of proline residues. The first-identified member of the cyclophilin family of PPI, cyclophilin A (CypA) was found to span 165 amino acids with an approximate SDS-PAGE mobility of 18 kiloDaltons [42]. Structural analysis of CypA revealed an eight-stranded antiparallel β -barrel core with two capping α -helices. A hydrophobic pocket on this barrel contains the PPI enzymatic site. As a PPI, CypA was thought initially to assist in nascent protein folding *in vivo*. CypA was also identified as a cytosolic receptor of the immunosuppressive drug cyclosporine A (CsA) [42-44].

INSERT – Box 2

Several lines of evidence, however, have recently demonstrated that PPIs directly function to modulate protein signal transduction, using their PPI activity to “switch” recognized signaling proteins into either an active or inactive conformer [45]. One such example is the regulation of the transforming growth factor-beta-receptor (TGFβR) by the PPI FKBP12. Engagement of FKBP12 of the proline-rich sequence with the intracellular domain of the TGFβR results in down-regulation of ligand-stimulated receptor transphosphorylation and signaling [46,47]. Other studies have revealed that CypA can modulate the tyrosine kinase activity of the Tec-family member Itk, by inducing a conformational switch within the SH2 domain of this kinase [48]. In addition to their regulation of cell surface receptors, PPIs can impact cell signaling by their interaction with transcription factors such as c-Myb [49], IRF4 [50], and Stat5 [51,52].

Role of cyclophilins and prolyl isomerization to PRLr function

Initial studies have revealed a significant role for cyclophilin family members in the regulation of PRLr-mediated signaling. Two members of the cyclophilin family, CypB and CypA have been respectively found to associate and modulate the function of Stat5 and Jak2. Triggered by PRL, CypB potentiates Stat5 function by inducing the release of the Stat repressor, PIAS3 [51,52], resulting in the enhanced PRL-induced gene expression, proliferation, cell motility, and the enhanced expression of the receptors for estrogen, progesterone, growth hormone, and prolactin [53]. In contrast, CypA constitutively associates with the PRLr and Jak2 [54], resulting in enhanced Jak2 and

Stat5 activity. These initial observations led to the hypotheses that cyclophilins may function as a proximate signaling switch during PRLr transduction (and CypB a distal switch), and that the pharmacologic blockade of the PPI activity of CypA/B may down-regulate PRLr signaling and function in breast cancer.

To further explore the role of CypA in the proximate activation of the PRLr a multi-pronged approach was utilized, that included: 1) overexpression of wild type CypA, 2) knockdown of CypA through the use of SiRNA 3) overexpression of a PPI-inactive, mutant CypA, 4) the development of a point-mutant of the PRLr incapable of binding CypA, and 5) the use of the cyclophilin PPI inhibitor cyclosporine A (CsA) [55]. Consistent with its role as a signaling switch, overexpression of wild type CypA resulted in significant increases in Stat5-driven gene expression from luciferase reporters consisting of singular (LHRE) and composite (CISH, cyclin D1) response elements, as well as the endogenous expression of both the Stat5-responsive CISH and cyclin D1 genes at the mRNA and protein levels. Conversely, 50% reductions in CypA levels mediated by CypA SiRNA resulted in 2-3 fold reductions in reporter and endogenous gene expression. To further establish a causal relation between the enzymatic activity of CypA and PRLr-mediated activation of Jak2, isomerase-deficient point mutant forms were overexpressed in T47D breast cancer cells, resulting in highly significant reductions in PRL-induced reporter and endogenous gene expression and two-three fold reductions in the levels of Jak2 phosphorylation. Finally, to prove that the actions of CypA associated with the PRLr was directly responsible for the activation of Jak2, the binding site for CypA on the PRLr was pin-pointed through targeted mutagenesis. These studies revealed that truncations of the PRLr shorter than constructs containing

the X-box motif were incapable of interacting with CypA. Analysis of the X-box motif revealed a proline at residue 334 that was conserved across all PRLr species. Given the affinity of cyclophilins for proline residues, it was hypothesized that P334 could play an important role in the engagement of CypA. To test this hypothesis a proline-to-alanine replacement mutation of the PRLr was generated (P334A), and the ability of this mutant PRLr to interact with CypA and mediate PRL-induced signaling was tested. These studies revealed that when expressed in T47D cells, the mutant P334A PRLr demonstrated significantly reduced association with CypA, while the ability of this mutant receptor to bind to Jak2 was not altered. Overexpression of the P334A mutant in T47D cells, however, resulted in a marked reduction of both Jak2 and Stat5 phosphorylation. Consistent with these findings were the markedly reduced ability of the the P334A PRLr to induce the expression of either PRL-responsive reporters or endogenous genes. These findings were viewed as notable, in that T47D cells in which these studies were conducted are known to express high levels of endogenous PRLr, indicating that the P334A mutant, perhaps through heterodimerization with the wt PRLr, functions as a dominant negative receptor. When assessed with the data presented above, these findings revealed that the direct action of the CypA on the PRLr is required for effective PRL-induced signaling, a process that is inhibited by P334A PRLr mutant.

INSERT – Box 3

Cyclosporine A (CsA) inhibits in vitro PRL-induced signaling and function in breast cancer cells

Given the notable effects on PRLr signaling through the manipulation of CypA levels, binding, and activity, it was hypothesized that the inhibitor of cyclophilin prolyl isomerase activity, cyclosporine A could have a significant effect on PRLr-mediated signaling. Furthermore, by acting on the PRLr and other cyclophilin-dependent receptors and signaling pathways, it was also hypothesized that CsA could significantly alter the growth and progression of human breast cancer [55]. To that end, treatment of a spectrum of ER-positive and –negative (and Her2+) breast cancer cell lines with CsA resulted in a significant dose-dependent inhibition of cell growth. At low-intermediate dose (1-10 µg CsA/ml), CsA treatment inhibited the proliferation, but did not induce the death of breast cancer cells. Treatment at higher doses (30 µg CsA/ml and above) resulted in the induction of cell death. These studies were extended to examine the effects of CsA treatment on anchorage-independent growth, as measured by soft agar colony formation. Treatment of CsA resulted in both a significant decrease in breast cancer cell colony number and colony size. In addition, both cell migration and invasion were inhibited by CsA. Taken together, these finding revealed that CsA inhibited many of the malignant properties of breast cancer cells in vitro.

Given the effects of CsA on breast cancer cell biology, the effects of CsA on PRL-induced breast cancer signaling were tested [55]. These studies showed that CsA pre-treatment of breast cancer cells resulted in a near complete inhibition of Jak2/Stat5 phosphorylation, and notable reductions in AKT and ERK activation. These reductions were found to correlate with significant decreases in PRL-induced gene expression at both the reporter and endogenous gene level. Interestingly, co-immunoprecipitation analysis revealed that while CsA had no effect whatsoever on the PRLr-Jak2

interaction, it completely abolished the interaction of CypA with the PRLr, consistent with the data obtained by mutagenesis of the PPI site in CypA. The inhibition of receptor signaling by CsA was found to be specific, in that while the PRLr- and GHR-associated signaling were reduced by CsA, no effect of this PPI inhibitor was noted for either the insulin or epidermal growth factor receptors.

In vivo relevance of CsA treatment of breast cancer

Given that CsA inhibited the growth, motility, invasion, and soft agar colony formation of human breast cancer, we hypothesized that CsA could inhibit the in vivo growth of human breast cancer [55]. This hypothesis was supported, as noted above, by precedent data demonstrating as much as a 90% decrease in the incidence of breast cancer in female allograft patients receiving CsA for immunosuppression [12]. To test this hypothesis, both the ER+ human breast cancer line MCF7 and the ER- line MDA231 were xenografted into the lactiferous ducts of nude mice and the growth and biologic characteristics of the resultant tumors assessed. Two biologic parameters were found to be significantly changed as a function of CsA therapy, notably central tumor necrosis and metastasis. First, central tumor necrosis was markedly increased, particularly in ER+ tumors. Second, and most remarkably, not a single metastasis to lymph nodes, brain, or visceral organs were noted in CsA treated mice. Indeed, in the experimental groups receiving varied doses of CsA (some as low as 10 mg/kg/day), numbering 150 mice, no metastasis were noted, whereas nearly 50% of the control mice demonstrated metastasis. Additional biochemical and immunohistochemical analysis of the CsA-treated and control primary breast cancers in the xenografted mice,

revealed that CsA therapy inhibited the in vivo association of CypA with the PRLr, and significantly reduced the PRL-responsive expression of cyclin D1. These highly significant results have several implications: 1) they support an in vivo biologic role for CypA and its attendant PPI activity, 2) raise interesting questions regarding the function of CypA in supporting the metastatic process and potentially central tumor angiogenesis, 3) indicate that the effects of CsA are direct and not a consequence of immunosuppressive actions (given our results in vitro and in the T/B cell deficient, nude mouse), and 4) raise the distinct possibility that the FDA-approved drug, CsA, or analogs thereof may be of use in the treatment of human breast cancer. The pharmacokinetics of CsA with respect to its immunosuppressive actions and toxic side effects are well understood and have been manageable in the clinical setting, a situation analogous to many of the effects clinically observed with the chemotherapeutic agents currently used to treat breast cancer. Thus, the actions of cyclophilins shed light not only on the mechanisms of proximal receptor-based signaling, they may provide significant translational opportunities for the development of novel PPI-inhibitory therapies directed against human breast cancer.

Conclusions

Collectively, this research has altered our vision of how PRLr, and GHr, signaling is mediated in breast cancer. Careful analysis has revealed that the conventional view of ligand-induced dimerization of the PRLr and GHr triggering receptor-associated signaling was largely based on the artifice of in vitro studies using only the extracellular domains of these receptors. Subsequent analysis using full length receptors in their in

vivo setting has revealed an entirely different picture, i.e. that the PRLr and GHr are pre-dimerized and that the activation of receptor-associated Jak2 kinases occurs as a consequence of conformational change in the receptors, in part mediated by prolyl isomerases, such as cyclophilin A. These findings raise a number of questions and research directions. For example, regarding the ligand-independent dimerization of the PRLr, what precise domains/residues are involved in PRLr predimerization? What would be the effects on signaling if these domains were disrupted? In terms of the actions of cyclophilins and CsA, what are the dynamic changes within the PRLr induced by cyclophilin following ligand binding? How does CsA stimulate tumorigenesis at multiple sites in the body, and yet, significantly inhibit the progression of breast cancer? Can CypA be targeted in a specific manner, such that the immunosuppressive and non-specific effects of CsA can be avoided? Is continuous therapy with CsA efficacious at preventing or inhibiting the progression of existent breast cancer in patients? While significant, the challenges poised by these questions present an opportunity to examine the fundamental mechanisms of proximal cytokine receptor-based signaling and translate them into novel therapies directed against disease states dependent upon the actions of prolyl isomerases.

BOX 1 – PRLr isoforms

Representing the first human PRLr isoform identified and containing the longest sequence, the long hPRLr represents a classic Type I single pass, cell membrane receptor consisting of an extracellular (ECD), a transmembrane (TM), and intracellular domains (ICD). The intermediate and $\Delta S1$ hPRLr isoforms are generated from alternative splicing, resulting in a deletion of a significant portion of the ICD or deletion of a portion of the ECD, respectively. Two short forms of the hPRLr, known as S1a and S1b, are also generated via alternative splicing. The PRL binding protein (PRLBP) represents the freely circulating ECD of the hPRLr, and arises from a proteolytic event rather than a splicing event. In addition, using an antibody specific for the PRLr ICD, our lab has recently observed a PRLr that consists of the TM domain and the full-length ICD, an isoform termed the ΔECD . This fragment is probably generated upon the proteolytic cleavage that releases the PRLBP.

BOX 2 – Classic functions of the CsA-CypA complex

In the cytosol the formation of the CypA-CsA complex results in the engagement of the calcineurin. In doing so, this complex inhibits the phosphatase activity of calcineurin. The loss of calcineurin activity blocks the dephosphorylation of the transcription factor NFAT. This precludes NFAT nuclear entry, resulting in a decrease in interleukin-2 expression and the clonal expansion of activated T-cells, which in part results in the immunosuppressive effects of CsA.

BOX 3 - Role of CypA in cancer

Several lines of recent evidence suggest that CypA may contribute to the pathobiology of human malignancy [1]. First, CypA is overexpressed in a large number of the primary lung cancers [2], in human pancreatic cancer cells [4], and in oral squamous cancer cells [6]. Second, CypA overexpression in lung cancer cells enhances tumor growth *in vivo*; conversely, stable suppression of CypA diminishes tumor growth *in vivo* [8]. Third, recent studies have demonstrated that overexpression of CypA in a variety of cancer cells confers resistance to oxidative stress-induced cell apoptosis *in vitro* [9]. Finally, significant epidemiologic data has implicated a role for cyclophilins in the pathogenesis of breast cancer. In a case cohort of 25,000 women who had received CsA as therapy for renal and cardiac allografts a significant increase in malignancies were noted at various sites, including skin, lymph tissues, kidney, and oropharynx. However, a reduction in the incidence of breast cancer of up to 50% in the CsA-treated group was noted over a ten-year follow-up period. Indeed, in the first year of therapy, the CsA-treated cohort demonstrated a 90% reduction in the incidence of breast cancer [12]. Taken together, these data suggest that CypA may play an important role in tumorigenesis at multiple sites within the body.

360

361

362

363

364 **Acknowledgements**

365 This work was supported by the Susan G. Komen Foundation BCTR05-03790 and NIH
366 grants CA69294 and CA102682. Additional support was received from the Zell
367 Scholar's Fund, the Lynn Sage Foundation, and the Avon Foundation.

368

References

- 1 Yao,Q. *et al.* (2005) Roles of cyclophilins in cancers and other organ systems. *World Journal of Surgery* 29, 276-280
- 2 Howard,B.A. *et al.* (2005) Stable RNA interference-mediated suppression of cyclophilin A diminishes non-small-cell lung tumor growth in vivo. *Cancer Res* 65, 8853-8860
- 3 Bachelot,A. and Binart,N. (2007) Reproductive role of prolactin. *Reproduction* 133, 361-369
- 4 Shen,J. *et al.* (2004) Protein expression profiles in pancreatic adenocarcinoma compared with normal pancreatic tissue and tissue affected by pancreatitis as detected by two-dimensional gel electrophoresis and mass spectrometry. *Cancer Res* 64, 9018-9026
- 5 Clevenger,C.V. *et al.* (2003) The role of prolactin in mammary carcinoma. *Endocrine Rev* 24, 1-27
- 6 Rey,O. *et al.* (1999) Differential gene expression in neoplastic and human papillomavirus-immortalized oral keratinocytes. *Oncogene* 18, 827-831
- 7 Harris,J. *et al.* (2004) Prolactin and the prolactin receptor: new targets of an old hormone. *Annals of Medicine* 36, 414-425
- 8 Campa,M.J. *et al.* (2003) Protein expression profiling identifies macrophage migration inhibitory factor and cyclophilin a as potential molecular targets in non-small cell lung cancer. *Cancer Res* 63, 1652-1656

391 9 Choi,K.J. *et al.* (2007) Overexpressed cyclophilin A in cancer cells renders resistance
 392 to hypoxia- and cisplatin-induced cell death. *Cancer Res* 67, 3654-3662
 393 10 Perks,C.M. *et al.* (2004) Prolactin acts as a potent survival factor for human breast
 394 cancer cell lines. *Br. J. Cancer* 91, 305-311
 395 11 Maus,M.V. *et al.* (1999) Prolactin as a chemoattractant for human breast carcinoma.
 396 *Endocrinology* 140, 5447-5450
 397 12 Stewart,T. *et al.* (1995) Incidence of de-novo breast cancer in women chronically
 398 immunosuppressed after organ transplantation. *Lancet* 346, 796-798
 399 13 Ormandy,C.J. *et al.* (1997) Null mutation of the prolactin receptor gene produces
 400 multiple reproductive defects in the mouse. *Genes and Development* 11, 167-178
 401 14 Horseman,N.D. *et al.* (1997) Defective mammopoiesis, but normal hematopoiesis, in
 402 mice with a targeted disruption of the prolactin gene. *EMBO J* 16, 6926-6935
 403 15 Hankinson,S.E. *et al.* (1999) Plasma prolactin levels and subsequent risk of breast
 404 cancer in postmenopausal women. *JNCI* 91, 629-634
 405 16 Wennbo,H. *et al.* (1997) Activation of the prolactin receptor but not the growth
 406 hormone receptor is important for induction of mammary tumors in transgenic mice. *J*
 407 *Clin Invest* 100, 2744-2751
 408 17 Clevenger,C.V. (2004) Role of Stat family transcription factors in human breast
 409 cancer. *Am J Pathol* 165, 1449-1460
 410 18 Miller,S.L. *et al.* (2004) Novel association of Vav2 and Nek3 modulates signaling
 411 through the human prolactin receptor. *Mol Endocrinol* 19, 939-949
 412 19 Miller,S.L. *et al.* (2007) Nek3 kinase regulates prolactin-mediated cytoskeletal
 413 reorganization and motility of breast cancer cells. *Oncogene* 26, 4668-4678

414 20 Brockman,J.L. *et al.* (2002) PRL activates the cyclin D1 promoter via the Jak2/Stat
 415 pathway. *Mol. Endocrinol.* 16, 774-784

416 21 Fang,F. *et al.* (2008) Quantification of PRL/Stat5 signaling with a novel pGL4-CISH
 417 reporter. *BMC Biotechnology* 8, 1-11

418 22 Sakamoto,K. *et al.* (2007) The Janus kinase 2 (Jak2) is required for expression and
 419 nuclear accumulation of Cyclin D1 in proliferating mammary epithelial cells. *Mol.*
 420 *Endocrinol.* 21, 2218-2232

421 23 Wagner,K.-U. *et al.* (2004) Impaired Alveologenesis and Maintenance of Secretory
 422 Mammary Epithelial Cells in Jak2 Conditional Knockout Mice. *Mol. Cell Biol.* 24, 5510-
 423 5520

424 24 Pezet,A. *et al.* (1997) The last proline of box 1 is essential for association with JAK2
 425 and functional activation of the prolactin receptor. *Mol. Cell. Endocrinol.* 129, 199-208

426 25 Pezet,A. *et al.* (1997) Tyrosine docking sites of the rat prolactin receptor required for
 427 association and activation Stat5. *J. Biol. Chem.* 272, 25043-25050

428 26 He,K. *et al.* (2003) Jak2 determinants for growth hormone receptor association,
 429 surface assembly, and signaling. *Mol. Endocrinol.* In Press

430 27 Xie,J. *et al.* (2002) Role of tyrosine kinase Jak2 in prolactin-induced differentiation
 431 and growth of mammary epithelial cells. *J. Biol. Chem.* 277, 14020-14030

432 28 Yamauchi,T. *et al.* (2000) Constitutive tyrosine phosphorylation of ErbB-2 via Jak2
 433 by autocrine secretion of prolactin in human breast cancer. *J. Biol. Chem.* 275, 33937-
 434 22944

435 29 Waters,M.J. *et al.* (2006) New insights into growth hormone action. *J. Mol.*
 436 *Endocrinol.* 36, 1-7

437 30 Harding,P. *et al.* (1996) Growth hormone and a GH antagonist promote GH receptor
438 dimerization and internalization. *Bioessays* 271, 6708-6712

439 31 Ross,R.J.M. *et al.* (2001) Binding and functional studies with the growth hormone
440 receptor antagonist B2036-PEG (Pegvisomant), reveal effects of pegylation and
441 evidence that it binds to a receptor dimer. *J Clin Endocrinol Metab* 86, 1716-1723

442 32 Gent,J. *et al.* (2002) Ligand-independent growth hormone receptor dimerization
443 occurs in the endoplasmic reticulum and is required for ubiquitin system-dependent
444 endocytosis. *Proc. Natl. Acad. Sci. USA* 99, 9858-9863

445 33 Brown,R.J. *et al.* (2005) Model for growth hormone receptor activation based on
446 subunit rotation within a receptor dimer. *Nature Structural & Molecular Biology* 12, 814-
447 821

448 34 Mellado,M. *et al.* (1997) Conformational changes required in the human growth
449 hormone receptor for growth hormone signaling. *Journal of Biological Chemistry* 272,
450 9189-9196

451 35 Constantinescu,S.N. *et al.* (2001) The erythropoietin receptor cytosolic
452 juxtamembrane domain contains an essential, precisely oriented, hydrophobic motif.
453 *Molecular Cell* 7, 377-385

454 36 Seubert,N. *et al.* (2003) Active and inactive orientations of the transmembrane and
455 cytosolic domains of the erythropoietin receptor dimer. *Molecular Cell* 2, 1239-1250

456 37 Gertler,A. *et al.* (1996) Real-time measurements of the interactions between
457 lactogenic hormones and prolactin-receptor extracellular domains from several species
458 support the model hormone-induced transient receptor dimerization. *J. Biol. Chem.* 271,
459 24482-24491

460 38 Biener, E. *et al.* (2003) Ovine Placental Lactogen-Induced Heterodimerization of
 461 Ovine Growth Hormone and Prolactin Receptors in Living Cells is Demonstrated by
 462 Fluorescence Resonance Energy Transfer Microscopy and Leads to Prolonged
 463 Phosphorylation of Signal Transducer and Activator of Transcription (STAT)1 and
 464 STAT3. *Endocrinology* 144, 3532-3540
 465 39 Tan, D. *et al.* (2005) Unmodified prolactin (PRL) and S179D PRL-initiated
 466 bioluminescence resonance energy transfer between homo - and hetero - pairs of long
 467 and short human PRL receptors in living human cells. *Mol. Endocrinol.* 19, 1291-1303
 468 40 Qazi, A.M. *et al.* (2006) Ligand-independent homo- and hetero-dimerization of human
 469 prolactin receptor variants: inhibitory action of the short forms by heterodimerization. *Mol.*
 470 *Endocrinol.* 20, 6748-6761
 471 41 Gadd, S. and Clevenger, C.V. (2006) Ligand-independent dimerization of the human
 472 prolactin receptor: functional implications. *Mol Endo* 20, 2734-2746
 473 42 Wang, P. and Heitman, J. (2005) The cyclophilins. *Genome Biology* 6, 226
 474 43 Colgan, J. *et al.* (2005) Cyclophilin A-Deficient Mice Are Resistant to
 475 Immunosuppression by Cyclosporine. *J. Immunol.* 174, 6030-6038
 476 44 Edlich, F. and Fischer, G. (2006) Pharmacologic targeting of catalyzed protein folding:
 477 The example of peptide bond cis/trans isomerases. *Handbook of Experimental*
 478 *Pharmacology* 172, 359-404
 479 45 Lu, K.P. *et al.* (2007) Prolyl *cis-trans* isomerization as a molecular timer. *Nature*
 480 *Chemical Biology* 3, 619-629
 481 46 Chen, Y.-G. *et al.* (1997) Mechanisms of TGF β receptor inhibition by FKB12. *EMBO J*
 482 13, 3866-3876

483 47 Lopez-Hasaca,M. *et al.* (1998) Effects of FK506-binding protein 12 and FK506 on
484 autophosphorylation of epidermal growth factor receptor. *J. Biol. Chem.* 273, 9430-9434

485 48 Brazin,K.N. *et al.* (2002) Regulation of the tyrosine kinase Itk by the peptidyl-prolyl
486 isomerase cyclophilin A. *Proc. Natl. Acad. Sci. U. S. A* 99, 1899-1904

487 49 Levenson,J.D. and Ness,S.A. (1998) Point mutations in v-Myb disrupt a cyclophilin-
488 catalyzed negative regulatory mechanism. *Molecular Cell* 1, 203-211

489 50 Mamane,Y. *et al.* (2000) Postranslational regulation of IRF-4 activity by the
490 immunophilin FKBP52. *Immunity* 12, 129-140

491 51 Ryszczyn,M.A. *et al.* (2000) Role of cyclophilin B in PRL signal transduction and
492 nuclear retrotranslocation. *Mol. Endocrinol.* 14, 1175-1186

493 52 Ryszczyn,M.A. and Clevenger,C.V. (2002) The intranuclear prolactin/cyclophilin B
494 complex as a transcriptional inducer. *Proc. Natl. Acad. Sci. USA* 99, 6790-6795

495 53 Fang,F. *et al.* (2009) Expression of cyclophilin B is associated with malignant
496 progression and regulation of genes implicated in the pathogenesis of breast cancer.
497 *Am. J. Pathol* 174, 297-308

498 54 Syed,F. *et al.* (2003) A novel and functional interaction between cyclophilin (Cyp) A
499 and the prolactin receptor. *Endocrine* 20, 83-89

500 55 Zheng,J. *et al.* (2008) Peptidyl-prolyl isomerase regulation of Jak2 activation and the
501 progression of human breast cancer. *Cancer Res.* 68, 7769-7778

502

503

FIGURE LEGENDS

Figure 1. Ligand-Dependent and -Independent Dimerization of the PRLr.

Schema 1 depicts the precedent ligand-dependent dimerization model. In this model, based on initial in vitro data using only ECD, the PRLr is in monomeric form at the cell membrane. In this sequential dimerization model, one molecule of PRL first binds to one PRLr monomer via binding site 1, and this 1:1 complex then recruits the second PRLr via binding site 2. The dimerization of the two PRLrs leads to activating changes in the intracellular domain, leading to PRL signal transduction, such as phosphorylation of the Jak2 kinase, phosphorylation of the PRLr, and the recruitment and phosphorylation of Stat5a. *Schema 2* depicts the ligand-independent model. In this model, the PRLr exists in dimeric form at the cell membrane in the absence of ligand. Recent data using full length and mutant truncated receptors in vivo indicate that this interaction involves the transmembrane domain. This ligand-independent interaction holds the receptors in an inactive form until the binding of PRL to this preformed complex induces activating changes in the intracellular domain, leading to phosphorylation of the Jak2 kinase, phosphorylation of the PRLr, and the recruitment and phosphorylation of Stat5a.

Figure 2. Regulation of the PRLr/JAK2 complex by PRLr-associated CypA.

CypA is constitutively associated with PRLr and Jak2 during unstimulated conditions. Upon binding of PRL to the PRLr, CypA positively regulates Jak2 activity by exerting its isomerase activity, presumably through its switching function of a cis-trans peptidyl prolyl isomerase. Ablation of CypA PPI activity by cyclosporine (CsA) and other

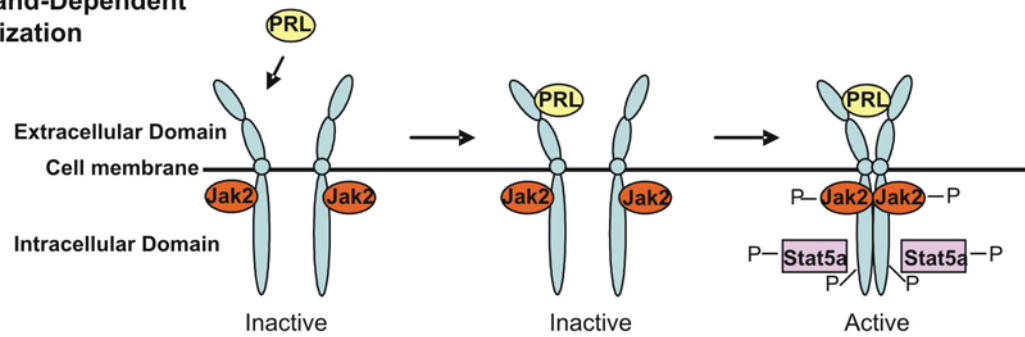
527 approaches inhibits PRL signaling and may be a novel therapeutic strategy in the
528 treatment of human breast cancer. *Red arrow* denotes *cis-trans* interconversion of
529 proline 334 in the X-box motif of the PRLr; *Red circle containing P* indicates
530 phosphorylation of JAK2 kinase, PRLr, and Stat5.

531

532

FIGURE 1

**1. Ligand-Dependent
Dimerization**



**2. Ligand-Independent
Dimerization**

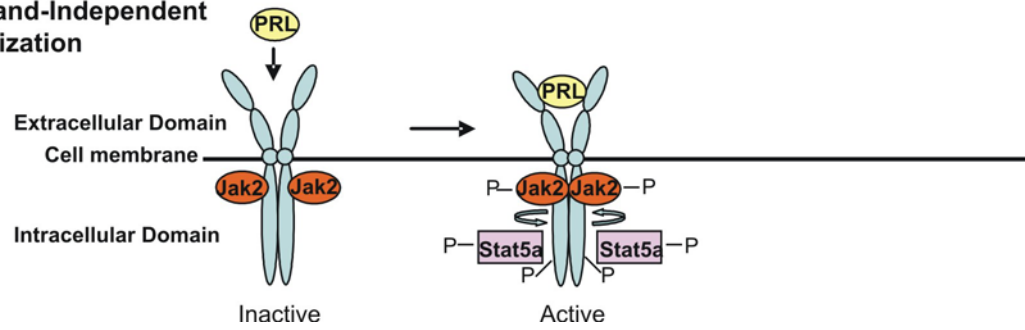
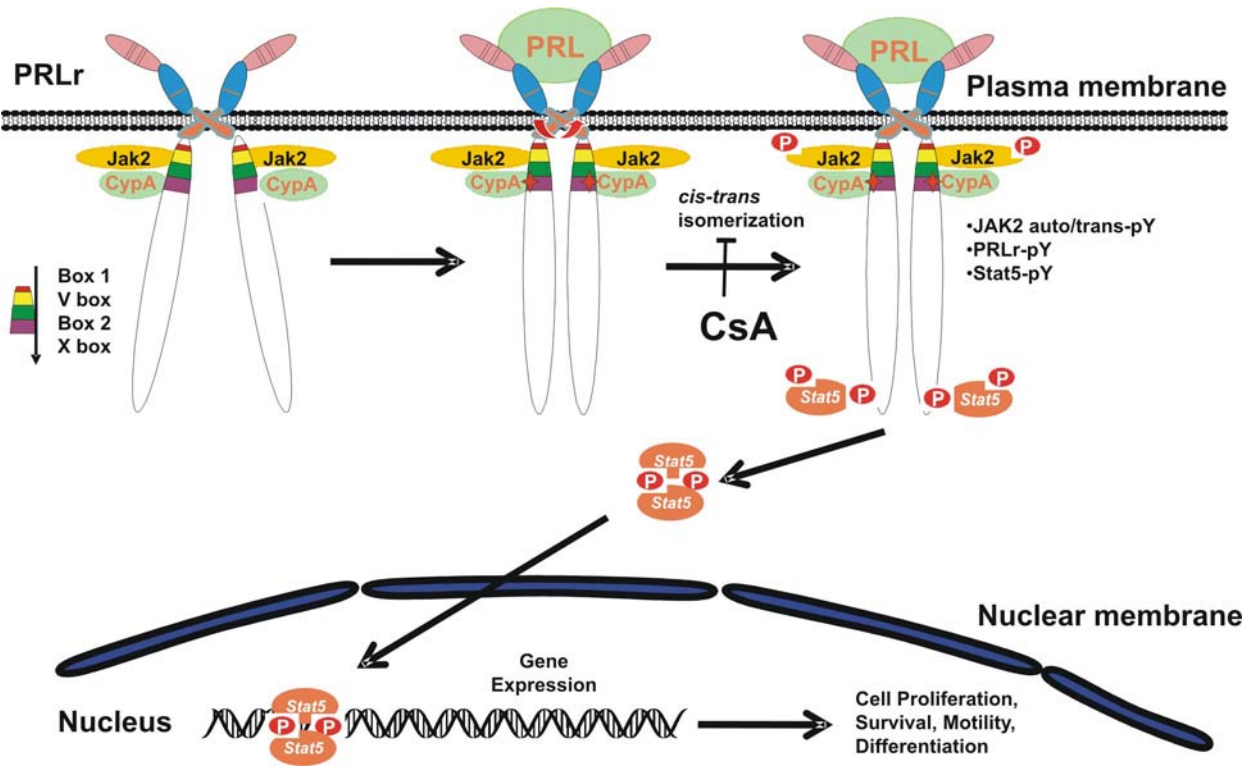


Figure 2



2009 Dod's The Leading Innovation and Knowledge Sharing (LINKS) Meeting

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

Jiamao Zheng¹, Anthony Kossiakoff², Charles Clevenger¹

¹Robert H. Lurie Comprehensive Cancer Center & Department of Pathology,
Northwestern University, Chicago, IL, United States, 60611. ²Department
of Biochemistry and Molecular Biology, University of Chicago, Chicago,
IL 60637

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 goal of the proposed project is to understand how cyclophilin A (CypA) modulates the prolactin (PRL) receptor (PRLr)-associated signaling and to determine the effects of altered CypA levels and activity on PRL signaling and breast cancer phenotype by combining biophysical structural investigations with studies in cell and animal models. 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. From a translation perspective, we have also shown that the X-box peptide significantly blocks PRLr signaling. 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.