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

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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 (PPIs) 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...
and Drug Administration–approved drug CsA, inhibits the \textit{in vitro} and \textit{in vivo} outgrowth of human breast cancer.

**Materials andMethods**

**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 \textit{CISH} gene. For luciferase assay, cells were transiently transfected with the LHRE and CISH reporters, in addition to either the empty vector (control)
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 retrovirals 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 postion 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 taut 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 counted 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 ± SE and analyzed using the appropriate 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 μg/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.
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 PRLr-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

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 transfecants was detected by Western blot analysis.

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Research.
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.
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

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×; bar, 50 μm). 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 μm) 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 communoprecipitation analysis.
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 \(\leq 10 \text{ mg/mL}\), this antiproliferative effect was reversible following washing and medium change; at concentrations of \(\geq 30 \text{ mg/mL}\), 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 vitro role for PPI inhibition in the treatment of breast cancer.

**Inhibition of PPI activity in vitro 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 cell 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. 6D, 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 1 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 NH\(_2\)-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 cycophilin 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 motilility 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 knockdown 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 epithelial, 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 allotraf 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 Paxil 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

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