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# A Novel and Functional Interaction Between Cyclophilin A and Prolactin Receptor

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**Precedent data have revealed that peptidyl isomerases can modulate the function of cell-surface receptors, but no such interactions have been previously shown for the members of the cytokine receptor superfamily. We demonstrate here that a functional interaction occurs between the prolactin receptor (PRLR) and peptidyl prolyl *cis/trans* isomerase cyclophilin A (CypA). CypA was coimmunoprecipitated with the PRLR in vivo from the breast epithelial cell line T47D and Chinese hamster ovary transfectants overexpressing transfected human PRLR. In addition, in vitro binding assays demonstrated a direct interaction of CypA with the PRLR, in the presence or absence of cyclosporine. Coimmunoprecipitation studies also showed an association of CypA with Jak2. Functional analysis revealed that overexpression of CypA inhibited PRL-induced Rac activation, while simultaneously prolonging Jak2 phosphorylation. These proximal actions had profound downstream effects: CypA overexpression significantly enhanced the basal and PRL-stimulated expression from a β-casein reporter construct. Hence, the interaction between CypA and the PRLR plays a differential regulatory role in the various signaling pathways leading from the PRLR.**

**Key Words:** Prolactin receptor; cyclophilin A; peptidyl prolyl isomerase; Rac1; Jak2; β-casein.

## Introduction

Prolactin (PRL) is a member of the cytokine/hematopoietin family, which includes growth hormone (GH), erythropoietin, granulocyte macrophage colony-stimulating factor, and interleukins 2–7 *(1)*. PRL is synthesized in the hypophysis, decidua, breast, and T-lymphocytes *(2–5)*. The pleiotropic effects of PRL include regulation of the growth and differentiation of mammary tissues and modulation of the immune response. These effects are mediated by the interaction of PRL with the PRL receptor (PRLR), a member of the cytokine receptor superfamily, which is expressed on numerous tissues including mammary epithelia, T- and B-lymphocytes, liver, kidney, ovary, and adrenal *(6,7)*. PRL signaling via the PRLR stimulates cell proliferation, survival, and cellular differentiation in a tissue- and microenvironment-dependent manner *(2,8)*.

The signaling activity of transmembrane receptors, such as the PRLR, is modulated by immunophilins. Members of the immunophilin family, which include cyclophilins and FK506-binding proteins (FKBPs), are peptidyl prolyl *cis/trans* isomerases (PPIs) *(9)*. This enzymatic activity is inhibited by but not necessary for the immunosuppressive effects of cyclosporin A (CsA). The PPI activity of the immunophilins has been implicated in cell-surface receptor transduction, at both the level of ligand engagement *(10)* and intracellular domain signaling *(11)*, through its putative modification of receptor structure by prolyl isomerization. For instance, FKBP12 has been shown to downregulate the signaling activity of transforming growth factor-β-receptor (TGF-βR) complex by binding to its proline-rich sequence and significantly downregulating ligand-stimulated receptor transphosphorylation and signaling *(10)*. These findings have been interpreted to indicate that the FKBP12 is not merely serving as a chaperone necessary for TGF-βR maturation, but is functioning as a switch, modifying receptor transduction via ligand-induced PPI activity. Recent work from our laboratory has also shown the role of cyclophilins, in particular cyclophilin B (CypB), during PRL signaling *(12–14)*. CypB interacts with PRL and potentiates PRL-driven cell proliferation via its interaction with the complex of Stat5 and PIAS3 *(13)*.

Several lines of data have indicated that the proline-rich box 1 motif within the intracellular domain of the PRLR is necessary for PRLR-associated signaling, possibly undergoing a structural change during such transduction *(15)*. These studies have revealed considerable mobility around the box 1 proline imide bonds, suggesting that a prolyl isomerase could significantly modify the structure of this domain necessary for the Jak family of tyrosine kinases *(16)*.

Here, we demonstrate an interaction between the PRLR and cyclophilin A (Cyp A), an 18-kDa protein with peptidyl prolyl *cis-trans* isomerase activity. Like other members of the cyclophilin family, it binds to CsA *(17)*, resulting in an inhibition of its PPIase activity. In addition, the CsA/CypA

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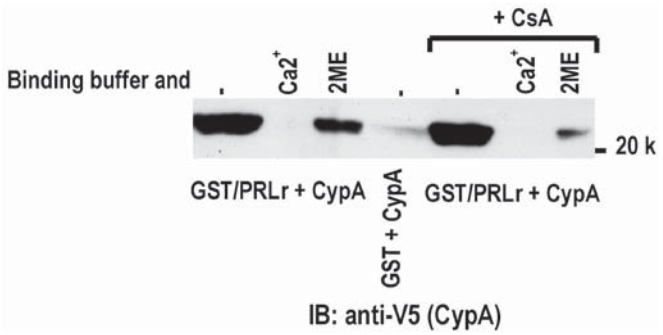


**Fig. 1.** In vitro interaction of CypA and PRLR. Purified epitope-tagged CypA in the presence or absence of 1 n*M* CsA, 5 m*M* $Ca^{++}$, or 1.5 m*M* 2-mercaptoethanol was admixed to a purified chimera of GST and the intracellular domain of the human PRLR (amino acids 235–599) long isoform or GST-only control. Following admixture of these proteins, the mixture was incubated with glutathione-Sepharose. After washing, the beads were subjected to 12% SDS-PAGE and immunoblot analysis was performed with anti-V5 antibody. Given its V5 epitope tag, the CypA used in this experiment migrates to >20 kDa.

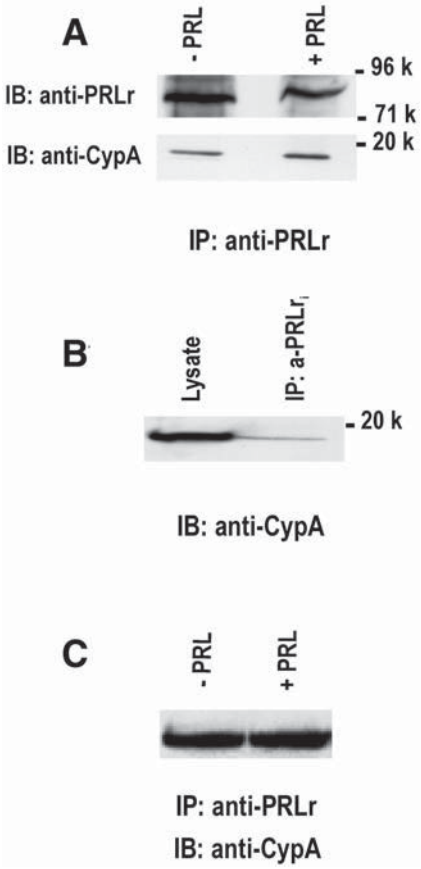


**Fig. 2.** In vivo interaction between CypA and PRLR. (**A**) T47D lysates stimulated with PRL (50 ng/mL) for 0 and 15 min were immunoprecipitated with anti-PRLR antibody. After washing, the immunoprecipitates were subjected to sequential immunoprecipitation and immunoblot analysis with anti-CypA and anti-PRLR antibody. (**B**) Lysates from T47D cells cultured in complete medium were immunoprecipitated with antisera specific to the hPRLR intermediate isoform (Zymed) and subjected to immunoblot analysis with anti-CypA antibody. (**C**) CHO cells transfected with 1 µg of both the long form of the PRLR and CypA were stimulated for 7.5 min with 100 ng/mL of PRL prior to anti-PRLR immunoprecipitation and immunoblot analysis with anti-CypA.

complex binds the cytoplasmic phosphatase calcineurin with high affinity and inhibits the action of this enzyme *(18)* and in turn the interleukin-2 promoter *(19)*. The functional significance of the interaction of CypA with the PRLR was examined by overexpression studies of CypA in the breast epithelial cell line T47D and Chinese hamster ovary (CHO). Those studies revealed an association between CypA and both the PRLR and Jak2. In parallel, overexpression studies utilizing a CypA expression construct resulted in down-regulation of PRL-induced Rac activity, prolongation of Jak2 phosphorylation, and enhanced PRL-induced gene expression.

## Results

### *In Vitro Interaction Between PRLR and CypA*

To investigate the direct interaction between CypA and PRLR, purified epitope-tagged CypA in the presence or absence of 1 n*M* CsA (a concentration of CsA at which it inhibits PPIase activity of CypA *[9]*) was admixed to a purified chimera of glutathione S-transferase (GST) and the intracellular domain of the PRLR (amino acids 235–599). Calcium ion and 2-mercaptoethanol were also included in some binding assays, given the role of calcium ion in activation of downstream targets of the CypA-CsA complex (i.e., calcineurin) and the reduced intracellular environment in which CypA resides. After extensive washing, the Sepharose conjugates from this "pull-down" experiment were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with anti-V5 antibody, as shown in Fig. 1. Additional analysis (not shown) revealed that of the input CypA, approx 90% was bound to the GST-PRLR. These data indicate that CypA can interact directly with the intracellular domain of the PRLR in vitro in the presence or absence of CsA. These findings suggest that the interaction does not involve the PPI pocket in CypA but instead a region of CypA outside of its core PPIase domain. The interaction between CypA and the PRLR was decreased by the inclusion of either divalent cation or reducing agent in the binding buffer, suggesting that calcium influx or alterations in the intracellular redox state may alter the association of CypA with the PRLR.

### *In Vivo Interaction Between PRLR and CypA*

To confirm the in vitro interaction between CypA and the PRLR in vivo, lysates from resting or PRL-stimulated T47D human breast cancer cells were immunoprecipitated with anti-PRLR antibody and subjected to sequential immunoblot analysis with anti-CypA and anti-PRLR antibodies. As shown in Fig. 2A, a constitutive interaction between

CypA and the PRLR in vivo was observed, further confirming the in vitro observation that ligand-induced dimerization is not required for CypA association with the PRLR. Semiquantitive analysis of these data revealed that approx 3% of endogenous CypA coimmunoprecipites with PRLR. To further map the binding region of PRLR and CypA, T47D cell lysates were used to coimmunoprecipitate the human PRLR intermediate isoform and CypA, using a newly developed antisera specific to the unique C-terminus of the intermediate isoform. As shown in Fig. 2B, CypA interacts with the intermediate isoform of PRLR, suggesting that the membrane-proximal domains within the PRLR are sufficient for the interaction with CypA. To confirm that the CypA-PRLR interaction was not unique to T47D cells, lysates from CHO cells transfected with a human PRLR long construct were immunoprecipitated with anti-PRLR antibody. Immunoblot analysis of these lysates also revealed a constitutive association of CypA with the PRLR in the presence or absence of ligand (Fig. 2C).

### *CypA Downregulates Activation of Rac1*

To address the question of the functional significance of CypA interaction with the PRLR, we initially studied the effects of PRL-induced Rac activation. The small GTP-binding protein Rac1 contributes to both cell motility and growth and is activated by Vav. Activated Vav, via its guanine nucleotide exchange factor activity, induces guanosine 5'-diphosphate/guanosine 5'-triphosphate (GDP/GTP) exchange on Rac1, thereby activating it. Activated (GTP-bound) Rac1 binds its downstream effectors such as p21-associated kinase (PAK) *(20)*. To measure activated GTP-Rac1, lysates from T47D cells transiently transfected with either empty vector and green fluorescent protein (GFP) expression construct or the CypA expression construct and GFP expression construct were incubated with GST-PAK and analyzed by anti-Rac1 immunoblot analysis (Fig. 3). Since the PAK kinase associates only with GTP-bound Rac1, GST-PAK/Rac1 precipitates can be used to assess the levels of activated Rac1 *(20)*. This analysis revealed a decrease in activated Rac1 on transfection with the CypA expression construct compared to the empty vector control, over a 30-min time course. These data indicate that CypA decreased and/or delayed PRL-induced Rac activation.

It was possible that the aforementioned effects of CypA were the trivial consequence of either nonphysiologic levels of CypA overexpression or the effect of CypA overexpression inducing decreased PRLR expression on the cell surface. To evaluate these possibilites, T47D transfectants (vector and GFP or CypA and GFP) were stained for cell-surface PRLR expression and examined using immunofluorescence microscopy. We found no difference in the staining of cells for PRLR that were GFP positive or GFP negative and transfected with empty vector or CypA expression construct (data not shown). To confirm that the results obtained with the Rac assays were not the consequence of massive, nonphysiologic overexpression of CypA, immunoblot analysis of the lysates from the transfectants (Fig. 3D) was also performed. These studies revealed that the levels of transfected CypA were only 20% that observed for the endogenous CypA. Thus, the decreased activation of PRL-induced Rac1 by overexpressed CypA did not appear to be the consequence of PRLR cell-surface downregulation or excessive overexpression of CypA.

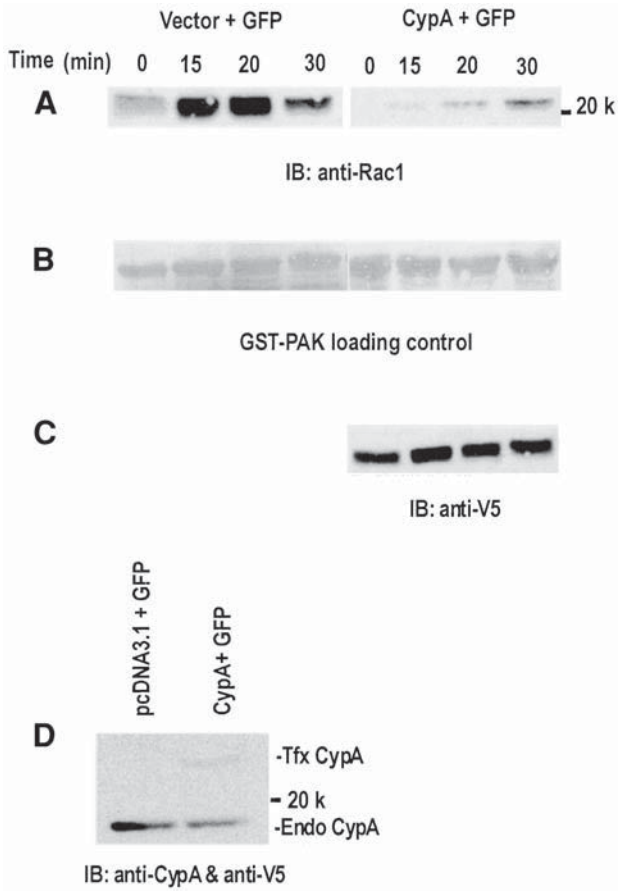


**Fig. 3.** Rac activation assay in T47D transfectants. T47D transfectants expressing GFP and CypA or GFP and control vector were rested in Dulbecco's modified Eagle's medium (DMEM)/ITS+ for 48 h and then stimulated with PRL at 100 ng/mL for the indicated times. The cells were lysed in lysis buffer containing GST-PAK, subjected to electrophoresis, and (**A**) immunoblotted with anti-Rac1 antibody. (**B**) The blots were stained with amido black staining solution to check for equal loading of GST-PAK. (**C**) Lysates from T47D cells transfected with CypA were subjected to electrophoresis and immunoblotted with anti-V5 antibody, to determine equal levels of CypA expression. (**D**) T47D cells transfected with pcDNA3.1 and GFP or expression construct of CypA and GFP were lysed, subjected to electrophoresis, and immunoblotted with anti-CypA and anti-V5 antibody to determine the levels of endogenous CypA and transfected CypA.

### *CypA Interacts with Jak2*

Having examined the effects of CypA overexpression on PRL-induced Rac activation, we wished to evaluate whether CypA could interact with and/or modulate the PRLR-associated Jak2/STAT signaling pathway. Precedent data have

suggested that immunophilins can also modulate the activity of tyrosine kinases *(21)*; however, no study to date has examined the association and/or effect of cyclophilins on Jak family activity. To examine whether there was an interaction between CypA and Jak2, T47D cell lysates were immunoprecipitated with anti-CypA or anti-Jak2 antibody and subjected to immunoblot analysis with anti-Jak2 and anti-CypA, respectively. As seen in Fig. 4B, an association between CypA and Jak2 is evident in both sets of immunoprecipitates. Notably, CypA appears capable of interacting with each of three species detected by anti-Jak2 immunoblot analysis.

### *CypA Prolongs Jak2 Phosphorylation and Enhances β-Casein Expression*

Jak2 is a protein tyrosine kinase known to be activated via autophosphorylation on ligand stimulation of the PRLR and is necessary for PRL-induced STAT activation *(22)*. To examine the effect of CypA levels on Jak2 activation, we overexpressed the CypA expression construct in CHO transfectants and examined the phosphorylation status of immunoprecipitated Jak2 after PRL stimulation from lysates by antiphosphotyrosine immunoblot analysis. CHO cells were used for this study because of their ease of transfection, enabling gene reporter assays. As seen in Fig. 4A, PRL-induced Jak2 phosphorylation in the CypA-CHO transfectants peaked at 7.5 min but was still appreciable at 15 min. By contrast, PRL stimulation of control transfectants resulted in a peak in Jak2 phosphorylation at 7.5 min with little appreciable phosphorylation at 15 min. Additional studies (not shown) extending this temporal analysis have revealed appreciable Jak2 activation in CypA transfectants, but not control transfectants, out to 30 min following PRL stimulation. As such, these data indicate that CypA potentiates the phosphorylation of Jak2, and possibly its activity.

To test whether the proximal effects of CypA on Jak2 and Rac activation had downstream effects, CHO cells were transfected with a luciferase reporter construct containing the β-casein promoter and *Renilla* control vector along with expression constructs for both the PRLR long isoform and CypA (or control vector). These transfectants were then assessed for their expression of the β-casein reporter, in the presence or absence of PRL, by dual color luminometry (Fig. 4C). We found a highly significant activation of the β-casein promoter by CypA overexpression in both basal and PRL-stimulated conditions.

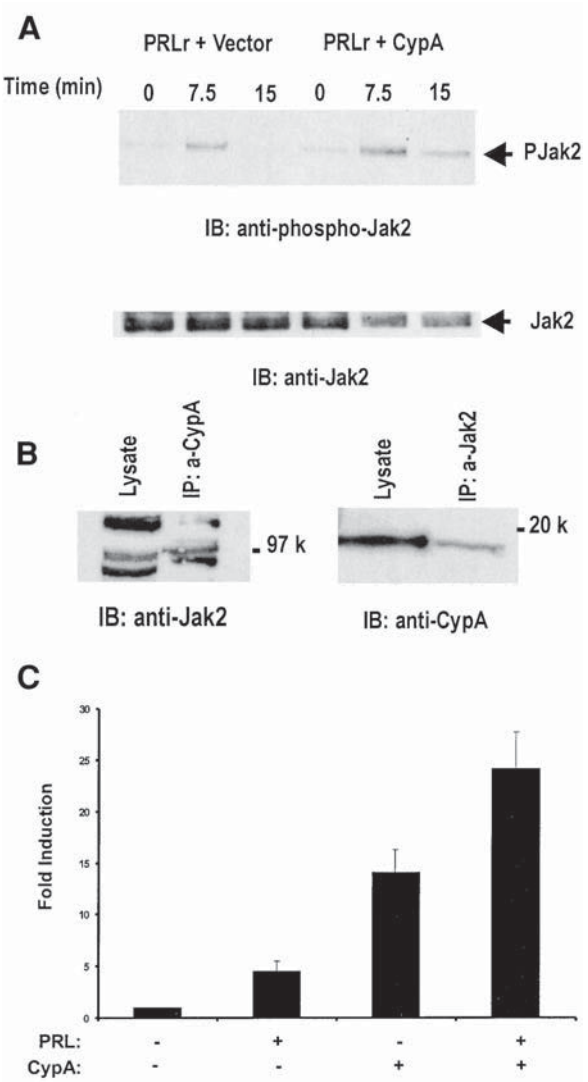


**Fig. 4.** Association and activation of Jak2 by CypA. (**A**) Jak2 activation is prolonged in the presence of CypA. CHO cells expressing human PRLR long isoform and control vector or PRLR long isoform and CypA were rested in DMEM + 0.1% bovine serum albumin (BSA) before stimulating with PRL at 100 ng/mL for the indicated times. Lysates were immunoprecipitated with anti-Jak2 antibody and subjected to immunoblot analysis with either anti-phospho-Jak2 antibody or anti-Jak2 antibody. (**B**) In vivo interaction between CypA and Jak2. T47D lysates were immunoprecipitated with anti-CypA or anti-Jak2 antibody. The immunoprecipitates were subjected to immunoblot analysis with anti-Jak2 or anti-CypA antibody. (**C**) Overexpression of CypA enhances expression from a β-casein reporter. CHO cells were transfected as described in Materials and Methods. Following transfection, the cells were washed, placed in defined medium, and stimulated for 24 h with 100 ng/mL of PRL. Total cell lysates were analyzed for luciferase activity and normalized to *Renilla* activity. Data are representative of one of four experiments. Error bars represent SEM. Demonstrated values are significant for all experimental pairings ($p < 0.05$).

## Discussion

Our data have demonstrated that CypA in vitro and in vivo is capable of interacting with human PRLR (see Figs. 1 and 2). Because the presence of CsA did not alter the interaction of CypA with the PRLR, these findings would indicate that the PPI pocket of CypA, blocked by CsA binding, is distinct from the region of CypA that interacts with the PRLR. This is not uncommon for cyclophilins; for example, CypB binds PRL via its C-terminus *(12)*. Given that CypA interacts with both the long and intermediate hPRLR isoforms (Fig. 2), these findings would suggest that the inter-

action motif for CypA resides between amino acids 237 and 312, a region common to both receptors, that encodes for the box 1/variable box/box 2 motif.

Our data indicate that CypA engages the PRLR constitutively in both T47D and transfected CHO cells and that overexpression of CypA in T47D cells resulted in a decrease in and/or delay of Rac activation (see Fig. 3). By contrast, overexpression of CypA in CHO cells resulted in an increased duration of Jak2 phosphorylation (see Fig. 4). In this context, CypA may be acting as a chaperone or a structural switch for the PRLR, altering structures necessary for PRLR transduction via prolyl isomerization. These necessary structures could represent a motif intrinsic to the PRLR such as the box 1 motif or a proline-rich motif found in PRLR-associated signaling proteins, or both.

Recent in vitro data have suggested that a PPI could significantly modify PRLR intracellular domain structure and thus function by regulation of the conformation of the proline-rich box 1 motif of the PRLR *(15)*. Nuclear magnetic resonance spectroscopic analysis of a peptide corresponding to this motif (box 1 amino acid sequence: IFPPVPGP) revealed that proline imide bonds in this motif assume preferred conformations. Most strikingly, the third proline residue of the box 1 motif was found in a predominantly *cis* position and underwent *cis-trans* isomerization slowly in the absence of PPI activity. By contrast, the remaining prolines in the box 1 were largely in the *trans* conformation and underwent rapid *cis-trans* isomerization. This suggests that the box 1 motif, which is necessary but not sufficient for PRLR-driven signaling and proliferation *(23,24)*, could modulate PRLR function by alterating of its inherent structure. Indeed, *cis-trans* isomerization of the third proline residue in the box 1 motif alters its conformation from a relatively linear strand into a "pseudo-cyclic" conformation *(15)*. Additionally, mutation of the corresponding proline residue in the box 1 motif of the GH receptor resulted in a receptor incapable of ligand-induced signaling and proliferation *(25)*. By contrast, a single publication has suggested that the C-terminal (fourth) proline of the box 1 motif may be necessary for Jak2 activation *(23)*. However, this observation was obtained using a highly nonconserved replacement of the fourth proline residue (i.e., a leucine-replacing proline) that may have induced significant and untoward structural changes. Nevertheless, when taken together, these observations suggest an important role for the proline residues in the box 1 motif, a domain that is highly conserved across the cytokine receptor superfamily.

The actions of CypA during PRLR signaling may also directly involve the signaling proteins associated with this receptor. Our findings indicate that CypA is able to bind with three different species reacting with anti-Jak2 antibodies (see Fig. 4B). Of note, we have observed the presence of multiple Jak2 bands with several different commercially obtained anti-Jak2 antibodies. Given the abundance of these proteins, and our frequent observation of ligand-induced tyrosine phosphorylation of the band migrating at 100 kDa, we believe that these species may represent alternatively processed and possibly functional forms of Jak2. The association between CypA and Jak2 in theory could contribute to the prolongation of Jak2 activation noted in CypA transfectants, although it is clear that in the absence of ligand, CypA does not increase the basal activity of Jak2 (Fig. 4A). Interestingly, the effect of CypA on Jak2 activation is entirely different from the effect of CypA on the member of the Tec tyrosine kinase family, Itk *(21)*, in which CypA appears to inhibit Itk activity.

It is also probable that CypA interacts with other components of the PRLR signaling network, given the significant increase in the expression from a transfected β-casein promoter luciferase construct noted in the presence and absence of ligand (Fig. 4C). We speculate that the modest increase in CypA obtained in the transfectants (see Fig. 3D) is capable of potentiating the low level of Jak2 activity observed in unstimulated cells over time, resulting in enhanced expression of this reporter. Whether this is the result of a reconfiguration of the PRLR complex, or the modulation of Stat regulatory proteins, such as phosphatases or suppressor of cytokine signaling-2/cytokine-inducible src homology-2–containing proteins, remains to be determined. As a whole, these observations reveal a previously unrecognized role for the association and function of CypA with respect to PRLR signal transduction.

## Materials and Methods

### *Cell Culture and Transfection*

Recombinant human PRL from *Escherichia coli* number AFP795 was obtained through National Hormone and Pituitary Program (NHPP), National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and Dr. A. F. Parlow. T47D cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin as previously documented *(6)*. When appropriate, T47D cells were rested in medium consisting of DMEM (Invitrogen) supplemented with sodium selenide, linoleic acid, insulin, and transferrin (ITS+; BD Biosciences). T47D cells ($5 \times 10^5$) were transfected with 1 μg each of pcDNA3.1 CypA-V5HisA or pcDNA3.1V5HisA and the vector encoding for GFP (pEGFP-N1); a gift from Dr. Nina Luning Prak, used as a transfection efficiency control) using lipofectamine (Invitrogen) as instructed. Briefly, 1 μg of pcDNA3.1 CypA V5HisA or pcDNA3.1V5HisA and 1 μg of pEGFP-N1 were complexed with the plus reagent and lipofectamine at room temperature. The mixture was added dropwise and added to the T47D cells in 800 μL of Optimem (Invitrogen). The cells were incubated for 3 h before adding 1 mL of 10% DMEM complete medium.

### *Immunoblot Analysis*

After harvesting, T47D cells were lysed in NP40 lysis buffer (10 m*M* Tris, pH 7.6; 150 m*M* NaCl; and 1% NP40).

Lysates were subjected to SDS-PAGE and transferred to polyvinyl difluoride (PVDF) membrane. Antigens were labeled with a 1:1000 dilution of rabbit anti-PRLR anti-serum developed by our laboratory *(6)* or anti-CypA (UBI) or anti-Rac1 (UBI) followed by a 1:1000 dilution of the antimouse or antirabbit horseradish peroxidase (HRP)–conjugated secondary antibodies (Sigma, St. Louis, MO). Anti-V5 antibody (Invitrogen) was purchased as an HRP conjugate and therefore did not require the use of a secondary antibody. Nonspecific interaction was blocked with 5% milk in phosphate-buffered saline (PBS)/Tween-20. Antigen-antibody complexes were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech) and exposure to Biomax film (Kodak).

### *Protein Expression and Purification*

V5HisA-tagged CypA was expressed in *Drosophila* S2 cells and purified using the TALON metal affinity resin (Clontech) per the manufacturer's instructions. Briefly, $2 \times 10^8$ cells were lysed in lysis buffer (50 m*M* Tris, pH 7.8; 150 m*M* NaCl; 1% NP40). Lysates were incubated with 200 μL of TALON metal affinity resin for 30 min at room temperature. The resin was washed three times with wash buffer (20 m*M* Tris-HCl, pH 8.0; 100 m*M* NaCl; 100 m*M* imidazole). The protein was eluted and dialyzed overnight against 5 m*M* HEPES, pH 8.0, at 4ºC. Protein concentration was estimated by spectrophotometry.

GST-PRLR chimeras were expressed using the GST expression system (Pharmacia Biotech) per the manufacturer's instructions. Briefly, 100-mL cultures of *E. coli* transformants were grown to midlog phase and induced with 0.1 m*M* isopropyl β-D-thiogalactoside for 4 h. Cells were pelleted, resuspended in lysis buffer, sonicated, and cleared of bacterial debris by centrifugation. Glutathione Sepharose beads (500 μL) were added to the supernatant and incubated at room temperature for 30 min. The beads were washed with PBS and incubated with glutathione elution buffer for 30 min at room temperature. The beads were then centrifuged to elute the protein.

### *In Vitro Binding Assay*

Ten nanograms of CypA (purified, V5 tagged) in the presence or absence of 1 n*M* CsA (a gift from Dr. Les Shaw) was admixed with 10 ng each of GST or GST-PRLR (amino acids 235–599) in binding assay buffer (10 m*M* Tris, pH 7.6; 125 m*M* NaCl; 10% glycerol) under various binding conditions (5 m*M* $CaCl_2$ or 1.5 m*M* 2-mercaptoethanol) in a final volume of 200 μL. The mixture was incubated at room temperature for 4 h before adding 20 μL of glutathione Sepharose bead slurry and incubated at room temperature for 20 min. The beads were washed with PBS and subjected to electrophoresis. The proteins were transferred to PVDF membrane and immunoblotted with anti-V5 antibody.

### *Immunoprecipitation*

T47D cells ($1 \times 10^6$) were rested overnight in DMEM/ITS+ and left unstimulated or stimulated with PRL at 50 ng/mL for 15 min and lysed and immunoprecipitated overnight as described previously *(26)* using 5 μL of anti-PRLR antibody. Antigen-antibody complexes were isolated by the addition of 50 μL of protein A agarose beads (Invitrogen). The immunoprecipitates were washed three times with lysis buffer, boiled in Laemmli buffer containing SDS and 2-mercaptoethanol *(4)*, subjected to 12% SDS-PAGE, and sequentially immunoblotted with anti-PRLR and anti-CypA antibodies.

Five microliters of anti-hPRLR intermediate antiserum (Zymed) was used to immunoprecipitate the intermediate isoform of the PRLR from $1 \times 10^6$ T47D cells. The antigen-antibody complexes were isolated using protein A agarose beads, washed, boiled, and subjected to 12% SDS-PAGE and immunoblotted with anti-CypA. The antisera used is specific to the intermediate hPRLR isoform since it is directed against the unique 13 amino acid sequence found only in the C-terminal tail of this isoform.

### *GTPase Assay*

T47D cells transiently transfected with pcDNA3.1 CypA V5HisA and pEGFP-N1 or pcDNA3.1V5HisA and pEGFP-N1 were rested for 48 h in DMEM/ITS+. Cells were stimulated with 100 ng/mL of PRL for 0, 15, 20, and 30 min and lysed in buffer containing 20 m*M* Tris (pH 7.4), 150 m*M* NaCl, 5 m*M* $MgCl_2$, 0.5% NP40, 5 m*M* β-glycerophosphate, protease inhibitors, and 25 μg of GST-PAK, obtained as previously described *(20)*. Lysates were incubated with 50 μL of glutathione Sepharose beads (Amersham Pharmacia Biotech) for 30 min at 4ºC. The beads were washed three times in lysis buffer, boiled in Laemmli buffer, subjected to 12% SDS-PAGE, and immunoblotted with anti-Rac1 antibody (UBI). The blots were stained with amido black staining solution for 1 min (Sigma) followed by destaining with 25% isopropanol/10% acetic acid for 30 min to check for equal precipitation of GST-PAK in samples.

### *Immunofluorescence Microscopy*

T47D cells ($10^5$) were seeded onto Chamber Slides (Nalge Nunc) and transfected with pcDNA3.1V5HisA and pEGFP-N1 or pcDNA3.1V5HisA and pEGFP-N1 using lipofectamine as previously described. Forty-eight hours posttransfection, the transfectants were washed with PBS at 4ºC and stained with anti-PRLR antiserum developed by our laboratory (1:200) in PBSA (1 mg/mL of BSA and 0.1% sodium azide) for 1 h at 4ºC. After washing three times with PBS, the cells were incubated for 45 min with a 1:200 dilution of Texas Red–conjugated donkey anti–rabbit secondary antibody in PBSA (a gift from Dr. Judy Meinkoth). The cells were washed three times with PBS and fixed in PBS 4% paraformaldehyde for 15 min at room temperature. After

adding Vectashield mounting solution (Vector) immunofluorescence was visualized using a Leica DM IL Immunofluorescence Microscope (Leica Microsystems).

### *Jak2 Phosphorylation and β-Casein Promoter Activation*

CHO cells ($2 \times 10^5$) were transfected with 1 μg each of pEF1 PRLR long V5HisA and pcDNA3.1V5HisA or pEF1 PRLR long V5HisA and pcDNA3.1 CypA V5HisA using Fugene 6 (Roche) per the manufacturer's instructions. Twenty-four hours posttransfection, the cells were rested in DMEM + 0.1% BSA for 24 h before stimulating with PRL at 100 ng/mL for 0, 7.5, and 15 min. The cells were harvested, lysed, and immunoprecipitated overnight using 5 μL of anti-Jak2 antibody (Santa Cruz Biotechnology) complexed with 50 μL of protein A agarose beads. The immunoprecipitates were washed, boiled in Laemmli buffer, subjected to 8% PAGE, and immunoblotted with anti-phospho-Jak2 (UBI). The blot was stripped and immunoblotted with anti-Jak2 (Santa Cruz Biotechnology) to check for equal loading. Dual luciferase assays following transfection with the aforementioned expression constructs along with the reporter constructs LHRE-TK-luc (containing the Stat5 DNA-binding sites of β-casein) and pCMV-Renilla were performed as previously described *(13)*.

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