Threonine 391 Phosphorylation of the Human Prolactin Receptor Mediates a Novel Interaction with 14-3-3 Proteins*§

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The prolactin receptor (PrlR) is a member of the cytokine receptor superfamily that lacks an intrinsic kinase domain and relies on the cytoplasmic Jak tyrosine kinases to transduce signals. Prolactin-induced Jak2 activation and consequent tyrosine phosphorylation of the receptor and downstream signaling molecules have been studied, but phosphorylation of the PrlR on serine or threonine residues has not been reported. Here we describe a novel interaction between the PrlR and the phosphoserine/phosphothreonine-binding 14-3-3 proteins. This association is mediated by the KCST391WP motif, which occurs in the major functional isoform of the human receptor and is conserved among a wide variety of species. Mutagenesis of threonine 391 to alanine significantly impaired 14-3-3 binding to the PrlR in a variety of species. Mutagenesis of threonine 391 to alanine greatly impaired 14-3-3 binding to the PrlR in both Vero and CHO cell lines. The T391A mutant exhibited increased basal and prolactin-induced tyrosine phosphorylation compared with the wild-type receptor and downstream signaling molecules. Interestingly, the T391A mutant exhibited increased basal and prolactin-induced tyrosine phosphorylation compared with the wild-type receptor. This was accompanied by a ligand-induced increase in protein kinase B and Erk activation but not of Stat5a. Phosphorylation of the receptor on Thr391 may therefore provide a new mechanism by which prolactin signaling is attenuated.

The prolactin receptor (PrlR) belongs to the superfamily of cytokine receptors and is expressed in most adult tissues. Its ligand, prolactin, is secreted by the anterior pituitary gland but is also produced by extrapituitary sources, including the mammary gland and lymphoid cells. Prolactin is essential for the development and maturation of the mammary gland as well as for modulating the immune response. Prolactin-deficient mice are infertile because of a complete failure of blastocysts to implant, whereas heterozygous mice display impaired mammary gland development and fail to lactate. Prolactin binding induces receptor dimerization, leading to auto-activation of associated Jak2 kinases. Jak2 then phosphorylates the receptor on tyrosine residues, thereby creating docking sites for various SH2 domain-containing cytoplasmic signaling molecules, the best studied of which are the signal transducer and activator of transcription (Stat) factors. Stat proteins are subsequently phosphorylated by Jak2, resulting in Stat dimerization and translocation to the nucleus where they bind to their target promoters. In addition to the Jak-Stat pathway, the PrlR engages other cytoplasmic kinases such as Src, Fyn, and Tec (7–9) and downstream effectors that include mitogen-activated protein kinase (MAPK) (10, 11) and phosphatidylinositol 3-kinase (PI3K) (12, 13).

Several distinct PrlR isoforms are generated by alternative mRNA splicing in a species-specific manner. The long form of the PrlR is the major functional isoform. In humans, an intermediate and several short isoforms have been characterized that share the same extracellular ligand binding and transmembrane domains as the long PrlR but vary in the length of their cytoplasmic tails (14–16). In addition, a AS1 PrlR variant that lacks part of the extracellular domain, resulting in a lower ligand binding affinity, has been described (17). Functional analyses have demonstrated a marked reduction in the ability of the intermediate PrlR to transduce signals and mediate prolactin-induced proliferation compared with the long form (14), whereas the short isoforms appear to act in a dominant negative manner when coexpressed with the full-length receptor (15, 16).

Although tyrosine phosphorylation of the PrlR has been well studied, phosphorylation of this receptor on serine or threonine residues has not been described. Here we report a novel threonine phosphorylation site that is specific to the long form of the PrlR and mediates binding to 14-3-3 proteins. These proteins are small acidic, ubiquitous proteins that recognize serine/threonine-phosphorylated residues in a context-specific manner (18, 19). In mammals, seven highly homologous family members have been described that bind to many different types of proteins, including cell cycle regulators, transcription factors, and proteins involved in signaling and apoptosis (20, 21).

In this study we provide evidence that threonine 391 phosphorylation of the human PrlR and accompanying 14-3-3 binding to this site may be involved in receptor desensitization, specifically affecting signaling to the MAPK and PI3K pathways. Furthermore, we demonstrate that okadaic acid stabilizes...
Thr\textsuperscript{391} phosphorylation, suggesting that phosphorylation of this site is regulated by a protein phosphatase 2A (PP2A)-like activity.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—PrlR cDNA in a pEF1A/V5-His vector containing carboxy-terminal V5 and His tags (Invitrogen) was generously provided by J. Cleverenger (9). Stat5a in pEF1A-Bos and HA-tagged PKB in pcDNA3 were gifts from W. Alexander and J. Testa, respectively. HA-tagged Erk2 in pcDNA3, Gal4-Sap1a, and GSE4-lux were kindly provided by Y. Nagamine. EE-tagged 14-3-3 ζ and γ CDNAs in pEF1A were a gift from A. Villunger. Site-directed mutagenesis was performed using the QuickChange kit (Stratagene) according to the manufacturer's instructions.

**Antibodies**—PrlR-specific polyclonal rabbit antiserum was kindly provided by J. Cleverenger. Commercially available antibodies used were: V5-specific mouse monoclonal antibody (Invitrogen), Glu-Glu-specific mouse monoclonal antibody (Babco), 14-3-3-specific rabbit polyclonal antibody (Upstate Biotechnology), phosphorytrosine-specific mouse monoclonal antibody (clone 4G10) (Upstate Biotechnology), anti-phospho-Erk1/2 (T202/Y204) rabbit polyclonal antibody (New England Biolabs), anti-phospho-PKB (T38) rabbit polyclonal antibody (New England Biolabs), HA-specific rat monoclonal antibody (Roche Applied Science), anti-phospho-Stat5a (Y694/Y699) mouse monoclonal antibody (Upstate Biotechnology), anti-Stat5a rabbit antiserum (Upstate Biotechnology).

**Generation of Anti-phospho-Thr\textsuperscript{391} PrlR Antibody**—The anti-phospho-Thr\textsuperscript{391} PrlR antibody was raised by immunizing New Zealand White rabbits with the GSKCS(pT)WPLP peptide conjugated to keyhole limpet hemocyanin. The antibody was first affinity purified with the immobilized peptide conjugated to Sepharose, and then non-phospho-specific antibodies were removed by adsorption to the non-phosphorylated peptide conjugated to Sepharose. The specificity of the affinity-purified anti-phospho-Thr\textsuperscript{391} PrlR antibody was verified by dot immunoblot against the immunizing phosphorylated peptide, the corresponding non-phosphorylated peptide, and an irrelevant phosphopeptide (CLG-XXX-XCLG). The peptides were synthesized by Mimotopes Pty. Ltd. (Clayton, Victoria, Australia).  

**Cell Culture**—SKBR3 and T47D cells were maintained in RPMI containing 10% fetal bovine serum (CSL) and 1 μg/ml insulin (Sigma). Cells were starved overnight in serum-free medium prior to stimulation with 1 μg/ml prolactin (kindly provided by G. Parlow). HC11 cells were grown in RPMI supplemented with 10% fetal bovine serum, 10 ng/ml EGF (Sigma), and 5 μg/ml insulin. For in vitro differentiation, cells were grown to confluence and maintained for 3 days, after which they were incubated in growth medium lacking EGF for 24 h prior to stimulation with 5 μg/ml prolactin and 1 μM dexamethasone (Sigma). 293T and NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. For transient transfections, 293T cells were transfected with FuGENE (Roche Applied Science) according to the manufacturer’s instructions. NIH3T3 and HC11 pools stably expressing wild-type and T391A PrlR were generated by transfection followed by selection in 0.75 and 0.2 μg/ml neomycin, respectively. Treatment of cells with okadaic acid (ICN Biomedicals) was at 1 μM.

**Protein Extraction of Cells**—Whole cell extracts were obtained by solubilizing cells in NEB (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 20 mM β-glycerophosphate plus complete (Roche Applied Science) protease inhibitors). Lysates were clarified by centrifugation at 16,000 × g for 10 min.

**Bacterial Expression of GST-14-3-3ζ**—An EcoRI-BamHI1-cDNA encompassing the coding region of 14-3-3ζ was cloned into pGEX-2T. E299 bacteria transformed with this plasmid were used to produce a glutathione S-transferase 14-3-3ζ fusion protein (GST-14-3-3ζ). An overnight culture was used to inoculate a 500-ml culture that was grown to an optical density of 0.8 at 600 nm. GST-14-3-3ζ protein expression was then induced with 0.1 μM isopropyl-β-D-thiogalactopyranoside for 3 h. Following induction, the bacterial cultures were harvested, and the pellets were washed with ice-cold phosphate-buffered saline (150 mM NaCl, 16 mM NaH2PO4, 4 mM NaH2PO4, pH 7.3) and resuspended in 10 ml of phosphate-buffered saline containing 1% Triton X-100, 5 mM EDTA, 1% aprotinin, 1 mM phenylmethanesulfonyl fluoride, and 1 μg/ml soybean trypsin inhibitor. The suspension was then sonicated three times for 10 s on ice and the lysate centrifuged for 10 min at 8,000 × g. Purification of GST-14-3-3ζ was performed by mixing with glutathione resin (Sigma) for 1 h at 4 °C. The resin was washed three times with phosphate-buffered saline. The purity and amount of bound GST-14-3-3ζ was then determined by SDS-PAGE and Coomassie staining.

**GST Pulldowns, Immunoprecipitations, and Western Blotting**—Pull-downs were performed by incubating protein lysates with GST or GST-14-3-3ζ coupled to glutathione beads for 2 h on ice. Beads were washed three times with NEB (see above). For immunoprecipitations, equal amounts of protein were incubated with specific antibodies for 2 h on ice. Immune complexes were collected by protein G-Sepharose (Amersham Biosciences) and washed three times with NEB. Precipitated proteins were released by boiling in sample buffer and were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using 4–20% gradient gels (Novex). The proteins were blotted onto polyvinylidene difluoride membranes (Millipore). After blocking with 20% horse serum (Horseradish peroxidase solution containing 0.1% Tween 20), filters were probed with specific antibodies. Proteins were visualized with peroxidase-coupled secondary antibody using the ECL detection system (Amersham Biosciences). Stripping of membranes was performed in SDS buffer (62.5 mM Tris, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) for 30 min at 60 °C. Membranes were then reprobed with the indicated antibodies.

**Luciferase Reporter Assays**—293T cells were grown in 6-well dishes and transfected with 0.25 μg each of wild-type PrlR or T391A PrlR and Gal4-Sap1a expression plasmids and the GSE4-luciferase reporter construct (22). pRL-TK, a Renilla luciferase plasmid under the control of the thymidine kinase promoter (50 ng/well) was cotransfected as an internal control. 24 h post-transfection, cells were either treated with 1 μg/ml prolactin or left untreated for 2 and 4 h. Luciferase activities were measured using the dual-luciferase reporter assay system (Promega).

**RESULTS**

A Conserved Motif within the Prolactin Receptor Mediates Interaction with 14-3-3 Proteins—Binding of 14-3-3 proteins to target proteins is generally mediated through RXXPSTXP or RXXPSTXP sequences in which pS/T is phosphorylated (18, 19). We noted the presence of a similar motif in the long form of the human PrlR (20, 21), which was found to be highly conserved among a wide variety of species (Fig. 1A). To test for a possible interaction between the receptor and 14-3-3 proteins, we transiently expressed the human PrlR in 293T cells, prepared whole cell lysates, and performed pulldown experiments using GST-14-3-3-392-395 and T391A PrlR. We initially selected the ζ isoform based on its ability to interact with the granulocyte-macrophage colony-stimulating factor receptor (23) and its abundant expression in various tissues, including the mammary gland (data not shown). Interestingly, the PrlR associated with GST-14-3-3ζ but not with GST alone (Fig. 1B). This interaction was further verified by coimmunoprecipitation of the PrlR with both the 14-3-3ζ and γ isoforms (Fig. 1C). The 14-3-3ζ isoform, whose expression is restricted to epithelial cells, was also found to associate with the PrlR (data not shown). Mutation of the central three amino acids within the putative 14-3-3 binding motif (GST\textsuperscript{391} to AAA) of the PrlR disrupted its association with GST-14-3-3ζ, demonstrating that the interaction was mediated via this consensus site (Fig. 1B).

In most cases, 14-3-3 proteins associate with target proteins in a phosphorylation-dependent manner. To explore whether Thr\textsuperscript{391} represented a novel phosphorylation site within the human PrlR and was essential for interaction with 14-3-3 proteins, we carried out binding studies with a mutant in which Thr\textsuperscript{391} was altered to alanine. The interaction between this mutant with GST-14-3-3ζ (Fig. 1B) and its coimmunoprecipitation with 14-3-3ζ and γ isoforms (Fig. 1C) was markedly reduced. Conversely, a S390A PrlR mutant still associated with 14-3-3ζ (data not shown). These results demonstrate an absolute requirement for Thr\textsuperscript{391} in the PrlR in vivo, we generated a polyclonal antibody that specifically recognized a phosphopeptide encompass-
CONSENSUS RSxTxP
human KSxSTW
mouse KSxTWP
rat KSxSTW
bovine KSxSTW
reindeer KSxSTW
sheep KSxSTW
rabbit KSxSTW
possum KSxSTW
chicken KSxSTW
xenopus KSxNMP

| GST | GST | GST | GST | GST |
|-----|-----|-----|-----|-----|
| WT  | A   | A   | A   | A   |
| T/A | T/A | T/A | T/A | T/A |

Fig. 1. Phosphorylation of the PrlR on Thr<sup>391</sup> mediates interaction with 14-3-3. A, sequence alignment of the PrlR from various species demonstrates conservation of a putative 14-3-3 binding motif. Phosphorylation of the threonine is predicted to be required for recognition. B, V5-tagged human wild-type (WT) and mutant PrlR<sup>391</sup> to AAA and Thr<sup>391</sup> to Ala) expression vectors were transiently transfected into 293T cells. Lysates were incubated with glutathione beads coupled to GST-14-3-3 or GST alone, and bound proteins were separated by SDS-PAGE. The PrlR was detected by Western blotting using a V5-specific antibody (left panel). The expression and levels of the PrlR constructs were verified by immunoblotting of whole cell lysates with V5-specific antibody (right panel). C, V5-tagged WT and T391A PrlR expression vectors were transiently transfected into 293T cells along with EE-tagged 14-3-3. WT and 14-3-3 isoforms were immunoprecipitated using EE-specific antibody and protein complexes immunoblotted with a PrlR-specific polyclonal antiserum (top panel). The membrane was reprobed with EE-specific antibody to verify 14-3-3 expression and immunoprecipitation (middle panel). Expression of the PrlR was verified by immunoblotting of whole cell lysates with V5-specific antibody (lower panel).

Fig. 2. In vivo phosphorylation of Thr<sup>391</sup> and 14-3-3 binding are stabilized by okadaic acid. A, whole cell extracts from transiently transfected 293T and from NIH3T3 and HC11 cells that stably express V5-tagged WT and T391A PrlR were immunoprecipitated with V5-specific antibody. HC11 cells were grown in proliferation medium containing EGF (+), or EGF was withdrawn for 24 h (-) prior to lysis. The receptor was resolved by SDS-PAGE and immunoblotted with a phospho-Thr<sup>391</sup>-PrlR-specific antibody (top panels). Immunoprecipitates from 293T cells were also immunoblotted with 14-3-3-specific antibody (middle panel, left). Membranes were reprobed with V5-specific antibody to confirm expression of the PrlR (bottom panels). B, NIH3T3 cells stably expressing V5-tagged WT PrlR were treated with 1 μM okadaic acid (OA) in MeSO for 1, 2, and 4 h. The PrlR was immunoprecipitated with V5-specific antibody, resolved by SDS-PAGE, and immunoblotted with phospho-Thr<sup>391</sup>-PrlR-specific (top panel) and 14-3-3-specific antibodies (middle panel). The membrane was reprobed with V5-specific antibody to confirm equal loading (bottom panel). MeSO alone had no effect on Thr<sup>391</sup> phosphorylation.

Thr<sup>391</sup> PrlR Phosphorylation Attenuates Signaling

Increased Tyrosine Phosphorylation of the T391A PrlR—Binding of prolactin to the PrlR activates Jak2 kinase, which in turn phosphorylates the receptor on tyrosine residues to generate docking sites for downstream signaling molecules. To investigate whether Thr<sup>391</sup> phosphorylation influenced activa-
Enhanced Signaling of PrlR T391A to the MAPK and PI3K Signaling Pathways—Stat transcription factors are one of the main targets of prolactin signaling. We therefore analyzed how the phosphorylation site mutant affects activation of Stat5 by cotransfecting 293T cells with expression vectors encoding Stat5a and either wild-type or mutant PrlR, followed by immunoblotting of lysates with a phospho-specific Stat5 antibody. Despite increased tyrosine phosphorylation of the T391A mutant (Fig. 4), no increase in the level of activated Stat5a was observed in response to prolactin (Fig. 5A). Even when the concentration of prolactin was titrated to ensure that signaling to Stat5a was not saturated, activation of Stat5a by the wild-type and T391A mutant PrlR were comparable after 30 min of prolactin treatment (Fig. 5B). However, a small but reproducible increase in basal Stat5a phosphorylation was evident prior to treatment with prolactin (Fig. 5, A and B). Comparable findings were made in two independent reporter assays using the Stat5-responsive β-casein promoter linked to a luciferase reporter. Although basal reporter activity was 1.5-fold greater for the T391A PrlR compared with wild-type PrlR, Thr391 phosphorylation did not influence Stat5a signaling following prolactin treatment (data not shown).

The PrlR has been reported to stimulate the PI3K and MAPK signal transduction pathways in addition to Stat transcription factors. To explore the activation of these pathways, we cotransfected PKB (also known as Akt) or Erk2 expression plasmids together with those encoding the wild-type or mutant PrlR and analyzed lysates from prolactin-stimulated cells by immunoblotting with phospho-specific antibodies that recognize activated PKB or Erk. Interestingly, PKB activation appeared to be prolonged following treatment with prolactin (Fig. 5C), whereas enhanced activation of Erk2 was observed by the mutant PrlR relative to that of the wild-type receptor (Fig. 5D).

To evaluate the increase in signaling at the transcriptional level, we employed a reporter assay dependent on endogenous Erk activity. Cells were transfected with a Gal4-Sap1a fusion protein in which the DNA-binding domain of Sap1a has been replaced by the DNA-binding domain of the yeast transcription factor Gal4. Upon phosphorylation of Sap1a by activated Erk, but not by related MAP kinases such as p38 or c-Jun NH2-terminal kinase (24), Gal4-Sap1 binds to the Gal4 binding sites in the promoter of G5E4-lux luciferase reporter (22). In agreement with the kinetics of Erk activation shown in Fig. 5D, whereas enhanced activation of Erk2 was observed by the mutant PrlR relative to that of the wild-type receptor (Fig. 5D).

The PrlR is one of the main targets of prolactin signaling. We therefore analyzed how the phosphorylation site mutant affects activation of Stat5 by...
MAPK and PI3K pathways, thereby affecting the magnitude of transcriptional responses.

**DISCUSSION**

In this study we identify a novel phosphorylation site within the human PrlR on Thr\(^{391}\) that mediates interaction with 14-3-3 proteins. This family of ubiquitously expressed proteins associates with a plethora of molecules involved in critical cellular processes such as the cell cycle and apoptosis. 14-3-3 binding to target proteins often regulates their subcellular localization but is also known to induce conformational changes affecting enzymatic activity or serve as a scaffold protein (20, 21). We have recently established that 14-3-3 proteins bind other members of the cytokine receptor superfamily, whereby the common β chain of the interleukin 3, interleukin 5 and granulocyte-macrophage colony-stimulating factor receptors was found to interact with 14-3-3ζ. Ligand stimulation promoted β chain phosphorylation on Ser\(^{585}\) and consequent asso-
cation with 14-3-3 (23), bridging the receptor to the p85 subunit of PI3K, and providing a critical survival signal for hemopoietic cells (25).

Phosphorylation of Thr\textsuperscript{391} in the PrlR appears to provide a negative regulatory mechanism by which to down-regulate receptor activity. The Thr\textsuperscript{391} phosphorylation mutant displayed increased basal and ligand-induced tyrosine phosphorylation. Moreover, this was found to selectively enhance signaling along the MAPK and PI3K pathways but did not affect activation of the Stat5a transcription factor by prolactin. The intracellular domain of the long human PrlR contains 10 tyrosine residues whose extent of phosphorylation and function remain to be established. The most carboxyl terminal tyrosine (Tyr\textsuperscript{587}) is known to be the primary site responsible for activation of Stat5 (26), although other tyrosines may be involved in engaging additional downstream effectors. The finding that the mutant PrlR induced Stat5 activation to a similar extent as the wild-type receptor suggests that tyrosine residues other than Tyr\textsuperscript{587} are affected by Thr\textsuperscript{391} phosphorylation. In contrast to Stat5a, increased Erk activation and Erk-dependent transcription were observed in the presence of the T391A receptor, while PKB activation was found to be prolonged. Thus, PrlR phosphorylation on the Thr\textsuperscript{391} site can influence both the intensity and duration of signaling of specific pathways activated by the PrlR.

Various mechanisms act in concert to control ligand-induced receptor activation. The PrlR is known to be regulated by negative feedback involving the induction of suppressor of cytokine signaling proteins that bind either the receptor itself or Jak2 (27). In vitro studies have implicated several members of the suppressor of cytokine signaling family in the control of prolactin signaling (28–30). A physiological function for suppressor of cytokine signaling proteins in the negative regulation of PrlR signaling in the mammary gland has been established for suppressor of cytokine signaling 1 (30). Dephosphorylation by site-specific phosphatases may also modulate the activation status of the receptor, although such phosphatases have not yet been identified for the PrlR. Nevertheless, it is possible that 14-3-3 proteins may recruit a phosphatase to the PrlR that dephosphorylates specific tyrosine residues. For example, the protein tyrosine phosphatase H1 was reported to associate with PrlR that dephosphorylates specific tyrosine residues. It is possible that Thr\textsuperscript{391} in the PrlR is dephosphorylated by PP2A. Alternatively, because the activity of many kinases is controlled by PP2A (36), the effect of okadaic acid on Thr\textsuperscript{391} phosphorylation could be indirect. Although the majority of human breast cancers are positive for the PrlR, both normal and neoplastic breast tissues are sources of extrapituitary prolactin, implying an autocrine-paracrine role for prolactin in breast cancer. An alternative means of modulating PrlR signal transduction in cancer cells may involve deregulated phosphorylation of the Thr\textsuperscript{391} residue described here. It is tempting to speculate that decreased phosphorylation of this site may prolong prolactin signaling and contribute to breast neoplasia.

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