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

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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 KCST^{391}WP 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 both glutathione *S*-transferase pulldown and coimmunoprecipitation assays. In breast carcinoma and mouse mammary epithelial cell lines, the endogenous receptor was found to associate with glutathione *S*-transferase-14-3-3 proteins independent of prolactin stimulation. A phospho-specific peptide antibody was generated and used to demonstrate phosphorylation of Thr^{391} *in vivo*. Phosphorylation of this site was found to be sensitive to okadaic acid, a specific inhibitor of serine/threonine protein phosphatases. Interestingly, the T391A PrlR 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 that of Stat5a. Phosphorylation of the receptor on Thr^{391} may therefore provide a new mechanism by which prolactin signaling is attenuated.

The prolactin receptor (PrlR)[1] 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 (1). Prolactin is essential for the development and maturation of the mammary gland as well as for modulating the immune response (2–4). PrlR-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 (5). 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 (6). 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 ΔS1 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

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[1] The abbreviations used are: PrlR, prolactin receptor; WT, wild-type; SH2, Src homology 2; Stat, signal transducer and activator of transcription; EGF, epidermal growth factor; HA, hemagglutinin; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; Erk, extracellular signal-regulated kinase; Jak, Janus kinase; PKB, protein kinase B; PP2A, protein phosphatase 2A.

Thr[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 pEF1/V5-His vector containing carboxyl-terminal V5 and His tags (Invitrogen) was generously provided by J. Clevenger (9). Stat5a in pEF1α-Bos and HA-tagged PKB in pcDNA3 were gifts from W. Alexander and J. Testa, respectively. HA-tagged Erk2 in pcDNA3, Gal4-Sap1a, and G5E4-lux were kindly provided by Y. Nagamine. EE-tagged 14-3-3 ζ and γ cDNAs in pEF1α were a gift from A. Villunger. Site-directed mutagenesis was performed using the QuikChange kit (Stratagene) according to the manufacturer's instructions.

Antibodies—PrlR-specific polyclonal rabbit antiserum was kindly provided by J. Clevenger. 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), phosphotyrosine-specific mouse monoclonal antibody (clone 4G10) (Upstate Biotechnology), anti-phospho-Erk1/2 (T202/Y204) rabbit polyclonal antibody (New England Biolabs), anti-phospho-PKB (T308) rabbit polyclonal antibody (New England Biolabs), HA-specific rat monoclonal antibody (Roche Applied Science), anti-phospho-Stat5a/b (Y694/Y699) mouse monoclonal antibody (Upstate Biotechnology), anti-Stat5a rabbit antiserum (Upstate Biotechnology).

Generation of Anti-phospho-Thr[391] PrlR Antibody—The anti-phospho-Thr[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 immunizing 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[391]-PrlR antibody was verified by dot immunoblots against the immunizing phosphorylated peptide, the corresponding non-phosphorylated peptide, and an irrelevant phosphopeptide (CLG-PPHSR(pS)LPDILG). 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 mg/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 *Eco*RI-*Bam*H1-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 0.6–0.8 at 600 nm. GST-14-3-3ζ protein expression was then induced with 0.1 M isopropyl-β-D-1-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 Na_2HPO_4, 4 mM NaH_2PO_4, 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 chloride, and 1 μg/ml leupeptin. 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—Pulldowns 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 with 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 polyvinylidine difluoride membranes (Millipore). After blocking with 20% horse serum (Hunter) in phosphate-buffered saline 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 G5E4-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 with 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 R*S*X*p*S/T*X*P or R*XXX*pS/T*X*P 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 ([388]KCSTWP[393]), which was found to be highly conserved among a wide variety of species (Fig. 1*A*). 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-Sepharose beads. 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. 1*B*). This interaction was further verified by coimmunoprecipitation of the PrlR with both the 14-3-3ζ and γ isoforms (Fig. 1*C*). The 14-3-3σ 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 (CST[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. 1*B*).

In most cases, 14-3-3 proteins associate with target proteins in a phosphorylation-dependent manner. To explore whether Thr[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[391] was altered to alanine. The interaction between this mutant with GST-14-3-3ζ (Fig. 1*B*) and its coimmunoprecipitation with 14-3-3ζ and γ isoforms (Fig. 1*C*) was markedly reduced. Conversely, a S390A PrlR mutant still associated with 14-3-3 (data not shown). These results demonstrate an absolute requirement for Thr[391] in the PrlR to create a binding site for 14-3-3 proteins.

In Vivo Phosphorylation of Thr[391] and 14-3-3 Binding Are Regulated by a PP2A-like Activity—To confirm phosphorylation of Thr[391] in the PrlR *in vivo*, we generated a polyclonal antibody that specifically recognized a phosphopeptide encompass-

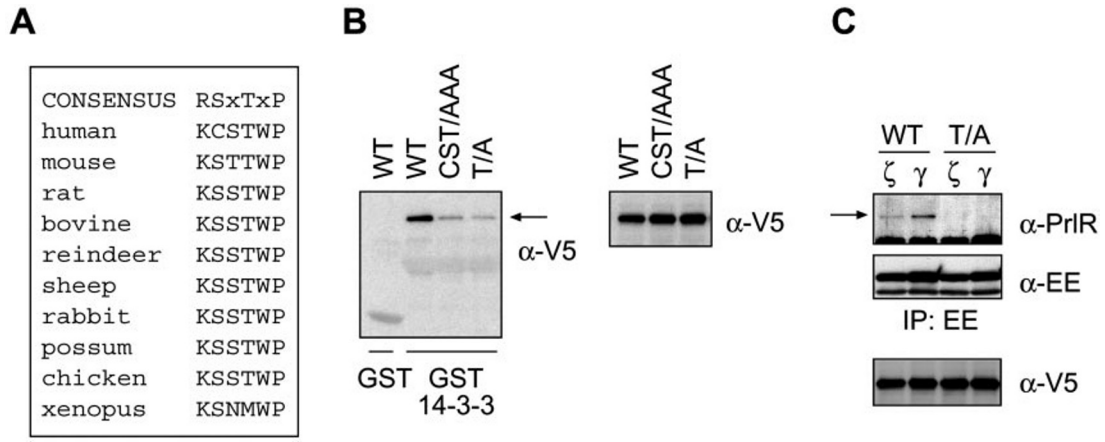

FIG. 1. **Phosphorylation of the PrlR on Thr[391] mediates interaction with 14-3-3.** *A*, sequence alignment of the PrlR from various species demonstrates conservation of a putative 14-3-3 binding motif. Phosphorylation of the threonine is predicted to be required for recognition. *B*, V5-tagged human wild-type (*WT*) and mutant PrlR ([389]CST[391] to AAA and Thr[391] to Ala) expression vectors were transiently transfected into 293T cells. Lysates were incubated with glutathione beads coupled to GST-14-3-3ζ 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 ζ and γ, respectively. 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*).

ing the 14-3-3 binding site in the human PrlR but did not cross-react with the corresponding unphosphorylated peptide. This antibody was used to demonstrate that the wild-type but not the T391A mutant receptor was phosphorylated in transiently transfected 293T cells (Fig. 2*A*). Phosphorylation of the wild-type receptor promoted association with endogenous 14-3-3 proteins, as determined by immunoblotting using an antibody that recognizes all 14-3-3 isoforms. Because of the lack of suitable antibodies for immunoprecipitation of 14-3-3 and PrlR proteins, we generated stable PrlR transfectants to address the interaction with endogenous 14-3-3 proteins as well as the phosphorylation of Thr[391]. Only the wild-type PrlR was found to be phosphorylated in NIH3T3 and EGF-dependent mouse mammary epithelial HC11 cells that stably expressed either the wild-type or the T391A mutant receptor (Fig. 2*A*). The presence of EGF had no effect on the level of Thr[391] phosphorylation. To gain insight into potential kinases or phosphatases that modify this site, we treated cells with various stimuli or inhibitors and analyzed their effect on Thr[391] phosphorylation. Activation of protein kinases A, B, and C was found to have little effect on phosphorylation of this site (data not shown). Interestingly, okadaic acid, a selective inhibitor of the serine/threonine phosphatase PP2A, significantly increased phosphorylation of Thr[391] in NIH3T3 cells expressing the wild-type PrlR (Fig. 2*B*). This increase was associated with further recruitment of endogenous 14-3-3 proteins (Fig. 2*B*).

Thr[391] Phosphorylation in Breast Epithelial Cells Is Independent of Prolactin Stimulation—We next investigated the association between 14-3-3 and the endogenous PrlR in human breast carcinoma cell lines, T47D and SKBR3, and in HC11 cells. GST-14-3-3ζ pulled down the receptor in all cell lines (Fig. 3, *A* and *B*), indicating that the PrlR is phosphorylated on Thr[391] in these cell lines. Phosphorylation of this site appears to be constitutive and independent of ligand binding because prolactin stimulation did not affect association of the PrlR with 14-3-3.

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[391] phosphorylation influenced activa-

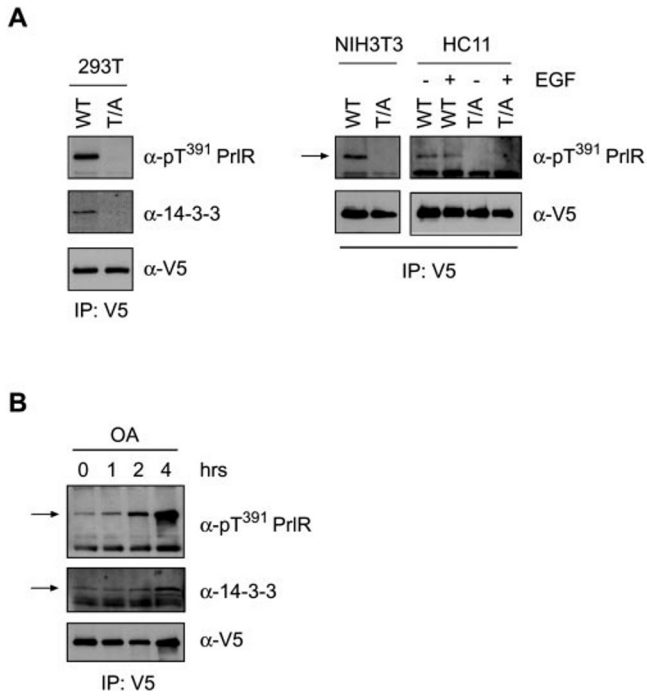

FIG. 2. ***In vivo*** **phosphorylation of Thr[391] and 14-3-3 binding are stabilized by okadaic acid.** *A*, whole cell extracts from transiently transfected 293T and from NIH3T3 and HC11 cells that stably express V5-tagged WT and T391A PrlR were immunoprecipitated with V5-specific antibody. HC11 cells 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[391]-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 Me_2SO for 1, 2, and 4 h. The PrlR was immunoprecipitated with V5-specific antibody, resolved by SDS-PAGE, and immunoblotted with phospho-Thr[391]-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*). Me_2SO alone had no effect on Thr[391] phosphorylation.

tion of the receptor, we transiently transfected vectors encoding wild-type or mutant receptor into 293T cells and then stimulated cells with prolactin for 10, 30, and 60 min. The receptor was immunoprecipitated from lysates and blotted with a phosphotyrosine-specific antibody (Fig. 4*A*). Interestingly, we observed increased basal as well as prolactin-induced tyrosine phosphorylation of the mutant receptor, suggesting that Thr[391] phosphorylation may modulate PrlR activation. Increased tyrosine phosphorylation of the T391A mutant compared with the wild-type receptor was found to be highly reproducible as shown by densitometric quantification of three independent experiments (Fig. 4*B*).

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. 5*A*). 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. 5*B*). 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, Thr[391] 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. 5*C*), whereas enhanced activation of Erk2 was observed by the mutant PrlR relative to that of the wild-type receptor (Fig. 5*D*). 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 NH_2-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. 5*D*, the mutant PrlR was able to sustain higher basal and prolactin-induced Erk-dependent transcriptional activation than the wild-type receptor (Fig. 5*E*). This was observed after 2 and 4 h of prolactin stimulation. Taken together, these results suggest that Thr[391] phosphorylation may negatively regulate signaling by the PrlR to specific downstream pathways such as the

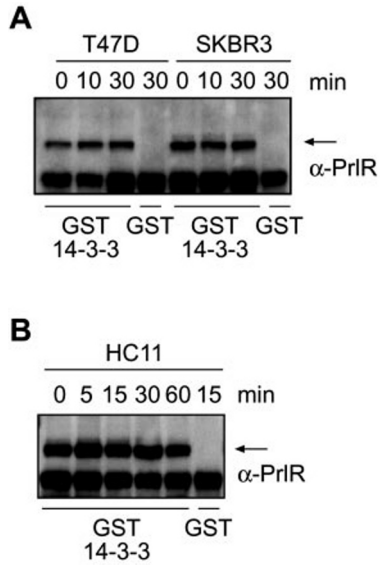

FIG. 3. **Interaction of 14-3-3 with the PrlR occurs independently of prolactin stimulation.** *A*, serum-starved T47D and SKBR3 cells were stimulated with prolactin for indicated times prior to lysis. *B*, HC11 cells were maintained at confluence, EGF was withdrawn for 24 h, and cells were then stimulated with prolactin and dexamethasone for indicated times. Pulldown experiments were performed using glutathione beads coupled to GST-14-3-3ζ or GST alone; the endogenous PrlR was revealed by Western blot with PrlR-specific polyclonal antiserum.

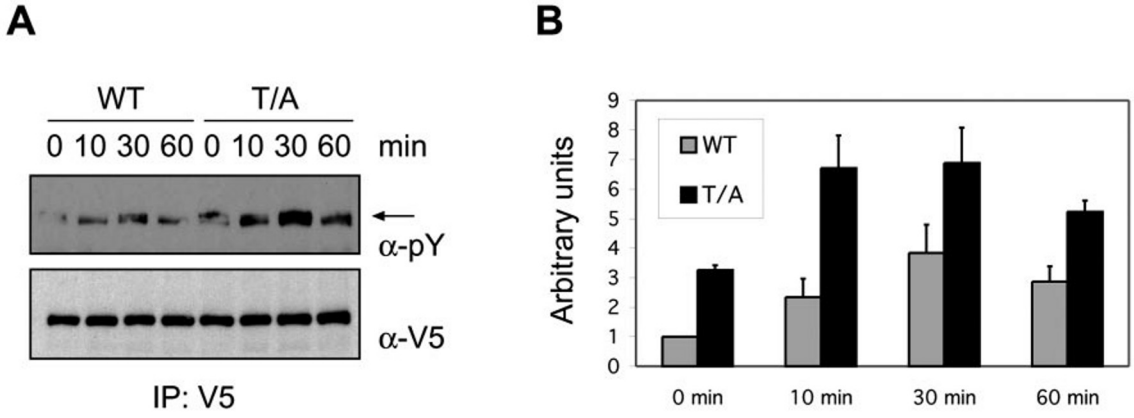

FIG. 4. **Tyrosine phosphorylation of the T391A mutant PrlR is increased compared with the wild-type receptor.** 293T cells were transiently transfected with V5-tagged WT or T391A PrlR expression vectors and stimulated with 1 μg/ml prolactin for indicated times prior to lysis. The PrlR was immunoprecipitated with V5-specific antibody and resolved by SDS-PAGE. Western blotting was performed using a phosphotyrosine-specific antibody (*pY*, *top panel*). The membrane was reprobed with V5-specific antibody to verify equal expression and immunoprecipitation of the PrlR (*bottom panel*). *B*, quantification of the tyrosine phosphorylation of the WT and T391A PrlR using ImageJ software (NIH). Mean values of three independent experiments normalized for the amount of immunoprecipitated protein are plotted. *Error bars* represent the mean ± S.E. The basal phosphorylation of the WT PrlR was set to 1 in all experiments.

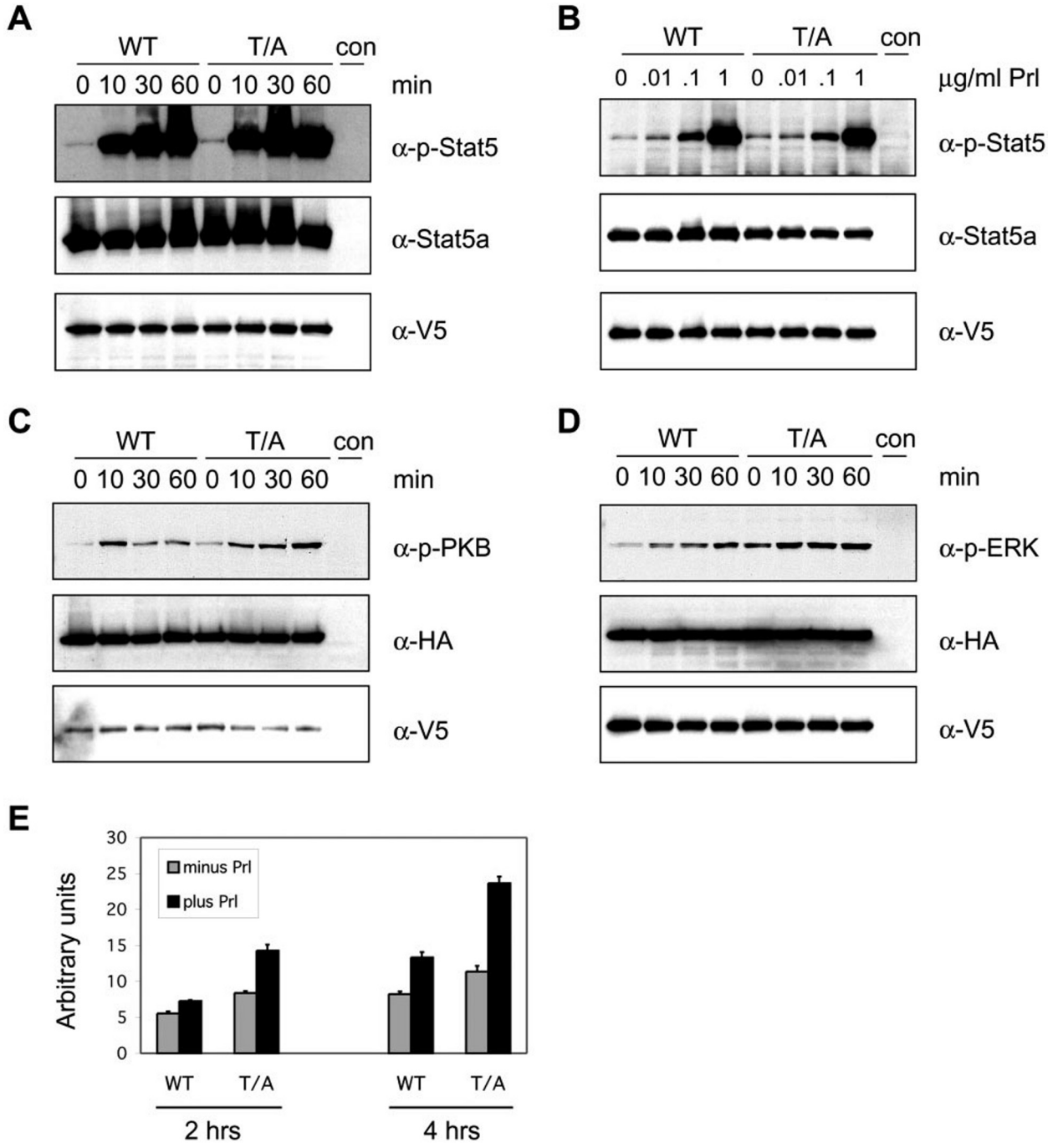

FIG. 5. **Thr[391] modulates signaling down the PI3K and MAPK pathways.** 293T cells were transiently transfected with expression vectors encoding V5-tagged WT or T391A PrlR together with Stat5a (*A* and *B*), HA-tagged PKB (*C*), or HA-tagged Erk2 (*D*). Cells were stimulated with 1 μg/ml prolactin for 10, 30, and 60 min (*A*, *C*, and *D*) or with 0.01, 0.1, and 1 μg/ml prolactin for 30 min (*Prl*, *B*). Whole cell lysates were separated by SDS-PAGE and immunoblotted with (*top panels*) phospho-specific Stat5 (*A* and *B*), PKB (*C*), and Erk antibodies (*D*). Membranes were reprobed with (*middle panels*) Stat5a-specific (*A* and *B*) and HA-specific antibodies (*C* and *D*), and also with V5-specific antibody to verify expression of each construct. Untransfected 293T cells were loaded as a control (*con*). *E*, 293T cells were transiently transfected with WT or T391A PrlR expression vectors together with a Gal4-Sap1a plasmid and the G5E4-lux reporter. 24 h post-transfection, cells were either left untreated or stimulated with 1 μg/ml prolactin for 2 and 4 h. Firefly luciferase activity was then determined and normalized using an internal *Renilla* luciferase control. Mean values of triplicate samples of one representative experiment are plotted. *Error bars* represent the mean ± S.E.

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-

ciation 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^{391} in the PrlR appears to provide a negative regulatory mechanism by which to down-regulate receptor activity. The Thr^{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^{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^{587} are affected by Thr^{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^{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 14-3-3β (31). Finally, serine/threonine phosphorylation has been implicated in attenuation of receptor signaling. In the case of ErbB1 (also known as EGFR or HER1), phosphorylation on Thr^{654} and Thr^{699}, via protein kinases C and D, results in down-regulation of EGF-induced c-Jun NH_2-terminal kinase activation, possibly involving receptor internalization. The Erk pathway, however, remained unaffected by phosphorylation of these sites (32). These findings parallel our observation that mutation of Thr^{391} in the PrlR only affects selective signaling pathways.

Stability and internalization of the PrlR do not appear to be altered by Thr^{391} phosphorylation. Pulse-chase labeling experiments indicated that the basal turnover rates of the wild-type and mutant receptors were comparable (data not shown). The PrlR has been shown to be down-regulated by internalization through clathrin-coated pits upon ligand binding (33). However, this mechanism may not be affected through 14-3-3 binding, because the rate of prolactin-induced internalization of the T391A mutant PrlR was similar to that of the wild-type receptor (data not shown).

The stimuli that modulate phosphorylation of the PrlR on Thr^{391} are yet to be established. Phosphorylation of the PrlR on Thr^{391} appears to be independent of prolactin stimulation in different cellular systems (Fig. 3). Although EGF is known to block PrlR signaling (34), it does not accomplish this by inducing phosphorylation of Thr^{391}, because EGF-starved mouse mammary epithelial cells showed no change in Thr^{391} PrlR phosphorylation status (Fig. 2*A*). We demonstrate here that okadaic acid treatment of cells leads to a marked increase in Thr^{391} phosphorylation, which was associated with increased 14-3-3 binding to the PrlR. Okadaic acid is an effective inhibitor of PP2A and can be used at concentrations up to 1 μM without any detectable inhibitory effect on protein phosphatase 1 or other major serine/threonine phosphatases (35). It is possible that Thr^{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^{391} phosphorylation could be indirect. Although the major cellular okadaic acid-sensitive phosphatase is PP2A, a role for other less abundant phosphatases such as protein phosphatases 4 and 5, which are also inhibited by okadaic acid (37, 38), cannot be excluded. Further understanding of the role of Thr^{391} phosphorylation in the PrlR will rely on examining its phosphorylation in different organs and at different developmental stages. Identification of the upstream kinase that modifies this site *in vivo* will provide insight into the pathways that cross-talk with PrlR signaling.

Prolactin and its receptor have been implicated in breast oncogenesis (39, 40). Higher prolactin levels have been associated with an increased risk of breast cancer (41), while 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^{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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