A single phosphoryl tyrosine residue of the prolactin receptor is responsible for activation of gene transcription

JEAN-JACQUES LEBRUN*, SUHAD AL†, VINCENT GOFFIN*, AXEL ULLRICH†, AND PAUL A. KELLY‡

*Institut National de la Santé et de la Recherche Médicale Unite 344, Endocrinologie Moléculaire, Faculté de Médecine Necker, 156 rue de Vaugirard, 75730 Paris Cedex 15, France; and †Department of Molecular Biology, Max-Planck-Institut für Biochemie, Am Klopferspitz 18A, 8033 Martinsried, Germany

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ABSTRACT Members of the cytokine/growth hormone/prolactin (PRL) receptor superfamily are associated with cytoplasmic tyrosine kinases of the Jak family. For the PRL receptor (PRLR), after PRL stimulation, both the kinase Jak2 and the receptor undergo tyrosine phosphorylation. To assess the role of tyrosine phosphorylation of the PRLR in signal transduction, several mutant forms of the PRLR in which various tyrosine residues were changed to phenylalanine were constructed and their functional properties were investigated. We identified a single tyrosine residue located at the C terminus of the PRLR to be necessary for in vivo activation of PRL-responsive gene transcription. This clearly indicates that a phosphoryl tyrosine residue in the cytoplasmic domain of a member of the cytokine/growth hormone/PRL receptor superfamily is directly involved in signal transduction.

The prolactin receptor (PRLR) is a member of the cytokine growth hormone (GH)/PRL receptor superfamily (1). Members of this family do not contain any intrinsic catalytic domain (such as tyrosine or serine/threonine kinase) but have been shown to associate with cytoplasmic tyrosine kinases from the Jak family that are necessary for signal transduction (for review, see ref. 2). For the PRLR, the receptor constitutively associates with the tyrosine kinase Jak2 (3–6). In addition, PRL-induced activation of Jak1 has also been reported in BAF-3 cells (5). We have shown (4) that upon PRL stimulation in Nb2 cells, the associated kinase Jak2 is activated, resulting in the tyrosine phosphorylation of both the kinase and the receptor, and that phosphorylation of both Jak2 and PRL was time- and dose-dependent in the Nb2 cell line. Moreover, in 293 cells (human embryonic kidney cells), transiently cotransfected with the cDNAs encoding Jak2 and PRLR, both the receptor and the kinase are basally tyrosine-phosphorylated, due to the high level of expression of Jak2. However, upon PRL stimulation, a significant increase in tyrosine phosphorylation of both Jak2 and PRLR is observed (36).

Recently, it has also been reported for several members of the family that upon ligand stimulation, the receptors themselves undergo tyrosine phosphorylation (7–12). The functional significance of this event still remains poorly understood. Phenylalanine replacement of the C-terminal tyrosine of the cytoplasmic domain of the interferon γ (IFN-γ) receptor, a member of the cytokine receptor superfamily, results in a nonfunctional receptor (13). Recent studies have indicated that growth factors and cytokines, including PRL, may regulate gene expression through direct phosphorylation of a group of Src-homology domain 2 (SH2)-containing latent cytoplasmic transcription factors known as Stat proteins (14–20). It has been suggested that signal specificity may be achieved through the activation of Stat molecules that can transactivate different sets of genes. Such a transcription factor termed mammary gland factor (MGF or Stat5) has recently been implicated in PRL-dependent transactivation of the β-casein gene promoter (21, 22). However, the possible involvement of receptor tyrosine phosphorylation in selecting specific signaling pathways remains unclear.

The PRLR is present in many different tissues and exists in several forms, depending on the species. In the rat, three natural forms of the PRLR have been identified: a short form of 291 aa (23), a long form of 591 aa, which differs from the short form by only the C-terminal tail (24), and the Nb2 form, which was identified in the Nb2 lymphoma cell line and represents an isoform of the long PRLR, lacking 198 aa due to a genetic deletion in the last exon encoding the receptor (25). Of these three forms of PRLR, only the long and Nb2 forms are able to transmit a lactogenic signal, as measured by stimulation of the β-casein or β-lactoglobulin promoters (26, 27), or to induce the interferon regulatory factor 1 promoter (28). We have recently shown that only the two (long and Nb2) forms were tyrosine-phosphorylated upon PRL stimulation (ref. 36 and Fig. 1). In addition, we demonstrated that deletion of the C-terminal part of the Nb2 form of the PRLR (mutant T22; Fig. 1) resulted in the loss of activation of the β-casein promoter and in the loss of tyrosine phosphorylation of the receptor itself, without affecting either association or activation of the kinase Jak2. This indicates a clear correlation between receptor phosphorylation and activation of gene transcription (36).

To examine the importance of single phosphoryl tyrosine residues present in the receptor itself, we constructed several mutant forms of the PRLR in which tyrosine residues have been changed to phenylalanine. Transfection of the cDNA encoding these mutants in the 293 cell line stably expressing the tyrosine kinase Jak2 (line LA; ref. 36) and precipitation of PRLR complexes using biotinylated PRL and streptavidin to increase sensitivity reveals that the tyrosine phosphorylation of the receptor is a necessary event for activation of gene transcription by PRLR. In addition, we show here that a single tyrosine residue at the C-terminal end of the receptor (Tyr-382 in the Nb2 form and Tyr-580 in the long form) promotes this activation of the β-casein promoter, in response to PRL.

MATERIALS AND METHODS

Mutagenesis Experiments. The construction of the tyrosine mutants used the Nb2, Δ296–322, and long PRLR cDNA and the pr/CMV vector (Invitrogen) as a template. Single-stranded DNA was generated by using the origin of replication of the M13 phages, present on the pr/CMV vector, in the (dut−, ung−) Escherichia coli C1236 strain, in the presence of the MKO7 M13 helper phage. Mutagenesis was done by the method of Kunkel (29). The mutated cDNAs were confirmed by sequencing using the Sanger technique (30), transformed in E. coli DH5α, and directly used for the expression experiments.

Abbreviations: GH, growth hormone; PRL, prolactin; PRLR, PRL receptor; IFN-γ, interferon γ; SH2, Src-homology domain 2.

†To whom reprint requests should be addressed.
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of SDS amid 4 (ref. 9450-1-7), amid of Proteins with Precipitation Cell Culture Tris HCl, glucose 4.5 (GIBCO) plasmid cDNAs different LA) cells were deprived of serum overnight, before being stimulated with ovine PRL (NIAMDD D-RPL-15) or recombinant nonlactosigenic bovine GH [a gift from W. Brumbach, American Cyanamid (ref. 9450-1-7)], both coupled to biotin (Calbiochem, catalog number 203-188). Stimulation was for 15 min at 37°C.

Precipitation of Proteins with Avidin-Agarose Beads. After stimulation, 293 cells (clone LA) were lysed in 1 ml of lysis buffer [10 mM Tris-HCl, pH 7.5/5 mM EDTA/150 mM NaCl/30 mM sodium pyrophosphate/50 mM sodium fluoride/1 mM sodium orthovanadate/10% (vol/vol) glycerol/0.5% Triton X-100] containing protease inhibitors [1 mM phenylmethylsulfonyl fluoride/pepsatin A (1 μg/ml)/leupeptin (2 μg/ml)/aprotinin (5 μg/ml)] for 10 min at 4°C. The insoluble material was discarded by centrifugation at 12,000 × g for 15 min at 4°C. The amount of protein was equalized in all the samples after measuring the protein concentration by using the Bradford technique. PRLR complexes were then incubated with 10 μl of avidin-agarose beads [10% (wt/vol) in lysis buffer; Calbiochem, catalog number 189742] for 2 h at room temperature as described (4). Samples were washed three times in 1 ml of lysis buffer and eluted in 20 μl of SDS loading buffer [20% glycerol/10% (vol/vol) 2-mercaptoethanol/4.6% (wt/vol) SDS/0.125 M Tris-HCl, pH 6.8]. Proteins were separated on a 7.5% polyacrylamide gel, transferred to nitrocellulose, and subjected to immunoblot analysis with an anti-phosphotyrosine monoclonal antibody [4G10; Upstate Biotechnology (Lake Placid, NY) 0.1 μg/ml] overnight at 4°C. Then, the membranes probed with this antibody were incubated with an anti-mouse antibody (Amersham, NA 931) coupled to peroxidase for 1 h at room temperature before being washed for four 20-min periods in the washing buffer (50 mM Tris-HCl, pH 7.6/200 mM NaCl/0.05% Tween 20) and labeled bands were revealed by chemiluminescence (ECL kit from Amersham) by following the manufacturer’s instructions. Membranes were stripped of probes overnight at 4°C in an acid solution (0.1 M glycine, pH 3/0.1 M NaCl) and reprobed with an anti-PRLR antibody (monoclonal antibody U5; ref. 4) at 0.5 μg/ml overnight at 4°C before being processed as described above.

Transcriptional Stimulation of the β-Casein Promoter. Wild-type 293 cells were plated in 6-well plates at 0.5 × 10⁶ cells per well before being transiently cotransfected as described below with 0.5 μg of pCH110 (β-galactosidase expression vector, Pharmacia). 0.2 μg of the fusion gene carrying the promoter region of the rat β-casein gene linked to the coding region of the luciferase gene, and 0.1 μg of plasmid pR/CMV, containing the different forms of PRLR: long, L Y237F, L Y580F, Nb2, Nb Y237F, Nb Y382F, Δ296–322, ΔY237F, and ΔY382F. One day after transfection, cells were incubated in the presence or the absence of ovine PRL (50 nM) and dexamethasone (250 nM) for 24 h before being lysed. Luciferase activity was measured in relative light units (Lumat LB 9501, Berthold, Nashua, NH) and normalized for β-galactosidase activity. Results are the mean ± SEM of four experiments.

RESULTS AND DISCUSSION

As summarized in Fig. 1, we recently observed a correlation between receptor tyrosine phosphorylation and the capacity of the receptor to transmit PRL signals to activate gene transcription (36). Of the three natural forms of the PRLR (long, short, and Nb2; refs. 23–25), only the long and Nb2 forms are able to activate the β-casein promoter (26, 27) and to be tyrosine phosphorylated. Although the short form is able to activate Jak2, it is not phosphorylated, despite the presence of four tyrosine residues in its cytoplasmic domain. The long form, which contains nine tyrosine residues, is phosphorylated, whereas the Nb2 form, which contains only three cytoplasmic tyrosine residues, still retains the ability to be

CELL CULTURE AND TRANSIENT TRANSFECTION. The human 293 clone stably expressing the tyrosine kinase Jak2 (clone LA) was grown in Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium (GIBCO) containing 10% (vol/vol) fetal calf serum (Sigma). Several hours before transfection, cells were plated in a rich medium (2 parts DMEM/F-12 and 1 part DMEM containing glucose at 4.5 g/liter) containing 10% fetal calf serum. Approximately 5 × 10⁶ cells were then transfected with the cDNAs encoding the different forms of PRLR (each at 4 μg) using the calcium phosphate technique. This amount of PRLR plasmid was selected as being adequate for observing PRL-dependent phosphorylation of the receptor (data not shown). After 24 h of expression, the LA cells were deprived of serum overnight, before being stimulated with ovine PRL (NIAMDD D-RPL-15) or recombinant nonlactosogenic bovine GH [a gift from W. Brumbach, American Cyanamid (ref. 9450-1-7)], both coupled to biotin (Calbiochem, catalog number 203-188). Stimulation was for 15 min at 37°C.

FIG. 1. (Upper) Representation of natural and mutant forms of PRLR. Illustrated are wild-type forms of the rat long, Nb2, and short PRLRs and the deletion mutants established from the Nb2 PRLR (Δ296–322 and T322). Transmembrane domains are represented by solid boxes. The juxtamembrane region containing the box 1 proline-rich motif is stippled. Numbers at the bottom of each receptor represent the last amino acid of the mature protein. (Lower) Ability of each receptor to associate and activate Jak2 (Jak2), to undergo tyrosine phosphorylation (PRLR PTyr), and to activate the β-casein promoter (Transcription) is indicated. PTyr, phosphoryrosine.

FIG. 2. Representation of the tyrosine mutant forms of PRLR. Illustrated are the long and the two tyrosine mutants established from its sequence (L Y237F and L Y580F), the Nb2 form and the two tyrosine mutants established from its sequence (Nb Y237F and Nb Y382F), the (Δ296–322) deleted form and the two tyrosine mutants established from its sequence (ΔY237F and ΔY382F). The tyrosine residues are indicated on the receptors. Transmembrane domains are represented by solid boxes.
tyrosine phosphorylated. In addition, a truncated mutant of the Nb2 form of the PRLR (T322), in which the last 70 C-terminal amino acids including the C-terminal tyrosine are missing, is unable to activate the β-casein promoter and to be itself tyrosine phosphorylated. In contrast, the deletion mutant Δ296–322, in which 27 aa, including Tyr-309, are missing, still activates the β-casein promoter and is itself phosphorylated (36). This indicates that phosphorylation of at least two cytoplasmic tyrosine residues (Tyr-237 and Tyr-382 in the Nb2 form) might play a role in signal transduction.

We constructed several mutant forms of the long, Nb2, and Δ296–322 PRLR, in which the two conserved tyrosine residues were separately mutated to phenylalanine (Fig. 2). The cDNAs encoding the natural and mutant forms of the Nb2 PRLR were transfected in the human 293 cell line stably overexpressing the tyrosine kinase Jak2 (LA line). Cells were stimulated with biotinylated hormones and the PRLR complexes were collected with avidin-agarose beads and processed. This approach greatly increases the sensitivity of the signal by trapping receptors at the cell surface. Moreover, by using this approach, the nonspecific signal generated by IgG heavy chains (55 kDa), during immunoprecipitation followed by Western blot analysis, is avoided, allowing a clear detection of the signal corresponding to the PRLR constructs with apparent molecular masses of 58 and 62 kDa. On the other hand, this method does not permit the examination of the state of phosphorylation of the kinase or the receptor in the absence of ligand. Constitutive activation of Jak kinases overexpressed in 293 cells has been reported (31). Nevertheless, despite the overexpression of Jak2, in the stable cell line (LA clone), no detectable phosphorylation of the kinase was observed in the absence of transfected PRLR (data not shown). When the cDNA encoding PRLR is transfected in the stable clone, both the receptor and Jak2 are basally phosphorylated, as in 293 cells cotransfected with the cDNAs encoding the kinase Jak2 and the PRLR (36). However, in both systems, this phosphorylation significantly increases when the cells are stimulated by PRL, indicating that tyrosine phosphorylation of Jak2 and PRLR is PRL-dependent. Since the primary goal of the study was to identify the sites of tyrosine phosphorylation of the receptor by comparing different mutants, the use of this technique is, therefore, appropriate.

The results shown in Fig. 3 indicate that PRL-stimulated wild-type Nb2 PRLR and the two tyrosine mutant forms (NbY237F and NbY382F) are able to activate Jak2, whereas receptor tyrosine phosphorylation was only observed with the Nb2 and NbY237F forms of PRLR. This indicates that of the three tyrosine residues present in the cytoplasmic domain of the Nb2 PRLR, only the most C-terminal tyrosine is phosphorylated. The cell surface expression of the different forms of receptor was confirmed by probing the membrane with a monoclonal antibody (U5; ref. 4) directed against the extracellular domain of the PRLR (Fig. 3B). To confirm this finding, we also mutated these two tyrosines in the deletion mutant Δ296–322. As shown in Fig. 3C, mutation of Tyr-237 to phenylalanine (Y237F) does not affect receptor

![Fig. 3](https://example.com/figure3.png)

**Fig. 3.** Anti-phosphotyrosine (A, C, and E) and anti-PRLR (B, D, and F) immunoblot analyses of avidin-agarose-recovered complexes from 293 LA cells transfected with wild-type and various mutant forms of PRLR. The 293 LA cells were transfected by using the calcium phosphate technique, with the cDNAs encoding wild-type and tyrosine mutant forms of the Nb2 (A and B), Δ296–322 (C and D), and long (E and F) PRLR. Cells were stimulated for 15 min with biotinylated recombinant bovine GH (−) or ovine PRL (+), lysed, and incubated with avidin-agarose. The proteins were separated on a 7.5% polyacrylamide gel and transferred to a nitrocellulose membrane before being incubated with the indicated antibodies. The position and the size of Jak2 (130 kDa) and of the phosphorylated receptors (62 kDa for the Nb2, 58 kDa for the Δ296–322, and 95 kDa for the long form) are indicated on the right. The smaller size of the Δ296–322 forms is due to the 27-aa deletion.
phosphorylation. In contrast, mutation of the last tyrosine residue (Y382F) completely abolished receptor phosphorylation. These results again confirm that only the C-terminal tyrosine (Tyr-382) of the Nb2 form of the receptor undergoes phosphorylation. Correct expression of all forms of PRLR is demonstrated in Fig. 3D.

Finally, we extended our analysis to the long form of the PRLR. For that purpose, we mutated Tyr-237 and Tyr-580 of the long form, homologous to Tyr-237 and Tyr-382 of the Nb2 form, to phenylalanine (Fig. 2). Interestingly, none of the mutations affected receptor phosphorylation (Fig. 3E). This indicates that, in contrast to the Nb2 form, the long form of the PRLR contains one or more tyrosine residues able to undergo phosphorylation.

To assess the functional capacity of these mutants to transmit a signal leading to transcriptional activation of PRL-responsive genes, we cotransfected cDNAs encoding different forms of the PRLR with the cDNA encoding the β-casein promoter coupled to the luciferase gene and the cDNA encoding for the β-galactosidase. The results shown in Fig. 4 clearly demonstrate that, for all the forms of PRLR examined including the long form, mutation of the C-terminal tyrosine to phenylalanine markedly reduces the capacity of the receptor to transmit the transcriptional response to PRL. In contrast, replacement of Tyr-237 has no effect on the ability of the different forms of PRLR to respond to PRL stimulation. These data closely parallel those reported for the IFN-γ receptor, by demonstrating that of the five intracellular tyrosines, the last was the sole tyrosine required for receptor activity (13). The fact that other tyrosine(s) of the long form of the PRLR are phosphorylated (Fig. 3E), whereas the ability of the receptor to activate β-casein transcription is markedly reduced, suggests that they are probably involved in some other signaling pathway, in agreement with the broad range of biological activities of PRL. Similar results have been observed with the IFN-γ receptor α chain, in which several tyrosine residues are phosphorylated but only the most C-terminal residue is required for IFN-γ receptor signal transduction (32).

This C-terminal phosphotyrosine is likely to be a binding site for a transducer molecule involved in the activation of milk gene transcription. Such transducer molecules usually contain one or several regions of homology to a noncatalytic domain of the Src molecule, called an SH2 domain. These SH2 domains recognize and bind to a phosphotyrosine residue within a specific stretch of amino acids (33). Recently, consensus binding sites of many SH2-domain-containing transducer molecules have been established (34). None of the SH2 binding peptides reported by Songyang et al. (34) obviously matches the Tyr-382/Tyr-580-Leu-Asp-Pro sequence flanking the C-terminal tyrosine of the PRLR. However, the optimal peptide sequences binding to the N-terminal SH2 domain of phospholipase C-γ (pY/LIV/E/LIV) or to Vav (pY/M/E/P) make those SH2-domain-containing proteins potential candidates for interacting with the Tyr-Leu-Asp-Pro sequence of the PRLR. We failed to detect any signal for phospholipase C-γ, in association with PRLR in Nb2 cells, by immunoblot analysis (data not shown). However, this could be due to the poor reactivity of our antibody directed against phospholipase C-γ. We have not yet investigated whether the C-terminal tyrosine interacts with Vav, but it seems unlikely to have a role in PRL-induced lactogenesis since its expression is restricted to hematopoietic cells. It has been shown that the cytokine/GH/PRL receptor superfamily activates a family of transcription factors, the Stat molecules (for review, see ref. 2). For the PRLR, two members of this family have been shown to be activated in response to PRL, Stat1 [also known as p91 (35)], and Stat5 [also known as mammary gland factor or MGF (21, 22)]. Stat1 is activated through Jak2 (36), but little is known about the activation of Stat5. It is possible that Stat5 could bind directly to the C-terminal phosphotyrosine of the receptor through its own SH2 domain, but this remains to be confirmed. This has been shown, for IFN-γ receptor, for which phosphorylation of the C-terminal tyrosine is necessary for binding and activation of Stat1 (32).

Thus, these data indicate that mutation of a single phosphotyrosine within the cytoplasmic domain of the PRLR is detrimental to activation of gene transcription. This strongly suggests that phosphotyrosines present in the cytoplasmic domains of the members of the cytokine/GH/PRL receptor superfamily might play a critical role in signal transcription. This observation provides one possible explanation for determining signal specificity for members of this receptor superfamily.

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**FIG. 4.** (Right) PRL-dependent induction of β-casein promoter/luciferase construct in wild-type (WT) 293 cells. Cells were cotransfected with the expression vectors containing the cDNAs encoding the forms of PRLR described in Fig. 2, the β-casein/luciferase construct, and a β-galactosidase expression vector, by using the calcium phosphate technique. Results are expressed as fold induction compared to cells not stimulated with PRL. Results represent the mean ± SEM of four experiments. (Left) The natural and mutant forms of the PRLR that were used.
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