Tyrosine Docking Sites of the Rat Prolactin Receptor Required for Association and Activation of Stat5*

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Prolactin (PRL) interacts with a single chain prolactin-specific receptor of the cytokine receptor superfamily. PRL triggers activation of Jak2 kinase which phosphorylates the PRL receptor itself and the mammary gland factor, Stat5, a member of the family of signal transducers and activators of transcription (Stat). Selection of the particular substrate (Stat 5), that is characterized by transcriptional responses to PRL, has been shown to be determined by specific tyrosine-based motifs common to many cytokine receptors. PRL-induced activation of Stat5 was abolished in 293 fibroblasts expressing PRL receptor mutants lacking all intracellular tyrosines. We have identified tyrosine phosphorylation sites of the PRL receptor (residues 580, 479, and 473) necessary for maximal Stat5 activation and subsequent Stat5-dependent gene transcription. Moreover, we have shown that none of the tyrosine residues of the PRL receptor are implicated in activation of Jak2. This study demonstrates that only specific tyrosines in the PRL receptor are phosphorylated and are in fact utilized differentially for Stat5-mediated transcriptional signaling.

The prolactin receptor (PRLR) is a member of the cytokine receptor superfamily (1). Members of this family are devoid of any catalytic domain in the cytoplasmic region and are known to associate with cytoplasmic tyrosine kinases of the Jak family that are necessary for signal transduction. The PRLR is constitutively associated with the tyrosine kinase Jak2 (2). In BaF3 cells, prolactin (PRL) also induces activation of Jak1 (3) and in Chinese hamster ovary cells Jak3 may interact with Jak2. Downstream of Jak2 activation, PRL induces tyrosine phosphorylation and activation of other cytoplasmic signaling proteins including signal transducers and activators of transcription (Stats). Stat1, Stat3, and Stat5 can all be activated by PRL in Nb2, 32D, and T-47D cells (4). Stat5, which was initially isolated as a factor that binds to DNA sequences essential for a lactogenic response (5), plays a critical role in regulating the expression of milk protein genes (6). However, the expression of Stat5 is not restricted to the mammary gland.

Recent studies indicate that there are at least two isoforms of Stat5 (a and b) (7–9) and that Stat5 can be activated upon stimulation with IL-2, IL-3, IL-5, granulocyte macrophage colony stimulating factor, EPO, GH, and epidermal growth hormone. It has also been shown that Stat1 and Stat3 can be activated by Jak kinases (7, 10, 11). Jak2 is also able to phosphorylate Stat5 in vitro (12). Stat proteins are presumed to be transiently recruited by activated cytokine receptor complexes via binding to specific phosphotyrosyl residues through their SH2 domain. Studies of the inhibitory activities of phosphotyrosine-containing peptides derived from the intracellular domain of the α chain of the interferon-γ receptor, the IL-4 receptor, the IL-10 receptor, or the EPO receptor provide evidence for a direct coupling of receptor and, respectively, Stat1, Stat6, Stat3, or Stat5 during the Stat activation cycle (13–16). In addition, Stat3 has been shown to associate with gp130, the signal transducing component common to IL-6, oncostatin M, ciliary neurotrophic factor, leukemia inhibitory factor, and IL-11 receptors. The regions of gp130 and leukemia inhibitory factor receptor β chain required for Stat3 activation have been defined as a small tyrosine containing motif YXXQ (17).

Studies of deleted or truncated mutant of IL-2, EPO, or GH receptor reported that cytoplasmic regions of this receptor other than the one required for Jak2 activation are necessary for a maximal Stat5 activation. This suggests that Stat5 activation may require binding of Stat5 to a receptor. Tyrosine phosphorylation of the PRLR is a necessary event for activation of gene transcription. A single phosphotyrosylated tyrosine in the Nb2 form of the PRLR (Nb2R), lacking 198 amino acids in the cytoplasmic domain, is required for PRL-mediated transcriptional induction of β-casein gene promoter. Indeed, mutation of the COOH-terminal tyrosine (Tyr385) of the Nb2R results in the absence of phosphorylation of the receptor and correlates with a complete loss of activation of gene transcription (18). However, mutation of the same residue in the long form of PRLR (Tyr386) leads to a variant receptor that retains 20% of transcriptional activity of wild type PRLR (18). These data are consistent with the hypothesis that the intermediary region of the cytoplasmic domain absent in the Nb2 form (amino acids 322–520) might contain other phosphorylated tyrosines involved in Stat5 recruitment, similar to the GH receptor where two regions of interaction with Stat5 have been identified; the region of residues 454–638 (19–22) and tyrosine 333 and 338 (23). Furthermore, it has been reported that a region in the human GHR between amino acids 520 and 540 regulates the inactivation of the Jak/Stat pathway and appears to implicate SHP-1 (24).

The cytoplasmic domain of the PRLR

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1 The abbreviations used are: PRLR, prolactin receptor; EPO, erythropoietin receptor; GHR, growth hormone receptor; IL-2Rβ, interleukin-2 receptor β chain; Nb2R, PRLR form expressed in Nb2 cells; EPO, erythropoietin; hGH, human growth hormone; IL, interleukin; PRL, prolactin; αPRL, ovine prolactin; IRS-1, insulin receptor substrate 1; Jak2, Janus kinase 2; Stat, signal transducer and activator of transcription; SH2, Src homology domain 2; WT, wild type; TK, thymidine kinase.
contains 9 tyrosines, among which 6 are found in the region that is deleted in the Nb2 form of the receptor and thus may be potentially specific to functions of the long form.

In this paper, we have used site-directed mutagenesis to investigate the role of these individual tyrosine residues in the Jak-Stat pathway and their requirement for Stat5 activation. This study demonstrates that PRL stimulation of 293 cells transfected with the PRLR plasmid activates Stat5 and that three of the four COOH-terminal tyrosine residues of the intracellular domain of the PRLR are required for its complete activation. Each of these tyrosine residues is able to induce transcriptional activation of the luciferase reporter gene controlled by the lactogenic hormone response element that has been shown to bind Stat5 protein (11). Additionally, each of these mutant receptors co-immunoprecipitated with tyrosyl-phosphorylated Stat5, suggesting a direct interaction between Stat5 and the PRLR. However, in 293 cells overexpressing the kinase Jak2, receptor mutants expressing normally inactive forms are able to transmit a lactogenic signal, suggesting a direct activation and interaction between Stat5 and Jak2.

MATERIALS AND METHODS

Pituitary Hormones—Recombinant human GH was kindly provided by Dr. A. Ythier, Serono Aeres, Geneva, Switzerland; recombinant bovine GH was kindly provided by Dr. W. Brumbach, American Cyanamid Co, Prinston, NJ. Ovine PRL was obtained from the National Hormone and Pitiitary Program/National Institute of Diabetes and Digestive and Kidney Diseases, Baltimore, MD.

Site-directed Mutagenesis—The construction of mutants was carried out using the cDNA encoding the long form of the rat PRLR subcloned into pRc/CMV expression vector using its M13 origin of replication and the MK07 M13 helper phage. Single stranded DNA was generated for oligonucleotide-directed mutagenesis in CJ236 cells. The mutated cDNAs were confirmed by sequencing.

Cell Culture and Transfection of Human 293 Fibroblasts—Cells were grown in complete medium (Dulbecco's modified Eagle's medium F-12 medium containing 10% fetal calf serum). Six hours before transfection, cells were plated in a rich medium (two-thirds Dulbecco's modified Eagle's medium F-12, one-third Dulbecco's modified Eagle's medium 4.5 g/liter glucose containing 10% fetal calf serum). Cells were co-transfected using the calcium phosphate technique with the indicated amount of cDNA encoding either wild type or different mutant forms of PRLR and Jak2 and incubated overnight in 3% CO2 at 37 °C. After 24 h of expression, the cells were deprived of serum for an overnight period.

Whole Cell Binding and Scatchard Analysis—293 cells were split into 6-well plates (0.5 × 10^6 cells/well) before being transiently transfected with, for each well, 0.5 μg of PRLR cDNAs. Transfected cells were deprived of serum for an overnight period. Then cells were incubated with 100,000 cpm of ^125I-hGH and increasing concentrations (0 to 50 μg) of unlabeled ovine PRL (oPRL) in 1 ml of phosphate-buffered saline containing 0.5% BSA. Incubations were carried out at room temperature for 4 h. Cells were washed twice with ice-cold phosphate-buffered saline, solubilized in 1 ml of 0.5 × NaOH and counted in a counter. ^125I-hGH was prepared using chloramine T; specific activity ranged from 75 to 120 μCi/μg.

Transient Transfection of 293 Cells for LHRE-TK-Luciferase Assay—293 cells were split into 6-well plates before being transiently transfected with 0.5 μg of pCH110 (β-galactosidase expression vector from Pharmacia), 0.1 μg of LHRE-TK-Luciferase (fusion gene carrying 6 copies of LHRE and the TK minimal promoter linked to the coding region of the luciferase gene) and 0.05 μg of plasmid pRc/CMV containing the different forms of PRLR cDNA.

Luciferase and β-Galactosidase Assay—Co-transfected cells were incubated in a serum-free medium with or without 18 ng of PRL, 24 h, and then lysed. Luciferase activity was measured in relative light units and normalized for β-galactosidase activity.

Purification of the PRLR Complexes, Immunoprecipitation, and Western Blot Analysis—Each 100-mm culture dish of 293 cells was co-transfected with 2 μg of either wild type or mutant forms of PRLR cDNA, and 1 μg of the cDNA encoding the human tyrosine kinase Jak2. Cells were stimulated by PRL (10^-7 m) for 10 min. Cells were subsequently 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 NaVO4, 10% glycerol, 0.5% Triton X-100) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml peptatin A, 2 μg/ml leupeptin, 5 μg/ml aprotinin) for 15 min at 4 °C. Lysates were cleared of debris by centrifugation at 15,000 × g at 4 °C for 10 min. The lysates were incubated with anti-Jak2 antibody (1 μg/ml) (Upstate Biotechnology, Inc.) or with a monoclonal anti-PRLR antibody (US, 5 μg/ml) and 15 μl of Protein A-Sepharose (Pharmacia Biotech, Inc.) overnight at 4 °C. Antibody complexes were washed in lysis buffer and boiled for 5 min in sample buffer (125 mM Tris, pH 6.8, 5% SDS, 10% β-mercaptoethanol, 20% glycerol). The amount of protein was equalized in all samples by measurement of protein concentration using a Bradford technique. Proteins, divided into 3 fractions, were separated on a SDS-polyacrylamide gel electrophoresis (7 or 6% gel), transferred onto polyvinylidene difluoride transfer membrane (Poly-screen™, NEN Life Science Products), and immunodetected with antibodies to anti-Jak2 (UBL, 1:5,000), anti-PRLR (mAb US, 5 μg/ml), anti-Stat5 (affinity 1:1,000), or anti-phosphotyrosine (UBI, 1:4,000) for 2 h at room temperature. Then, the membranes were incubated with an anti-rabbit or anti-mouse IgG-conjugated horseradish peroxidase (1:8,000) for 1 h at room temperature and revealed by ECL detection system (Amersham).

RESULTS

Site-directed Mutagenesis of PRLR—To investigate individually the role of each tyrosine residue in the cytoplasmic domain of the PRLR regarding Stat5 recruitment and the corresponding signaling pathway, several PRLR mutants were prepared (Fig. 1): A, mutant 9F in which all tyrosines were replaced by phenylalanine; B, individual substitution of each phenylalanine for tyrosine (8F-Ynn). The cDNA encoding the natural and mutant forms of the PRLR were transiently transfected in human 293 fibroblast cells to determine their binding capacity along with their ability to transmit a lactogenic signal by the activation of the LHRE-TK-Luciferase reporter construct. Additionally, mutants were assayed for PRL-stimulated tyrosyl phosphorylation of Jak2, which is associated with the PRLR and Stat5.

Expression and Binding Activities of PRLR Variants in 293 Cells—To confirm that the PRLR constructs encoded proteins of the expected sizes, we analyzed the receptor proteins in solubilized membranes of transfected 293 cells. Western blots were performed after immunoprecipitation of the extracts with the anti-PRLR antibody US. As shown in Fig. 2, the mutated PRLRs migrated as a protein of the expected size (~92 kDa) and showed no evidence of unusual receptor cleavage that could contribute to any loss of activity. In order to determine the possible effect of tyrosine replacement on cell surface PRLR expression, similar experiments using biotinylated PRL to purify specifically PRLR of cytoplasmic membrane were performed. The level of PRLR precipitated was similar for each PRLR variant (data not shown), suggesting that mutations of tyrosine residues have no effect on processing of PRLR.

Specific binding of ^125I-hGH to transiently transfected 293 cells demonstrated again that wild type and variant receptors were normally expressed. The number and affinity of PRL-binding sites were obtained with competition assays followed by Scatchard analysis (Table 1). All clones exhibited a single class of binding sites with an apparent affinity of 2 to 5 nM. All clones exhibited a single class of binding sites with an apparent affinity of 2 to 5 nM. Then, each clone was transfected in human 293 fibroblast cells to determine its binding capacity along with their ability to transmit a lactogenic signal by the activation of the LHRE-TK-Luciferase reporter construct. Additionally, mutants were assayed for PRL-stimulated tyrosyl phosphorylation of Jak2, which is associated with the PRLR and Stat5.

Functional Activity of Rat PRLR Using LHRE-TK-Luciferase Gene Reporter Transcription—To assess the transcriptional activity mediated by Stat5, we used the LHRE-TK-Luciferase reporter gene. In this construct, six copies of the lactogenic hormone response element (LHRE) were fused to the minimal promoter of the herpes simplex virus thymidine kinase gene (TK promoter) which governs the expression of the luciferase gene. LHRE is an element of the β-casein promoter that has been used for the affinity purification of Stat5 (6), in addition, it is a PRL responsive enhancer (19). The cDNAs encoding...
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FIG. 1. Schematic representation of wild type and mutated forms of PRLR. Upper panel, all the PRLRs shown contain 210 amino acids in the extracellular, 24 amino acids in the transmembrane (TMD), and 357 amino acids in the cytoplasmic domain. The mutant PRLRs are named with the number of the tyrosine residue (Y) that is not replaced by phenylalanine. The underlined numbers represent the position of tyrosines conserved in PRLR of different species. Lower panel, the ability of each receptor to associate with Stat5 and to activate Stat5 (Stat5 circled P) or Jak2 (Jak2 circled P), to undergo tyrosine phosphorylation of PRLR (PRLR circled P), and to activate the LHRE-TK promoter (LHRE) is indicated. Circled P, phosphotyrosine.

FIG. 2. Expression of the mutant PRLRs. Cell lysates of 293 cells expressing the mutant PRLR constructs were immunoprecipitated with anti-PRLR antibody (U5) and analyzed by immunoblotting with the same antibody. The migration positions of molecular mass standards (in kDa) are denoted on the right. The arrow indicates the migration of the PRLR.

TABLE I

Association constants of PRL receptor mutants expressed in 293 cells

| PRLR   | $K_a$ (nM$^{-1}$) | Sites/cell |
|--------|------------------|------------|
| WT     | 4.8 ± 0.5        | 55,800 ± 4,100 |
| 9F     | 6.3 ± 0.9        | 56,100 ± 3,800 |
| 8F-Y580| 3.6 ± 0.9        | 41,700 ± 2,800 |
| 8F-Y515| 4.4 ± 1.0        | 32,800 ± 2,900 |
| 8F-Y479| 3.3 ± 1.3        | 45,600 ± 1,600 |
| 8F-Y473| 5.8 ± 1.7        | 37,200 ± 3,700 |
| 8F-Y402| 4.7 ± 0.5        | 45,500 ± 1,900 |
| 8F-Y346| 3.7 ± 1.0        | 61,300 ± 3,100 |
| 8F-Y330| 4.9 ± 1.1        | 51,400 ± 2,500 |
| 8F-Y309| 4.4 ± 0.4        | 66,000 ± 2,400 |
| 8F-Y237| 5.5 ± 0.8        | 37,200 ± 1,900 |
| 4PA    | 4.9 ± 0.6        | 33,800 ± 2,500 |

mutated or wild type PRLRs were transiently cotransfected with the cDNA encoding the reporter gene in 293 cells. As shown in Fig. 3, the activity associated with the wild type PRLR corresponds to a 27-fold luciferase induction in the presence of PRL. The mutated PRLR SF-Y580 displayed ~80% of the wild type receptor activity and the PRLR mutants SF-Y479 and SF-Y473 each retained ~12% of the wild type receptor activity. In contrast, the other forms, particularly the 9F variant, failed to respond to PRL. The 4P-A mutant which is unable to interact with Jak2 (25) is not able to induce transcription of the reporter gene in response to PRL stimulation.

Activation of Stat5 in Response to PRL—To determine which PRLR tyrosines are involved in Stat5 activation, we analyzed the phosphorylation level of mutant PRLRs. In these experiments, the PRLR was immunoprecipitated with anti-PRLR antibody from cells expressing either the wild type or mutant receptors following PRL stimulation. The immunoprecipitates resolved by SDS-polyacrylamide gel electrophoresis were transferred to polyvinylidene difluoride filters and probed with the 4G10 anti-phosphotyrosine monoclonal antibody. As indicated in the Western blot in Fig. 4, two major bands of 92 and 92 kDa are co-immunoprecipitated with anti-PRLR antibody from PRL-treated 293 transfected cell lysates. To confirm that the 92- and 95-kDa tyrosine-phosphorylated bands are indeed phosphorylated PRLR and Stat5, respectively, and not other co-immunoprecipitated proteins, parallel samples of the same immunoprecipitate were probed with anti-PRLR or anti-Stat5. The PRLR monoclonal antibody revealed the 92-kDa protein (Fig. 2), while Stat5 antiserum detected a 95-kDa protein (Fig. 4, lower panel). The 8F-Y580 mutant, which retains 80% of transcriptional activity of wild type PRLR, also displayed a
level of tyrosine phosphorylation of Stat5 is that it is comparable to that observed with the wild type PRLR. Stat5 phosphorylation was seen for mutants 8F-Y479 and 8F-Y473, which have reduced transcriptional activation, but to a lesser extent than that seen with wild type and 8F-Y580. No induction of Stat5 phosphorylation was detected with the other mutated receptors, which failed to induce transcriptional activity upon PRL stimulation. These results support the notion of an interaction between Stat5 and the PRLR, suggesting that 8F-Y479 and 8F-Y473 variants have a lower affinity for Stat5 than wild type and 8F-Y580 form.

Jak2 Overexpression Restored the Functional Activity of Mutated Receptor—To determine whether Jak2 can restore PRL signaling of the mutated PRLRs, cDNAs for Jak2 were cotransfected with the receptor mutants and the LHRE-TK-Luciferase construct in 293 cells. As shown in Fig. 5 (upper panel), the basal level of luciferase activity was increased in cells overexpressing Jak2. However, the 4P-A mutant was still unable to transmit a lactogenic signal (50 versus 60 RLU/μg) in response to PRL stimulation, representing a 1.2-fold induction of the system was close to saturation. On the other hand, the three active mutants and the wild type receptor showed a lower fold induction when Jak2 was overexpressed, implying that the system was close to saturation. These results suggest that Jak2 is able to complement the absence of receptor tyrosines and interacts with and activates Stat5.

To assess the importance of Jak2 for PRL-induced Stat5 activation, we compared the ability of wild type PRLR and 9F or 8F-Y479 mutant forms to mediate PRL-induced tyrosine phosphorylation of Stat5 (Fig. 5 lower panel). Overexpression of Jak2 in cells expressing mutated 8F-Y479 PRLR resulted in complexes that retained the ability to stimulate Stat5 at levels comparable to those observed with wild type receptor, while the 9F PRLR variant showed Stat5 activation, but at a decreased level. Based upon these observations, it appears that phosphotyrosyl residues of the receptor complex outside of the receptor, likely in Jak2, also contribute to the recruitment of Stat5 in response to Jak2. However, the decrease in phosphorylation intensity of Stat5 in cells expressing 9F mutant suggests that Jak2 is less efficient than the receptor to activate Stat5.

Tyrosine Phosphorylation of Jak2 in Response to PRL Stimulation—To further investigate the mechanisms involved in the Jak-Stat pathway, we evaluated whether different forms of the PRLR were able to phosphorylate Jak2. This was measured in 293 cells which were cotransfected with the cDNA encoding various PRLRs and Jak2. Lysates from unstimulated (-) or PRL-stimulated (+) 293 transfected cells were immunoprecipitated with Jak2 antibody, and further analyzed by Western blotting using anti-phosphotyrosine antibody. Fig. 6 shows that Jak2 is tyrosyl-phosphorylated in response to PRL, except for the mutant 4P-A. As described previously, the presence of Jak2 in the immunoprecipitated complexes from unstimulated cells suggests preassociation of the kinase with the PRLR. Parallel samples of the same immunoprecipitates were immunoblotted with an antibody to Jak2, confirming the 130-kDa band as Jak2. In addition, it can be seen that comparable levels of Jak2 were present in all samples. Therefore, the defect associated with the 4P-A mutant is not due to an absence of Jak2.

DISCUSSION

The prevailing hypothesis of signal transduction by members of the cytokine receptor family is that upon binding of ligand to its receptor, association and activation of one or more members of the Jak family of tyrosine kinases occurs. The Jak kinases trans-phosphorylate each other as well as their associated membrane receptors. The latent cytoplasmic Stat proteins are recruited by the phosphotyrosines and serve as substrates for the Jak kinases. Subsequent to phosphorylation, the Stat proteins homo- or heterodimerize, translocate to the nucleus, and induce receptor-associated gene expression. For various tyrosine kinase receptors such as epidermal growth hormone or platelet-derived growth factor, the tyrosine residues in the cytoplasmic region recruit specific signal transducers containing SH2 domains, (28, 29). Based on the ability of phosphotyrosine-containing peptides derived from the intracellular domain of different cytokine receptors to inhibit DNA complex formation with Stats, it has been proposed that activation of Stats occurs through direct receptor-Stat interactions. These include the activation of Stat1 by the interferon-γ (13), Stat2 by the interferon-α (31), Stat6 by the IL-4R (14), and Stat5 by the β chain of the IL-2R (32), or the EPO (15). Functional analysis of systematic truncated and point mutated forms of receptors for GH and EPO have indicated that Stat proteins are required for hormone-induced gene transcription. In addition, in vitro experiments demonstrate an interaction between Stat5 and the COOH-terminal phosphotyrosine residues (Ty549, Tyr551) of the rbGH receptor (19). This association takes place between the SH2 domain of Stat5 and phosphorylated tyrosine of the GHr. When a mutated form of Stat5 was used in which a conserved arginine in the floor of the SH2 phosphotyrosyl pocket is replaced by glycine, no interaction between mutated Stat5 and GHr was detected. (19). Previous results indicated that the Y237F and Y580F (18) and the Y309F and Y402F (data not shown) mutant...
Fig. 5. Restoration of activity of inactive mutated PRLR in cells overexpressing Jak2. Upper panel, 293 cells were co-transfected with reporter cDNA (100 ng), wild type or different mutated forms of PRLR cDNA (50 ng), and with or without Jak2 cDNA (50 ng). After incubation in serum-free medium in the presence or absence of 18 nM oPRL for 24 h, cells were lysed and assayed for luciferase and β-galactosidase activities. Results are the means of ± S.E. of four independent experiments expressed in relative light units (RLU), normalized for β-galactosidase activity (RLU/βGal/h). Lower panel, 293 cells were co-transfected with wild type or different mutated forms of PRLR cDNA (2 μg), Stat5 cDNA (1 μg), and with the indicated amounts of Jak2 cDNA. Lane U untransfected cells; lane V cells transfected with Stat5 cDNA alone. Cells were incubated for 10 min at 37 °C without (−) or with (+) 18 nM oPRL. Cellular proteins were immunoprecipitated with denoted antibodies and Western blotted with anti-PY antibody. Molecular masses of the protein standards are indicated between the two panels in kDa. The arrows indicate the migration of Stat5 and PRLR.

Fig. 6. The ability of mutated PRLR to elicit PRL-dependent tyrosyl phosphorylation of Jak2. Upper panel, 293 cells expressing various forms of PRLR and Jak2 were unstimulated (−) or stimulated (+) with 18 nM oPRL for 10 min. Lysates were immunoprecipitated and Western blotted with anti-PY antibody. Lower panel, half of the precipitates were analyzed by Western blot with anti-Jak2 antibody. Molecular masses of the protein standards are indicated on the right in kDa. The arrows indicate the migration of the Jak2 protein.

forms of PRLR, in which one tyrosine was replaced by phenylalanine, are able to activate PRLR-associated tyrosine kinase Jak2 and to mediate the transcriptional activation of PRL responsive gene. Only the Y580F mutation reduced the capacity of the receptor to activate the transcription of the PRL responsive promoter. This data showed that the last tyrosine of the cytoplasmic domain is important for signal transduction. However, there are probably other tyrosines implicated in this function which account for the partial activity of the Y580F mutant. The present results show that the amino acids surrounding specific tyrosine residues of PRLR are involved in transducing PRL signals. Experiments using the 9F mutant established that tyrosine phosphorylation of the PRLR facilitates Stat5 activation and LHRE induction. Our results with the 8F-Y580 extended this observation by showing a PRLR retaining only Tyr580 is capable of both Stat5 activation and LHRE induction. Our results with the 8F-Y580 extended this observation by showing a PRLR retaining only Tyr580 is capable of both Stat5 activation and LHRE induction. Our results with the 8F-Y580 extended this observation by showing a PRLR retaining only Tyr580 is capable of both Stat5 activation and LHRE induction. Our results with the 8F-Y580 extended this observation by showing a PRLR retaining only Tyr580 is capable of both Stat5 activation and LHRE induction. Our results with the 8F-Y580 extended this observation by showing a PRLR retaining only Tyr580 is capable of both Stat5 activation and LHRE induction. Our results with the 8F-Y580 extended this observation by showing a PRLR retaining only Tyr580 is capable of both Stat5 activation and LHRE induction. Our results with the 8F-Y580 extended this observation by showing a PRLR retaining only Tyr580 is capable of both Stat5 activation and LHRE induction. Our results with the 8F-Y580 extended this observation by showing a PRLR retaining only Tyr580 is capable of both Stat5 activation and LHRE induction. Our results with the 8F-Y580 extended this observation by showing a PRLR retaining only Tyr580 is capable of both Stat5 activation and LHRE induction. Our results with the 8F-Y580 extended this observation by showing a PRLR retaining only Tyr580 is capable of both Stat5 activation and LHRE induction. Our results with the 8F-Y580 extended this observation by showing a PRLR retaining only Tyr580 is capable of both Stat5 activation and LHRE induction.

of the wild type receptor. Thus, Tyr580 appears to be the primary site responsible for Stat5 activation, however, residues Tyr479 and Tyr473 compensate, providing minimal functional activity. Among the eight intracellular tyrosines of the pGHR, five are phosphorylated upon GH stimulation and four of them, which are part of the five COOH-terminal tyrosine residues, provide the site(s) for Stat5 docking (20–22). The results reported in this study as well as past studies in several laboratories are summarized in Fig. 7 as a model comparing tyrosine docking sites of the GHR and PRL that are involved in the association of Stat5.

The prolactin receptor in the red deer and cows although truncated at the carboxyl-terminal region and lacks residues 558–591 containing the distal conserved tyrosine (Tyr580 for the rat PRLR) is functional (35). Moreover, the red deer receptor is able to induce the phosphorylation of the receptor-associated Jak 2 kinase. Thus, the cervine receptor seems to mediate the effect of prolactin on transcriptional activation through an alternative tyrosine residue or an alternative docking protein. Four out of seven tyrosine residues in the cytoplasmic domain of the red deer receptor are conserved in other species including tyrosine 479 (Tyr488 in red deer). Other tyrosine residues could play a similar role. Residue Tyr351 of the red deer, also present in the human PRLR receptor, has a motif close to that of the GH receptor containing a docking site for Stat5 protein (Lys-X-Tyr351-X-X-Glu (PRLR) versus Asn-X-Tyr338-X-X-Glu (GHR)).

Whereas phosphotyrosyl regions recognized by the SH2 domains of several signaling proteins have been identified (36), limited knowledge exists concerning the phosphotyrosyl residue preference of Stat5 SH2 domains. Regarding Stat5 recruitment by the long PRL, no similarity is seen between the sequences surrounding the three phosphorylated tyrosines in-
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Figure 7. Schematic representation showing the comparison of the tyrosine docking sites of the PRLR and GHR involved in the association of Stat5. For the two receptors several intracellular tyrosines were found to be redundantly used for the recruitment of Stat5. Five tyrosines of the GHR and six tyrosines of the PRLR are phosphorylated upon hormone stimulation. Stat5 can be recruited via its SH2 domain to four of the five COOH-terminal phosphotyrosines of the GHR: Tyr469 (487), Tyr516 (534), Tyr548 (566), and Tyr609 (627) whereas it can be recruited to three of the four COOH-terminal phosphotyrosines of the PRLR: Tyr473, Tyr479, and Tyr580. In the case of the rat GHR, tyrosine 314 (333) and/or inability to respond to interferon-α were shown to lack either Tyk2 or Jak2 kinase (39, 40). Gouilleux et al. (11) have shown that Jak kinases act both as a docking protein and tyrosine kinase for Stats as well as other phosphotyrosyl-binding signaling proteins is based upon several independent observations. To date, no case of Jak-independent signaling has been reported for any receptor. Concerning the PRLR, the COOH-terminal tyrosine seems to be the predominant docking site. In addition, Stat5 can be directly recruited by Jak2 in the model of the PRLR. A SH2 containing adaptor may be involved in the interaction processes (dashed arrows). Numbers in parentheses represent the position of the tyrosine residues according to Refs. 20–22. The positions of all intracellular tyrosine residues of the receptors are presented as a letter Y followed by a number, p, phosphotyrosine residues; bold line, nonphosphorylated tyrosine residues.
Comparison of sequences surrounding phosphorylated tyrosines involved in Stat5 recruitment present in cytoplasmic domains of members of the cytokine/PRL/GH receptor superfamily

| Receptor | Tyrosine | Consensus |
|----------|----------|-----------|
| IL-2R   | 392      | D A Y C T F P | (37) |
|         | 510      | D A Y L S L Q | (37) |
|          | 510      | D A Y x x x x | |
| EPOR    | 343      | D T V L V L D | (33,34) |
|         | 401      | E F V T I L D | (33,34) |
|         | 431      | Y L Y L V V S | (33,34) |
|         | 479      | P G Y V A C S | (33,34) |
|          | 479      | x x x x x x | |
| GHR     | 315 (333) | D S Y K P E F | (23) |
|         | 339 (338) | E F V N D D S | (23) |
|         | 469 (487) | D F V A Q V S | (19-22) |
|         | 516 (534) | N A V F C E A | (19-22) |
|         | 566 (548) | D I V I T T E | (19-22) |
|         | 609 (627) | C G Y V S T D | (19-22) |
|          | 609      | - x x x x x | |
| PRL     | 473      | T V Y V K K P | |
|         | 479      | P D Y V E I H | |
|         | 479      | x x x x x x | |
|          | 580      | L D V L D P T | |

Amino acid residues are represented by single letters. The location of the tyrosine in the primary sequence is indicated in bold, surrounded by a box. The number indicating the position of the tyrosine residue is based on the first amino acid being the first residue of the mature protein, without signal peptide; in parentheses with the signal peptide according to Refs. 20–23. Amino acid residues that are not conserved among tyrosine motifs of each receptor are represented with x in the consensus. Φ represents hydrophobic amino acid residues; –, acidic amino acid residues; h, uncharged polar amino acid residues.

action as determined in Chinese hamster ovary cells that over-expressed Jak1, Jak2, or Jak3 cDNA (44). In addition, some reports suggest that the regulation of Jak3, a member of Jak family preferentially expressed in hematopoietic cells, is probably important in the differentiation and activity of these cells. Thus, the level of expression of Jak3 in normal human peripheral blood B cells (45) and in human monocytes (46) was found to be markedly up-regulated following stimulation. However, Jak3 expression was strongly induced during activation by interferon-γ. Finally, Fujitani et al. (47) found that the JH2 domain of Jak1, Jak2, or Jak3 could specifically associate with carboxyl-terminal portion of Stat5. These results indicate that Stat5 is activated through cytokine receptors by two distinct mechanisms, one dependent on receptor tyrosine phosphorylation and the other mediated by the direct Jak-Stat interaction (Fig. 7).

Multiple sites of phosphorylation with different affinities for various SH2 domains would provide a mechanism by which PRL could initiate several signaling pathways simultaneously. Thus, receptor sequences involved in Stat1, Stat2, Stat3, Stat5, and Stat6 activation appear to be different and could explain the specific activation of a Stat and the diversity of effects induced by a cytokine. Thus, it is important to determine whether tyrosines of the intracellular domain are associated with other cytoplasmic proteins. Accordingly, it should be noted that the sequence surrounding Tyr[402] which is phosphorylated in PRLR is in good agreement first, with the sequence of the phosphorylation site for tyrosine kinase receptors (36, 48), second, with the YXXQ motif involved in Stat3 activation (17), and third, with the YXXP motif described for Stat1 binding (49). In addition, the sequence surrounding Tyr[402] (Arg-Ser-X-Tyr[402] -Ser-X-Ala-Asp) is very close to the specific recognition site of the SH2 domain of IRS-1 (Asn-Pro-X-Tyr[IRS-1]-X-Ser-X-Ser-Asp) (50). Other tyrosines of the PRLR that are not implicated in Stat5 recruitment are phosphorylated upon PRL stimulation and could serve as docking sites for other proteins containing an SH2 domain. The identification of the putative tyrosines used by the PRLR to recruit the Stat1, Stat3, and IRS-1 proteins (51) involved in the PRL signaling pathway is currently under investigation.

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