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Identification of JAK protein tyrosine kinases as signaling molecules for prolactin. Functional analysis of prolactin receptor and prolactin-erythropoietin receptor chimera expressed in lymphoid cells

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The mechanism of action of prolactin (PRL) was studied in murine lymphoid BAF-3 cells transfected with either the long form of the PRL receptor (PRL-R), or a chimeric receptor consisting of the extracellular domain of the PRL-R and the transmembrane and intracellular domain of the erythropoietin receptor (PRL/EPO-R). PRL sustained normal and long-term proliferation of BAF-3 cells expressing either the PRL-R or the hybrid PRL/EPO-R. Upon [^{125}I]PRL cross-linking, both types of BAF-3 transfectants were shown to express two [^{125}I]PRL cross-linked species differing in size by 20 kDa. These cross-linked complexes, after denaturation, were recognized by antibody against the PRL-R, indicating that they contain the transfected receptor. PRL induced rapid and transient tyrosine phosphorylation of both the PRL-R and the PRL/EPO-R in BAF-3 transfectants. Furthermore, PRL induced rapid tyrosine phosphorylation of the Janus kinase 2 (JAK2) which was already physically associated with the PRL-R or the PRL/EPO-R in the absence of ligand. JAK1 was also associated with PRL-R and PRL/EPO-R in the absence of ligand. However, only in BAF-3 cells expressing the PRL-R does PRL induce rapid and transient tyrosine phosphorylation of JAK1. These results demonstrate that JAK protein tyrosine kinases couple PRL binding to tyrosine phosphorylation and proliferation.

Key words: cytokines/JAKs/kinases/lymphocytes/prolactin/receptors

Introduction

The pituitary hormone prolactin (PRL) is involved in a variety of biological functions, including lactation, reproduction and osmoregulation in male and female vertebrates (Nicoll, 1980). In addition, an increasing body of evidence indicates that PRL plays an immunomodulatory role in mammals (for a review see Gala, 1991; Hooghe *et al.*, 1993). Two types of PRL receptors (PRL-R) have been cloned from different organs and species, differing markedly in the size of their cytoplasmic domains (Kelly *et al.*, 1991). These intracellular domains vary from 57 residues in the rat liver (Boutin *et al.*, 1988) to 358 residues in the rabbit mammary gland (Edery *et al.*, 1989). Interestingly, PRL-Rs are members of the recently described cytokine receptor superfamily which includes, apart from the growth hormone (GH) receptor, numerous hematopoietic receptors such as receptors for interleukin (IL)-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, erythropoietin (EPO), granulocyte-(G-CSF) and granulo-macrophage colony-stimulating factor (GM-CSF), leukemia inhibitory factor, mpl and the receptor for ciliary neurotrophic factor (Bazan, 1990; Cosman *et al.*, 1990; Taga and Kishimoto, 1992). All these receptors exhibit conserved motifs in their extracellular domains, including four cysteine residues and a tryptophan-serine (WSXWS) motif. They all have low sequence homology in their cytoplasmic domain, in particular no consensus sequences for serine/threonine or tyrosine kinases; however, they contain a proline-rich sequence located in their juxtamembrane domain. These receptors are distantly related to the receptors for interferons (IFNs) (Taga and Kishimoto, 1992). Numerous cytokines have been shown to induce rapid and transient tyrosine phosphorylation of specific proteins in target cells including their own receptors, indicating the involvement of specific tyrosine kinase(s) activation in their signaling pathways (reviewed by Miyajima *et al.*, 1992; Taga and Kishimoto, 1992). Thus, there has been considerable interest in identifying tyrosine-phosphorylated proteins induced by ligand binding, as well as protein-tyrosine kinase(s) that associate(s) with ligand-activated receptors. Src family tyrosine kinases were shown to be activated by IL-2, IL-3 and IL-7 (Miyajima *et al.*, 1992, 1993; Venkitaramn and Cowling, 1992; Kobayashi *et al.*, 1993), and a tyrosine kinase of 97 kDa has been reported to be activated by a number of cytokines (Linnekin *et al.*, 1992).

A major advance in this field has come from the recent discovery that members of a newly described family of cytoplasmic tyrosine kinases, the Janus kinases (JAK) family (Wilks *et al.*, 1991), are involved in signal transduction by several receptors lacking tyrosine kinase domains. Genetic complementation of mutant cell lines defective in both interferons alpha/beta (IFNα/β) and/or interferon gamma (IFNγ) signaling have demonstrated the involvement of the three known members of the JAK family: Tyk2, JAK1 and JAK2 (Velazquez *et al.*, 1992; Muller *et al.*, 1993; Watling *et al.*, 1993). JAK2 has been shown to participate in the signaling pathways elicited by EPO, GH and IL-3 (Argetsinger *et al.*, 1993; Silvennoinen *et al.*, 1993; Witthuhn *et al.*, 1993).

Despite the cloning of PRL-Rs and extensive studies in various PRL target cells, the mechanism of signal transduction involved in PRL action remains poorly understood. One PRL-responsive cell line, the PRL-

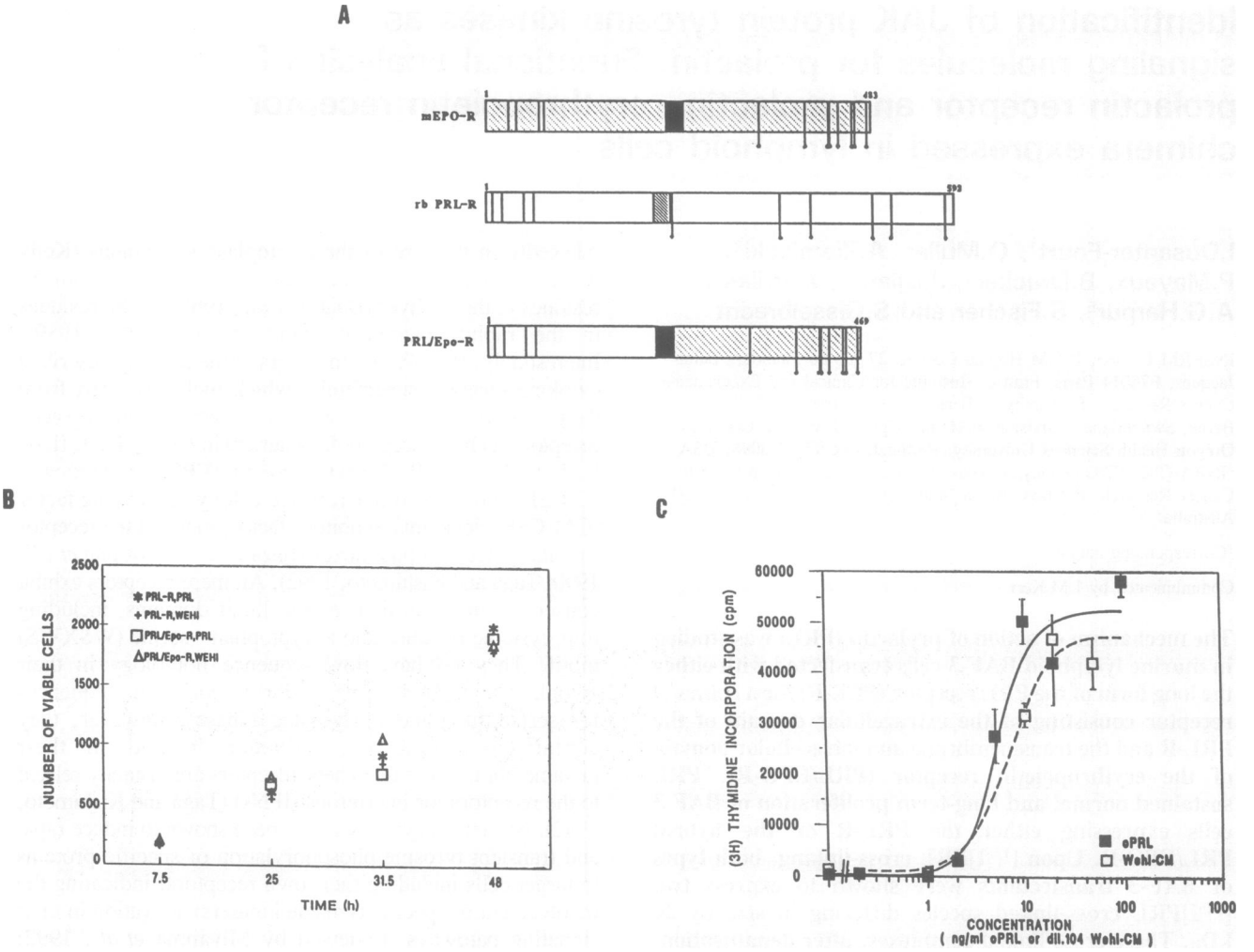

Fig. 1. PRL responsiveness of the PRL-R-transfected BAF-3 cells. (**A**) Schematic representation of PRL-R, EPO-R and the chimeric PRL/EPO-R. Numbers indicate the first and the last amino acid of each coding sequence. Transmembrane domains are indicated by hatched (PRL-R) or black (EPO-R and PRL-EPO-R) boxes. Bars in the extracellular domains indicate positions of conserved cysteine residues. Positions of tyrosine residues in the intracellular receptor domains are also indicated (↓). (**B**) BAF-3 cells expressing PRL-R (+, *) or PRL/EPO-R (□, △) were incubated in RPMI-1640 supplemented with either PRL (*, □, 0.5 nM) or Wehi-CM (+, △, 5%) for various periods of time and the number of viable cells was determined by trypan blue exclusion. (**C**) PRL-R-transfected BAF-3 cells were incubated in the presence of increasing concentrations of PRL or Wehi conditioned medium (Wehi-CM) as a source of IL-3, and pulse-labeled with [^{3}H]thymidine. Cell-associated radioactivity was determined. Mean ± SD (n = 3).

dependent rat T lymphoma cell line Nb2, has been used. In Nb2 cells, PRL was shown to induce the activation of specific immediate-early genes and rapid tyrosine phosphorylations of a few proteins (Yu-Lee *et al.*, 1990; Rillema *et al.*, 1992; Rui *et al.*, 1992). However, these cells exhibit an abnormally high responsiveness to PRL which may be related to the expression of a truncated form of the PRL-R, having a deletion of 198 amino acids in the middle of its cytoplasmic domain (Ali *et al.*, 1991).

Given the structural similarity between PRL-R and hematopoietic receptors, and to assess the biological functionality of the long form of the PRL-R in the hematopoietic system, we introduced the rabbit mammary PRL-R cDNA (Edery *et al.*, 1989) into the BAF-3 IL-3-dependent pro-B lymphoid murine cell line. In contrast to parental cells, PRL-R transfectants were shown to proliferate at a high rate in the presence of PRL and in the absence of IL-3. Since BAF-3 cells expressing the PRL-R responded well to PRL stimulation, they were used as a model system to assay the signaling activity of the full-length long-form PRL-R. In addition, in order to more precisely analyze which domain of the PRL-R is involved in transducing mechanisms and to compare the PRL-R signaling pathway to the better characterized EPO receptor, a hybrid receptor made of the PRL-R extracellular domain and the EPO-R transmembrane and cytoplasmic domains was constructed (PRL/EPO-R) and transfected into BAF-3 cells. The signaling pathways induced by PRL triggering these two PRL-responsive receptors—either the PRL-R containing the normal long PRL-R cytoplasmic domain or the chimeric receptor containing the EPO-R cytoplasmic domain—were analyzed and compared.

Results

PRL mitogenic action

The bone marrow-derived murine pro-B lymphoid BAF-3 cell line is strictly dependent on the presence of IL-3 for its proliferation. These cells do not express endogenous PRL receptors and do not proliferate in the presence of PRL alone

(not shown). To examine the functionality of the long form of the PRL-R expressed in these cells, the rabbit PRL-R cDNA (Figure 1A) cloned in the pBabeNeo retroviral vector was introduced into the BAF-3 cells. All PRL-R-transfected cells grew rapidly in the presence of 0.5 nM ovine PRL (oPRL) as opposed to parental or mock-transfected BAF-3 cells which died in <3 days. The rate of proliferation of the PRL-R transfectants was quantitated by counting the number of viable cells in the presence of either PRL or IL-3. PRL-R-transfected cells grew as fast as the parental cells when cultivated in the presence of either IL-3 or PRL with a doubling time of 12 h (Figure 1B). The dose−response of the mitogenic signal transduced by the full-length PRL-R was characterized by a [^{3}H]thymidine incorporation assay (Figure 1C). As expected, transfected cells showed stimulation of DNA synthesis in response to PRL and IL-3, the EC_{50} being 0.1 nM for PRL. No [^{3}H]thymidine incorporation was detectable in the absence of PRL or IL-3, indicating that the transfectants were strictly dependent on IL-3 or PRL for their growth. PRL-R transfectants have been maintained in the presence of PRL exclusively (0.5 nM, 10 ng/ml) and have remained PRL and IL-3 responsive for more than a year.

BAF-3 cells transfected with the EPO-R proliferate normally in response to EPO (D'Andrea *et al.*, 1991 and personal observations). Therefore, to better understand how PRL stimulates the proliferation of BAF-3 cells and to compare the signaling pathways triggered by PRL, a chimeric receptor composed of the extracellular part of the rabbit PRL-R and the transmembrane and intracellular part of the EPO-R was constructed (PRL/EPO-R; Figure 1A), subcloned in the same vector as PRL-R and introduced in the IL-3-dependent BAF-3 cells. When culture medium was supplemented with PRL instead of IL-3, cells expressing the chimeric receptor were able to proliferate and exhibited a 12 h doubling time, similar to parental cells in the presence of IL-3 (Figure 1B).

Characteristics of the PRL and PRL/EPO receptors in BAF-3 cells

Transfected cells were characterized for PRL binding properties. PRL-R-transfected BAF-3 cells (Figure 2) and PRL/EPO-R-transfected cells (data not shown) expressed 2000−4000 PRL binding molecules/cell, which were of low affinity for PRL [K_d = 2.0 ± 0.4 nM (n = 4), see the insert in Figure 2]. PRL binding was displaced by two different monoclonal antibodies, M110 and A917, directed against the rabbit mammary PRL-R. These transfected receptors exhibited the same strict specificity as the mammary receptor for binding lactogenic hormones: the human growth hormone and the ovine placental lactogen bound to these receptors, as opposed to bovine growth hormone or insulin (data not shown).

Expression of the full-length PRL-R was examined by immunoprecipitation from metabolically labeled cells (Figure 3A). The PRL-R appeared as a protein of ~100 kDa, recognized by both monoclonal A917 (lane 2) and polyclonal S46 (lane 3) anti-PRL-R antibodies. Cross-linking experiments were undertaken to confirm the identity of the PRL binding molecules and to study interactions with accessory proteins (Figure 3B). Using the very efficient carbodiimide cross-linker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), a single PRL binding

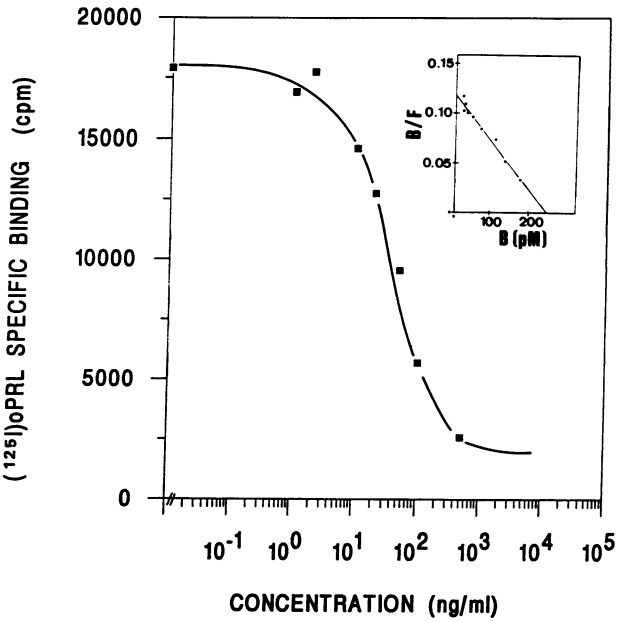

Fig. 2. PRL binding characteristics of the PRL-R expressed on BAF-3 cells. PRL receptor-transfected BAF-3 cells (5×10^6 cells/point) were incubated with [^{125}I]PRL in the presence of increasing concentrations of the unlabeled hormone. Specific cell-associated radioactivity was measured. Results were subsequently analyzed by the method of Scatchard (insert). One representative experiment among four others.

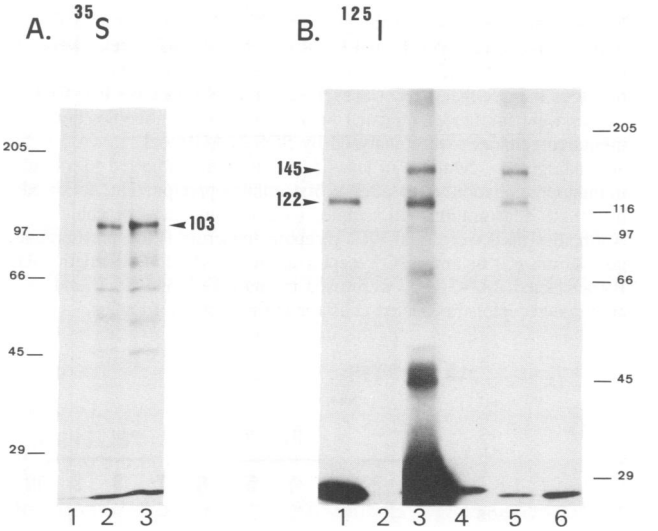

Fig. 3. Expression of PRL-R in BAF-3 cells. **(A)** PRL-R-transfected BAF-3 cells were metabolically labeled and PRL-R immunoprecipitated with the indicated antibodies. Immunoprecipitates were subjected to SDS−PAGE under reducing conditions followed by fluorography. Lane 1: pre-immune serum; lane 2: monoclonal anti-PRL receptor antibody A917; lane 3: anti-PRL receptor antiserum S46. Positions of size standards (in kDa) are shown on the left, and the relative molecular weight of the indicated band on the right ($\times 10^{-3}$). **(B)** PRL-R-transfected BAF-3 cells were incubated with [^{125}I]PRL in the absence (lanes 1, 3 and 5−6) or presence (lanes 2 and 4) of a 200-fold excess of unlabeled PRL. Cross-linking was allowed to proceed in the presence of either the EDC cross-linker (lanes 1−2) or the DSS cross-linker (lanes 3−6). All cross-linked PRL-R complexes were first immunoprecipitated with anti-PRL-R antibody S46 and solubilized in SDS sample buffer. Samples in lanes 5 and 6 were subjected to a second immunoprecipitation assay and re-incubated with either anti-PRL-R antiserum S46 (lane 5) or pre-immune serum (lane 6). All samples were analyzed by SDS−PAGE under reducing conditions, followed by autoradiography. Positions of size standards (kDa) are shown on the right. The relative molecular weights of the ^{125}I-labeled complexes are indicated on the left ($\times 10^{-3}$).

protein of 122 kDa was detected, corresponding to the cross-linked complex of receptor (100 kDa) and one molecule of PRL (22 kDa) (Figure 3B, lane 1). Using the cross-linker disuccinimidyl suberate (DSS), however, a second [^{125}I]PRL–receptor complex of 145 kDa was identified in addition to the 122 kDa complex. Similar results were also obtained using the cross-linker ethylene-glycolbis(succinimidyl)succinate (EGS) (data not shown). Both the 122 and 145 kDa complexes were precipitated by the polyclonal anti-PRL-R antibody S46 (Figure 3B, lane 3), and by the monoclonal antibody A917 (data not shown). The relationship between these two receptor complexes was further substantiated by re-precipitation of SDS-solubilized anti-PRL-R immunoprecipitates with anti-PRL-R serum (lane 5).

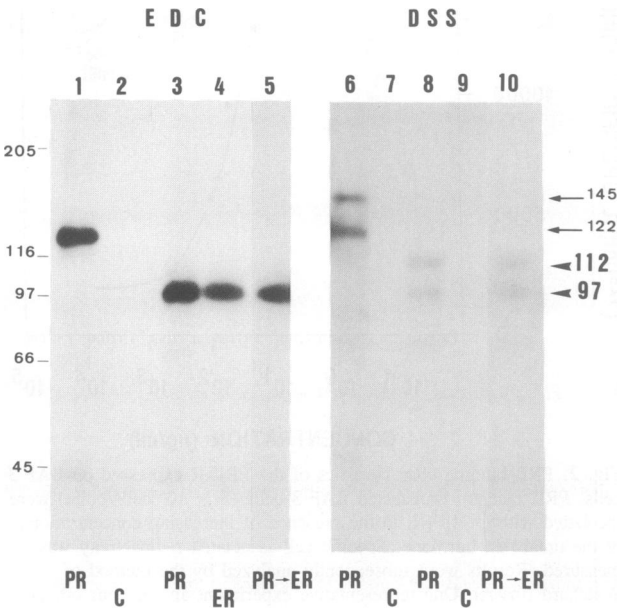

Fig. 4. Characterization of the chimeric PRL-R/EPO-R expressed in BAF-3 cells. PRL/EPO-R- or PRL-R-transfected BAF-3 cells were incubated with [^{125}I]PRL and further subjected to cross-linking reactions using either EDC (lanes 1–5) or DSS (lanes 6–10). Cell lysates were immunoprecipitated with the indicated antibodies and immunoprecipitates were analyzed by SDS–PAGE and autoradiography. Samples in lanes 5 and 10 were subjected to a double immunoprecipitation assay after a first immunoprecipitation, followed by a SDS denaturation. All samples were run on the same gel. Antibodies used were: anti-PRL receptor antiserum (PR), pre-immune goat serum (C) or anti-EPO receptor antiserum (ER). Full-length PRL-R-transfected BAF-3 cells were used in lanes 1–2 and 6–7, and chimeric receptor-transfected cells for the rest of the samples.

The expression of the chimeric PRL-R/EPO-R receptor was also studied by similar cross-linking experiments (Figure 4). Using the cross-linker EDC, a single [^{125}I]PRL–receptor complex of the expected size was detected (lanes 3–5), corresponding to the chimeric receptor (80 kDa) and one molecule of PRL (22 kDa). This receptor size is much smaller than that of the full-length PRL-R because of the smaller intracellular domain of EPO-R (237 amino acids) as compared with that of PRL-R (358 amino acids) (see Figure 1A). When DSS was used to cross-link, a second [^{125}I]PRL-bound receptor complex was identified ~20 kDa larger (Figure 4, lanes 8–10). Both [^{125}I]PRL–receptor complexes were immunoprecipitated by antibodies directed against either the PRL-R extracellular domain (lane 8) or the EPO-R intracellular domain (not shown). Furthermore, immunoprecipitation with anti-PRL-R antibodies, followed by solubilization in SDS sample buffer and re-precipitation with anti-EPO-R antibodies, yielded the same two [^{125}I]PRL complexes (lane 10). These results show that each of the two DSS-cross-linked complexes contained the chimeric receptor, and the higher-sized complex contained an additional molecule of 20 kDa.

PRL-dependent tyrosine phosphorylation of the PRL receptors

Since BAF-3 cells expressing the full-length PRL-R and PRL/EPO-R chimera respond well to PRL stimulation, they were used to study the PRL signaling pathways triggered

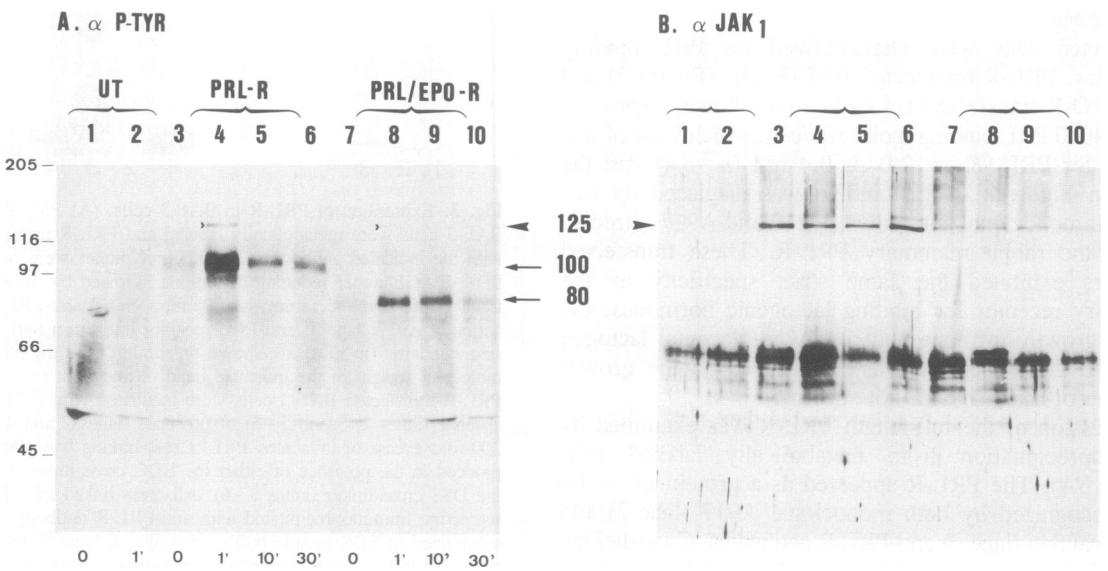

Fig. 5. PRL induced tyrosine phosphorylation of its receptor. Untransfected (lanes 1–2), PRL-R-transfected (lanes 3–6) or PRL/EPO-R-transfected BAF-3 cells (lanes 7–10) were treated with PRL (1 μg/ml) for the indicated time and cells lysates were immunoprecipitated with anti-PRL-R antibody S46. Samples were analyzed by SDS–PAGE under reducing conditions, followed by immunoblotting with (**A**) anti-phosphotyrosine antibody and then (**B**) stripping and re-blotting with anti-JAK1 antibody. Molecular weight standards are indicated on the left (kDa).

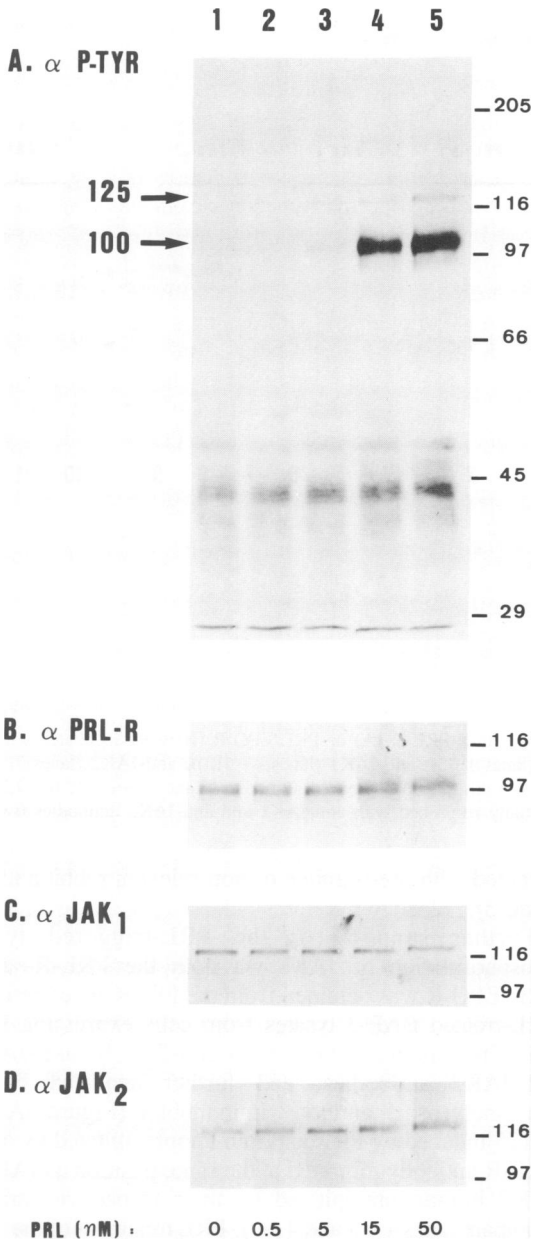

Fig. 6. Dose dependence of PRL-induced tyrosine phosphorylations of the PRL receptor and its associated proteins. PRL-R transfectants were incubated with various concentrations of PRL for 1 min at 37°C. Cell lysates were immunoprecipitated with anti PRL-R antibody (S46) and analyzed by SDS−PAGE and immunoblotting. Blots were incubated with (**A**) anti-phosphotyrosine; (**B**) anti-PRL-R S46; (**C**) anti-JAK1 and (**D**) anti-JAK2 antibodies. Concentrations of PRL used were: 0 (lane 1), 0.5 nM (lane 2), 5 nM (lane 3), 15 nM (lane 4) and 50 nM (lane 5).

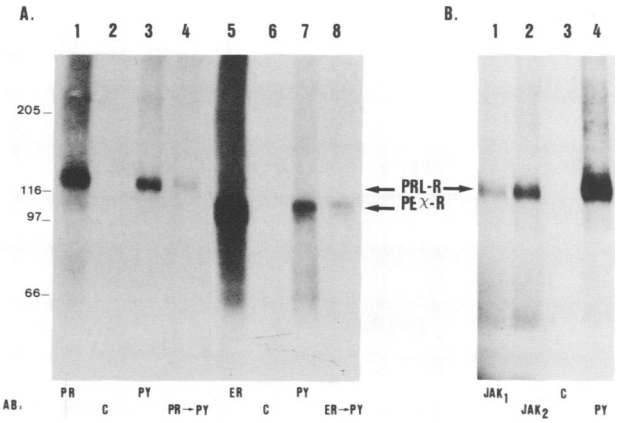

Fig. 7. The [^{125}I]PRL cross-linked complexes are tyrosine phosphorylated and associated with JAKs. (**A**) PRL-R- (lanes 1−4) or PRL/EPO-R-transfected cells (lanes 5−8) were incubated with [^{125}I]PRL for 10 min, cross-linked with EDC, and cell lysates were immunoprecipitated with either pre-immune goat serum (C) or anti-PRL-R (PR), anti-EPO-R (ER), anti-phosphotyrosine (PY) antibodies followed by solubilization in boiling SDS sample buffer. Samples were either analyzed directly on SDS−PAGE (lanes 1−3 and 5−7) or further subjected to a second immunoprecipitation assay using anti-phosphotyrosine antibodies (lanes 4 and 8). All samples were run on the same gel. Radioactive complexes were detected by autoradiography. (**B**) PRL-R-transfected BAF-3 cells were processed as indicated in (A) and immunoprecipitated with either a non-relevant rabbit antiserum (C) or anti-JAK1 (JAK1), anti-JAK2 (JAK2) or anti-phosphotyrosine (PY) antibody. PE-R = PRL/EPO-R.

by these two different PRL-responsive receptors. First, we investigated whether PRL induced tyrosine phosphorylation of proteins associated with the PRL-R or PRL/EPO-R (Figures 5 and 6). Proteins solubilized from PRL-treated BAF-3 transfectants were immunoprecipitated with the anti-PRL-R antibody S46, the proteins resolved by SDS−PAGE and Western immunoblotted with anti-phosphotyrosine antibody. PRL-dependent tyrosine phosphorylation of two proteins, of 100 and 125 kDa in the full-length PRL-R transfectants, and 80 and 125 kDa in the chimeric receptor transfectants, were reproducibly detected at times as early as 1 min (Figure 5A, lanes 3−10). These phosphorylations were transient, returning to control values after 60 min. No tyrosine-phosphorylated proteins were detected in anti-PRL-R immunoprecipitates from untransfected parental BAF-3 cells treated with PRL (Figure 5A, lanes 1−2). These tyrosine phosphorylations were dose dependent on PRL, and were detected at concentrations as low as 1 nM PRL (20 ng/ml) (Figure 6A). The 100 and 80 kDa species were identified as the PRL-R and the PRL/EPO-R, respectively, by reprobing the blots with anti-PRL-R antibody S46 (Figure 6B and data not shown).

PRL-induced tyrosine phosphorylation of the PRL-R and the PRL/EPO-R was further confirmed by cross-linking and immunoprecipitation (Figure 7A). BAF-3 cells were incubated with [^{125}I]PRL, cross-linked with EDC, solubilized and immunoprecipitated with either anti-PRL receptor or anti-phosphotyrosine antibodies. The radioactivity immunoprecipitated by both antibodies revealed that ~30% of the occupied receptors were tyrosine phosphorylated after 10 min of PRL incubation (compare Figure 7A, lanes 1 and 3). When [^{125}I]oPRL cross-linked complexes were first immunoprecipitated with anti-PRL-R antibodies, denaturated in SDS sample buffer and re-incubated in the presence of anti-phosphotyrosine antibodies (double immunoprecipitation experiment), the PRL receptor could still be immunoprecipitated by anti-phosphotyrosine antibodies (lane 4), indicating that the PRL receptor itself was tyrosine phosphorylated following ligand binding. Similar results were obtained with the chimeric receptor-expressing cells (Figure 7A, lanes 5−8), indicating that PRL can trigger the tyrosine phosphorylation of the chimeric receptor.

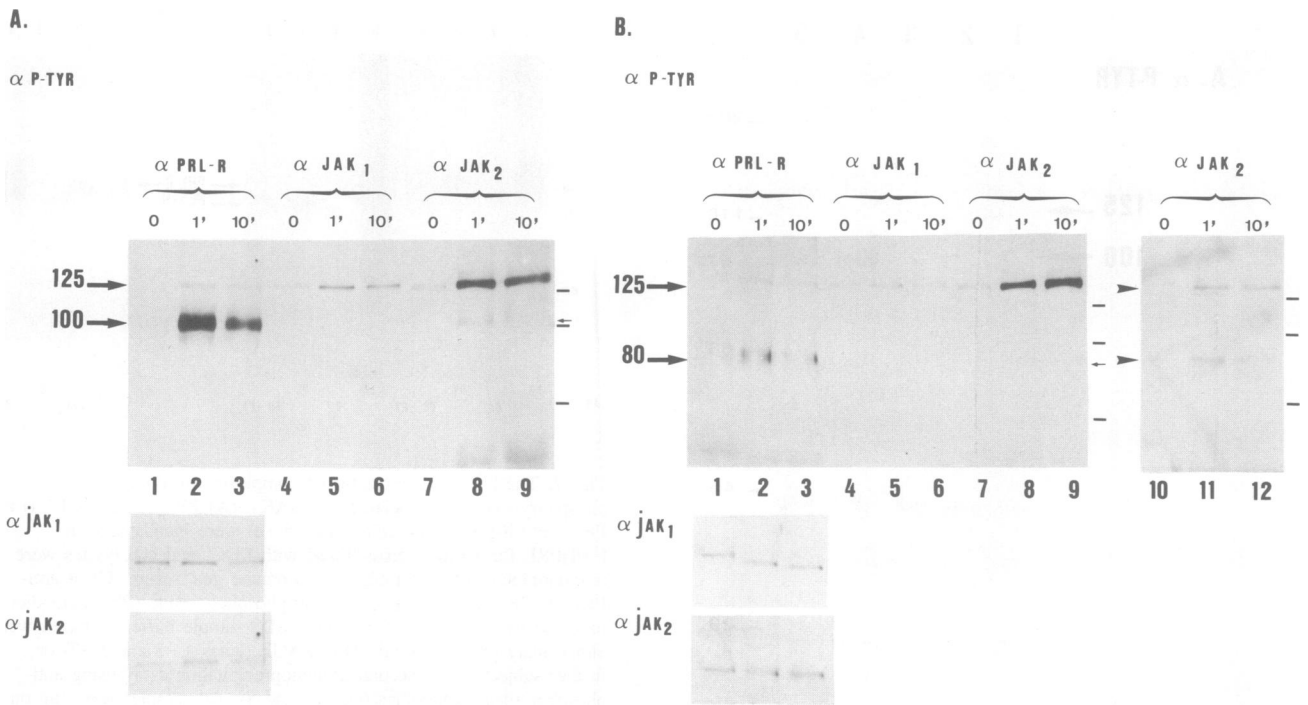

Fig. 8. PRL induces the tyrosine phosphorylation of JAKs. BAF-3 cells expressing either PRL-R (**A**) or PRL/EPO-R (**B**) were incubated with PRL (1 μg/ml) for the indicated time, lysed and immunoprecipitated with anti-PRL-R (lanes 1–3), anti-JAK1 (lanes 4–6), or anti-JAK2 (lanes 7–12). Immunoprecipitates were analyzed by SDS–PAGE followed by immunoblotting with anti-phosphotyrosine antibodies. Lanes 7–9 and 10–12 in (B) represent two separate experiments. Subsequently, blots (lanes 1–3) were sequentially re-probed with anti-JAK1 and anti-JAK2 antibodies (two lower panels).

JAK1 and JAK2 are associated with the PRL receptor and tyrosine phosphorylated following ligand binding

The size of the second tyrosine-phosphorylated protein (125 kDa, pp125) induced by PRL and co-precipitated with the full-length or the chimeric PRL receptor suggested that it could be a member of the recently described JAK family of protein tyrosine kinases. To test this hypothesis, we first investigated whether the JAK(s) were associated with PRL-R. Anti-PRL-R immunoprecipitates from PRL-treated BAF-3 lysates were probed for the presence of JAK1 or JAK2 by sequentially immunoblotting the same filter (Figures 5A and 6A) with anti-JAK1 or anti-JAK2 antibodies (Figures 5B, 6C and D). The antibodies we used have been shown to be totally specific for the respective JAK and do not cross-react (Muller *et al.*, 1993; Silvennoinen *et al.*, 1993). As shown in Figure 5B, lane 3, or Figure 6C and D, lane 1, even in the absence of PRL both JAK1 and JAK2 were present in the anti-PRL-R immunoprecipitates from BAF-3 cells expressing PRL-R, indicating that these kinases are pre-associated with the PRL-R. However, the amount of JAK2 associated with the receptor was increased somewhat in the presence of PRL, depending on the PRL concentrations (compare Figure 6D, lanes 1–5) and the time of action (Figure 8A, bottom panel). As expected, anti-PRL-R immunoprecipitates from untransfected parental BAF-3 cells did not contain any JAKs (Figure 5B, lanes 1–2). To strengthen these results, we next checked whether anti-JAK1 or anti-JAK2 antibodies could immunoprecipitate [^{125}I]PRL cross-linked receptor complexes. Both anti-JAK1 and anti-JAK2 antibodies were able to immunoprecipitate [^{125}I]PRL–receptor complexes, although anti-JAK1 antibodies were less effective (Figure 7B, lanes 1–2). No precipitation of any [^{125}I]PRL–receptor complexes was detected with pre-immune or non-relevant rabbit antiserum (lane 3).

Further support for the PRL-triggered tyrosine phosphorylation of JAKs via both the PRL-R and the PRL/EPO-R was obtained from the following experiments. PRL-treated BAF-3 lysates from cells expressing PRL-R were immunoprecipitated with anti-PRL-R, anti-JAK1 or anti-JAK2 antibodies, and further analyzed by anti-phosphotyrosine antibody immunoblot (Figure 8A). The pp125 induced by PRL and immunoprecipitated by an anti-PRL-R antibody migrated at the same position as JAK1 and JAK2 immunoprecipitated by their respective antibodies (compare lanes 1–3 and 4–9). PRL treatment of the BAF-3 transfectants induced rapid tyrosine phosphorylation of both JAK1 and JAK2, with similar kinetics as seen for the PRL-R (compare lanes 1–3 and 4–9 in Figure 8A). The PRL-induced tyrosine phosphorylation of JAK2 was significantly higher than that of JAK1. In addition to JAK2, the anti-JAK2 antibodies co-precipitated another tyrosine-phosphorylated protein of exactly the same size as the pp100 PRL-R (see the black arrow on the right-hand side of Figure 8A, lanes 7–9). This band probably represents co-precipitating PRL-R. However, in our experimental conditions only a small amount of pp100 was associated with JAK2, as suggested in the co-precipitation assays (compare pp100 from lanes 3 and 9 in Figure 8A) and considering that the anti-PRL-R antibody has low efficiency in immunoblot, we could not detect the presence of PRL-R in the JAK2 immunoprecipitates by immunoblotting.

Using the same approach, we analyzed the action of PRL on the JAKs in cells expressing the PRL/EPO-R chimera (Figure 8B). In these cells, in addition to the chimera (lanes 1–3), PRL triggered the rapid tyrosine phosphorylation of

JAK2 (Figure 8B, lanes 7–9 and 10–12). In addition, anti-JAK2 antibodies co-precipitated an 80 kDa protein that was tyrosine phosphorylated after PRL action, of the same size as the chimeric receptor (lanes 8–10). As shown in Figure 8B, lanes 7–9 and 10–12, although this co-precipitation was sometimes difficult to see, it was always detectable. Interestingly, under our experimental conditions PRL did not induce a significant increase in the tyrosine phosphorylation of JAK1 (Figure 8B, lanes 4–6). Similar to our findings with the full-length PRL-R, JAK1 and JAK2 were found pre-associated with the chimeric receptor, as indicated by sequentially reprobing the blots with anti-JAK1 and JAK2 antibodies (see Figure 5B, lanes 7–10, and Figure 8B, lane 1, in the two lower panels).

Discussion

Our results provide clear evidence that the cDNA coding for the long form of the mammalian PRL-R expressed in the pro-B lymphoid BAF-3 cells allows long-term proliferation of these cells in the presence of PRL. Similar results were recently obtained upon transfection of the PRL-R in the FDCP1 IL-3-dependent myeloid cell line (Fuh *et al.*, 1993), indicating that the functionality of an exogenously expressed PRL-R in hematopoietic cells is not restricted to a lymphoid context. On the contrary, the epidermal growth factor receptor (EGF-R), possessing intrinsic tyrosine kinase activity and normally expressed on epithelial cells like PRL-R, was unable to induce mitosis when introduced and activated in BAF-3 cells (Shibuya *et al.*, 1992). PRL was also able to stimulate BAF-3 cells to proliferate when these cells expressed the chimeric PRL/EPO-R, indicating that regions from PRL-R and another cytokine receptor, despite their low structural similarities, can be functionally interchangeable for inducing mitosis. Similar results were previously reported for chimeric receptors made of the GH-R extracellular domain and the GCSF-R intracellular domain (Ishizaka-Ikeda *et al.*, 1993) or chimeric IL-3-R/IL-2-R receptor (Sakamaki *et al.*, 1993), indicating that this complementarity between cytokine receptor domains is a more general phenomenon.

Despite its biological activity, the PRL-R expressed on the BAF-3 transfectants exhibited a low affinity for PRL. Accordingly, the half-maximal mitogenic activity of PRL in BAF-3 cells (EC_{50} = 0.1 nM, see Figure 1) reflects 5% of the PRL receptors' occupancy. The transfected PRL-R was identified by immunoprecipitation with a polyclonal and two monoclonal anti-PRL-R antibodies both from ^{35}S-labeled cells, or from [^{125}I]PRL cross-linked to the receptor, as a 100 kDa protein. This size is 30 kDa larger than that predicted for the cloned rabbit mammary PRL receptor, but is in agreement with the size reported for the rabbit PRL-R expressed in transfected Cos-7 and insect cells (Lesueur *et al.*, 1991; Cahoreau *et al.*, 1992). Using the longer-armed cross-linking agents DSS or EGS, a second 145 kDa [^{125}I]PRL-bound complex was identified in addition to the complex formed between the 100 kDa PRL-R and [^{125}I]PRL (22 kDa). As far as we are aware, such a complex has not been reported in any PRL-R-expressing cells (transfectant or not). In order to understand the nature of this second PRL cross-linked complex, we carried out a number of double immunoprecipitation experiments. All our experiments indicated that the higher-sized PRL-bound complex contained the transfected PRL-R linked to one molecule of [^{125}I]PRL plus an additional 20 kDa molecule. Because of the mol. wt of the PRL (22 kDa), one attractive possibility would be that PRL dimers are formed and bound to the receptor. However, the exact identity of this added molecule is presently unknown. Whether it participates in PRL signal transduction or receptor dimerization is currently under investigation.

The PRL-responsive lymphoid cell lines established by us have provided a simple and sensitive assay system to elucidate the signal transduction mechanism mediated by the normal long PRL-R form, as well as the chimeric PRL/EPO-R. Using two different approaches, either cross-linking experiments followed by double immunoprecipitation with anti-phosphotyrosine antibodies (Figure 7), or immunoprecipitation followed by immunoblotting (Figures 5, 6 and 8), we show clearly that PRL induces rapid tyrosine phosphorylation of both the full-length PRL-R and the chimeric PRL/EPO-R. Thus, PRL can bind to the PRL-specific extracellular receptor domain and trigger the tyrosine phosphorylation of either the PRL-R or the EPO-R intracellular domain. As far as we are aware, this is the first observation that PRL induces the rapid and transient tyrosine phosphorylation of its own receptor. Two recent reports, both working with the highly PRL-responsive Nb2 lymphoma cells, have indicated that PRL induces low levels of tyrosine phosphorylation of its own receptor which are barely detectable and only evident after a long time (>60 min) of PRL incubation (Rillema *et al.*, 1992; Rui *et al.*, 1992). Many cytokines, including EPO (Dusanter-Fourt *et al.*, 1992) and GH (Carter-Su *et al.*, 1989; Silva *et al.*, 1993), have been shown to induce tyrosine phosphorylation of their own receptors and numerous other cellular proteins, despite being devoid of kinase activity. For receptors with intrinsic tyrosine kinase activity, these phosphorylated tyrosine residues become potential docking sites for many intracellular transducing molecules containing specific src homology domain 2 motifs (SH2 domains) (Cantley *et al.*, 1991). Similar events might take place on the phosphorylated cytokine receptors and this justifies efforts to identify PRL-R-associated molecules and the tyrosine kinase(s) involved.

A tyrosine-phosphorylated protein of ~125 kDa was reproducibly observed induced by PRL and co-precipitated with the full-length or the chimeric PRL receptor. Because of its size, we focused our attention on the kinases of the JAK family. Using three different experimental approaches, our data clearly indicate that JAK2 associates with the PRL-R and the PRL/EPO-R both in the absence and presence of the ligand: (i) immunoblotting with anti-JAK2 antibody detected JAK2 in anti-PRL-R immunoprecipitates (Figure 6D); (ii) tyrosine-phosphorylated molecules of the same size as PRL-R, PRL/EPO-R and JAK2 were co-precipitated with either anti-PRL-R or anti-JAK2 antibodies (Figures 5, 6 and 8); and (iii) anti-JAK2 antibody immunoprecipitated [^{125}I]PRL cross-linked receptor complexes (Figure 7B). In addition, we show that in both PRL-R- and PRL/EPO-R-transfected BAF-3 cells, PRL triggers high and transient tyrosine phosphorylation of JAK2, with kinetics similar to the tyrosine phosphorylation of the PRL-activated receptor. PRL also triggers the recruitment of some additional JAK2 molecules to the full-length PRL-R. These results provide the first evidence that the JAK2 kinase participates in the PRL signaling pathway through

association with either the PRL-R intracellular domain or the EPO-R intracellular domain of the chimeric receptor. Preliminary results indicate that JAK2 is also involved in the PRL signaling pathway in a Chinese hamster ovarian (CHO) cell line transfected with the PRL-R (J.Djiane, unpublished data), as well as in the PRL-dependent rat lymphoma cell line Nb2 and mammary organ culture (C.Carter-Su, personnal comunication). These data are consistent with recent reports indicating that IFN α/β and γ, EPO, GH and IL-3 activate and/or are dependent on the expression of at least one member of the JAK family of kinases for their functional activity (Velazquez *et al.*, 1992; Argetsinger *et al.*, 1993; Silvennoinen *et al.*, 1993; Muller *et al.*, 1993; Watling *et al.*, 1993; Witthuhn *et al.*, 1993). G-CSF and GM-CSF have also been mentioned as activators of JAK2 (Silvennoinen *et al.*, 1993).

PRL also seems to induce tyrosine phosphorylation of JAK1, although to a much lower extent, without affecting the number of JAK1 molecules associated with the activated receptor. This PRL-induced phosphorylation of JAK1 was only detected in cells expressing the full-length PRL-R, although JAK1 was shown to be pre-associated with both PRL-R and PRL/EPO-R (Figure 5B), suggesting that the JAKs can be activated differentially depending on the intracellular receptor domain to which they are associated. Although this tyrosine phosphorylation detected was quite low, it seems that both JAK1 and JAK2 are associated with the receptor and are involved in the PRL transducing signal, but that PRL triggers differentially their respective tyrosine phosphorylation and association. Similar results were reported for cells activated by IL-3 (Silvennoinen *et al.*, 1993), and very recently for cells activated by IFN α/β, IFN γ and EGF (Shuai *et al.*, 1993). Our data are consistent with very recent studies showing that JAK1 is absolutely required for the IFN α/β-induced activation of Tyk2 and for the IFN γ-induced activation of JAK2 (Muller *et al.*, 1993; Watling *et al.*, 1993).

Overall, our results support the hypothesis that JAKs couple PRL binding to tyrosine phosphorylation and proliferation. Since two types of JAKs were shown to be associated with the PRL-responsive receptors, it is tempting to speculate that the kinases may be reciprocally activated upon receptor dimerization after ligand binding. Whether these kinases will activate gene expression through pathways involving members of the interferon-stimulated gene factor 3 (Veals *et al.*, 1992), or related proteins, also including molecules such as interferon regulatory factor (IRF) 1 and 2 (Yu-Lee *et al.*, 1990), is currently under investigation.

Materials and methods

Hormones and antibodies

Ovine prolactin (NIDDK-oPRL-19, 31 IU/mg) was a gift from the National Hormone and Pituitary Program (Baltimore, MD). The goat anti-PRL-R antiserum S46 was raised against a rabbit mammary PRL-R preparation (Dusanter-Fourt *et al.*, 1987) and monoclonal anti-PRL-R antibodies M110 and A917 were selected for their ability to block PRL binding to its mammary receptor (Katoh *et al.*, 1985). Rabbit anti-EPO-R antibodies were raised against a recombinant protein containing the intracellular domain of the murine EPO-R, as described previously (Mayeux *et al.*, 1991). Monoclonal anti-phosphotyrosine antibodies were 4G10. Rabbit-raised anti-JAK1 antibodies (Wilks *et al.*, 1991) and anti-JAK2 antibodies (UBI) were used.

Plasmids and DNA constructs

The full coding sequence of the rabbit mammary PRL-R cDNA (Edery *et al.*, 1989) was inserted (−3 to +1950 nucleotides from the ATG start codon) in the *SalI* polylinker cloning site of the pBabeNeo retroviral vector. In this vector, the long terminal repeats (LTR) from the murine Moloney leukemia virus drive the expression of exogenous cDNA and the SV40 promoter directs the expression of the neomycin resistance gene (Morgenstern and Land, 1990). The PRL/EPO-R chimeric receptor was constructed using the Kunkel (1985) mutagenesis procedure. The 27-mer mutagenic oligonucleotide 5′-ACCATGAAAGACCTCATCTTGACGCTG-3′ included the last 12 nucleotides coding for the extracellular domain of the rabbit PRL-R and the first 15 nucleotides coding for the transmembrane domain of the murine EPO-R (D'Andrea *et al.*, 1989). The chimeric construct was introduced in the pBabeNeo retroviral vector.

Cells

Murine IL-3-dependent BAF-3 cells (Palacios and Steinmetz, 1985) were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) (Dutscher) and 5% culture medium from the IL-3-producing Wehi-3B cell line (Wehi-CM). BAF-3 cells (10^7) were electroporated (Biorad apparatus, 956 μF, 350 V) in the presence of 50 μg of the plasmid vector. Cells were selected in medium supplemented with Wehi-CM and FCS and containing G418 (800 μg/ml, Gibco). Cells were next incubated in FCS-supplemented RPMI-1640 medium containing oPRL (10 ng/ml, 0.5 nM) instead of IL-3. All transfectants were maintained for >6 months in these conditions.

Biosynthetic labeling

For studies on DNA synthesis, cells (10^4 cells/well) were incubated in 96-well microtiter plates in RPMI-1640 medium containing the indicated growth factor at various concentrations. After 36 h, the cells were pulsed for 3 h with [^{3}H]TdR (2 μCi/well, Amersham Inc.) and [^{3}H]TdR incorporated into DNA was determined using an automated cell harvester. The results are the mean and SD of three independent experiments.

For protein biosynthetic labeling, cells were pre-incubated for 1 h at 37°C in culture medium without methionine and cysteine, and further incubated for 3 h in the same medium supplemented with a mixture of L-[^{35}S]methionine and L-[^{35}S]cysteine (250 μCi/ml, cell labeling mix, Amersham Inc.). After washing, cells were solubilized and immunoprecipitated as described below.

Immunoprecipitations and immunoblotting

Cells (2×10^7) were deprived of PRL and FCS by incubation for 5−6 h in Iscove medium (Gibco) supplemented with 1% detoxified bovine serum albumin (BSA Fraction V, Sigma) and 75 μg/ml iron-saturated transferrin (Sigma). In these conditions, viability of the cells exceeded 90%. oPRL (10 ng−1 μg/ml) was added to the cell suspension and incubation was allowed to proceed for 1−30 min at 37°C. The reaction was stopped by adding an excess of cold phosphate-buffered saline (PBS) and immediately pelleting the cells. Cells were solubilized in a mild lysis buffer [20 mM Tris−HCl (pH 7.4), 150 mM NaCl, 1% NP40, 10% glycerol, 1 mM sodium-orthovanadate, 1 mM phenylmethylsulfonylfluoride, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 10 μg/ml aprotinin] and insoluble material was pelleted at 20 000 *g* for 20 min. Cell lysates were first pre-incubated with pre-immune goat serum (for anti-PRL-R immunoprecipitation assays) or rabbit serum (for anti-JAKs immunoprecipitation assays) and protein A−Sepharose beads for 2 h at 4°C. Pre-cleared supernatants were incubated with the indicated antibodies for 18 h at 4°C. Immune complexes were collected by incubation with protein A−Sepharose beads and eluted by boiling in SDS sample buffer [0.0625 M Tris−HCl (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol]. Where indicated, samples were 10 times diluted in NP40 lysis buffer and re-immunoprecipitated with a second antibody (double immunoprecipitation assay). All samples were subjected to SDS−PAGE (8%) under reducing conditions and, when indicated, transferred to nitrocellulose membrane (Schleicher and Schuell, BA 85) for immunoblotting. Nitrocellulose filters were blocked in TBS−Tween [10 mM Tris−HCl (pH 7.5), 137 mM NaCl, 0.1% Tween] containing 5% low-fat dry milk for 2 h on a rocking platform. Blots were incubated with the indicated antibodies in blocking buffer, washed and incubated with a second horseradish peroxidase-conjugated anti-species-specific antibody (Amersham) for 30 min at room temperature. Immune complexes were detected by ECL chemiluminescence according to the manufacturer's instructions (Amersham).

PRL binding and cross-linking assays

oPRL was iodinated using chloramine T to a specific activity of ~30 μCi/μg. Cells were deprived of PRL and FCS as described above. A total of 5×10^6 cells/point were incubated at 37°C for 45 min with increasing amounts of [^{125}I]PRL in the presence or absence of a 200-fold excess of

unlabeled hormone in RPMI-1640 supplemented with 0.25% BSA and 0.1% sodium azide. Cells were washed in cold PBS and radioactivity associated with the pellets was determined in a gamma-counter. Results were analyzed by the method of Scatchard. Alternatively, cells were incubated with a fixed amount of [^{125}I]PRL (100 000 c.p.m.) and increasing concentrations of various unlabeled hormones. Similar results were obtained in both conditions. For cross-linking experiments, $2-5 \times 10^7$ cells/point were incubated with [^{125}I]PRL (2.5 nM) at 37°C in the presence or absence of a 200-fold excess of unlabeled PRL. The reaction was stopped by adding an excess of cold PBS and immediatly spinning down the cells. Cells were resuspended in PBS containing the cross-linking agent, either DSS (0.5 mM, Pierce) or EDC (10 mM, Pierce) and *N*-hydroxysulfosuccinimide (sulfoNHS, 20 mM, Pierce), for 20 min at 4°C. The reaction was stopped by adding 0.1 M ethanolamine and cells were solubilized in the NP40 lysis buffer as described above.

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