Stimulation of c-Src by prolactin is independent of Jak2

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Interaction of prolactin (PRL) with its receptor (PRLR) leads to activation of Jak and Src family tyrosine kinases. The PRL/growth hormone/cytokine receptor family conserves a proline-rich sequence in the cytoplasmic juxtamembrane region (Box 1) required for association and subsequent activation of Jaks. In the present work, we studied the mechanisms underlying c-Src kinase activation by PRL and the role that Jak2 plays in this process. PRL addition to chicken embryo fibroblasts (CEF) expressing the rat PRLR long form resulted in activation of c-Src and Jak2 and in tyrosine phosphorylation of the receptor. Receptor phosphorylation was due to associated Jak2, since in cells expressing either a Box 1 mutated PRLR (PRLR_{4P-A}), which is

unable to interact with Jak2, or a kinase-domain-deleted Jak2 (Jak2Δk), PRL did not stimulate receptor phosphorylation. Interestingly, addition of PRL to cells expressing PRLR_{4P-A} resulted in an activation of c-Src equivalent to that observed with the wild-type receptor. These findings indicate that PRL-mediated stimulation of c-Src was independent of Jak2 activation and of receptor phosphorylation. Our results suggest that PRL-activated Src could send signals to downstream cellular targets independently of Jak2.

Key words: cytokine signalling, prolactin receptor, proto-oncogenes, tyrosine kinases.

INTRODUCTION

Prolactin (PRL) is a polypeptide hormone that modulates a variety of physiological functions, such as development of the mammary gland and lactation or immune regulation (for a review see [1,2]). The receptor for prolactin (PRLR) is a member of the type I cytokine receptor family, which includes receptors for growth hormone, interleukins-2 to -7, erythropoietin, granulocyte colony stimulating factor, granulocyte-macrophage colony stimulating factor, etc. [3–5]. Three different forms of PRLR have been described so far. The short and the long forms, generated by alternative RNA splicing, differ in length and sequence of the cytoplasmic domain. The intermediate form of the receptor, found in the rat thymoma cell line Nb2, was generated from the long form through a partial in-frame deletion of 198 amino acids of the cytoplasmic domain [2]. Only the long and the Nb2 forms are capable of mediating lactogenic signals [6,7]. The function of the short PRLR is less clear [7–9].

The PRLR has no intrinsic enzymic activity. PRL binding results in receptor dimerization [10,11], activation of Jak and Src family kinases [12–15] and tyrosine phosphorylation of cellular proteins, including the receptor and additional molecules implicated in signalling cascades, such as Vav, the Grb2/Sos-Ras-Raf-Mapk, the Jak-Stat, etc. [16–20], that stimulate cells to proliferate and/or differentiate. As a structural feature of the cytokine receptor family, the PRLR conserves a proline-rich sequence in the cytoplasmic juxtamembrane region, named Box 1, which seems to be essential for signal transduction as well as for association and activation of Jak2. Mutations or deletions in this domain that prevent binding of Jak2 lead to functional inactivation of the PRLR [21–24].

Activation of Src family kinases by different cytokine receptors is well documented (for a review see [25]). Yet, the contribution of these tyrosine kinases to the signalling mechanisms induced by cytokines remains unclear. The interaction of Src family members such as c-Src and Fyn with the PRLR, as well as their activation in response to PRL stimulation has been described before [14,15]. In the present work we have studied the mechanisms responsible for Src kinase activation and analysed the role of Jak2 kinase activity in the phosphorylation of PRLR. The results demonstrate that the association and stimulation of c-Src by PRLR are independent of Jak2 activity. Moreover, PRLR tyrosine phosphorylation seems to be mediated by Jak2 and not by other receptor-associated kinase.

EXPERIMENTAL

Materials

Ovine prolactin (NIDDK-o-PRL-20; 31 units/mg) was a gift from Dr A. F. Parlow (National Hormone and Pituitary Program, Harbor-UCLA Medical Center, Torrance, CA 90509, U.S.A.). Mouse monoclonal antibody (mAb) to haemagglutinin (HA) epitope 12CA5 was purchased from Boehringer Mannheim. The mAb U6 to the PRLR was a gift from Dr P. A. Kelly (INSERM U344, Faculté de Médecine, Paris, France [26]). Goat antiserum to the PRLR S46 was provided by Dr J. Djiane (INRA, Jouy en Josas, France [27]). The mAb 327 to c-Src was a gift from Dr J. S. Brugge (Harvard University Medical School, Boston, MA 02115, U.S.A. [28]). SRC-2 rabbit polyclonal antibody to Src was purchased from Santa Cruz Biotechnology. The 4G10 mAb

Abbreviations used: PRL, prolactin; PRLR, prolactin receptor; GM-CSF, granulocyte-macrophage colony stimulating factor; CEF, chicken embryo fibroblast; DMEM, Dulbecco's modified Eagle's medium; mAb, monoclonal antibody; HA, haemagglutinin; RCAS, replication-competent retroviral vector.

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to phosphotyrosine and the rabbit polyclonal antibody to Jak2 were purchased from Upstate Biotechnology Inc.

Plasmids and DNA constructs

The cDNA for the long form of PRLR from rat ovary was kindly provided by Dr P. A. Kelly [29]. The four proline residues in the Box 1 of the wild-type form of the receptor were substituted with alanine residues using the site-directed-mutagenesis kit Sculptor (Amersham) with the oligo, 5'-GACCTGGATCTTTGCGGC-CGTTGCTGGGGCAAAAATAAAGGATTT-3', to generate the PRLR_{4P-A} mutant. The wild-type and the mutant forms of PRLR were additionally modified to encode the HA epitope, YPYDVPDYA, between the first and second amino acid residue of the mature protein, by site-directed mutagenesis *in vitro* (as above), using the following primer: 5'-ATGGGCTACCCCTA-TGACGTGCCCCGATTACGCCAGCCTGGGCGCC-3'. Construction of the kinase-inactive mutant of Src (SrcK⁻, Lys-295 → Met) has been described previously [30]. The Jak2ΔK, a Jak2 form with the C-terminal kinase domain deleted, was generated from the original Jak2 cDNA provided by Dr J. N. Ihle (St. Jude Children's Research Hospital, Memphis, TN 38105, U.S.A. [31]) as described elsewhere [32]. The RCAS(A) and RCAS(B) replication-competent retroviral expression vectors, provided by Dr S. H. Hughes (NCI-Frederick Cancer Research and Development Center, Frederick, MD 21702-1201, U.S.A. [33]) were used in chicken embryo fibroblasts (CEF). The HA-tagged cDNAs of wild-type and mutant forms of PRLR were inserted in the *Cla*I site of the RCAS(B) and the cDNAs of wild-type and mutant forms of c-Src and Jak2 were subcloned into the *Cla*I site of the RCAS(A) vector.

Cell culture, transfection and metabolic labelling

CEF were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) newborn-calf serum, 1% (v/v) chicken serum and antibiotics at 37 °C in a 5% CO₂ incubator. Exponentially growing cells were transfected with the specific RCAS constructs using the calcium phosphate precipitation method. After 4 cell passages, the medium was collected, stored frozen at -70 °C as virus stocks, and used later to infect CEF to obtain a rapid expression of recombinant proteins [34]. Infected cells at 90% confluence were stimulated with 1 µg/ml ovine PRL or with vehicle for 5 min at 37 °C. For biosynthetic cell labelling, cultures from 60-mm Petri dishes were preincubated in 2 ml of DMEM, lacking methionine and cysteine, for 30 min at 37 °C, and further incubated for 2 h under the same conditions with 200 µCi of Trans³⁵S-Label ([³⁵S]-methionine/[³⁵S]cysteine; ICN Pharmaceuticals).

Immunoprecipitation and Western blotting

Cell cultures were placed on ice, washed twice with ice-cold TBS [10 mM Tris/HCl (pH 7.4), 130 mM NaCl] and scraped from the dish with 1 ml of lysis buffer (LB) [10 mM Tris/HCl (pH 7.4), 130 mM NaCl, 10 mM sodium pyrophosphate (pH 7.4), 10 mM NaF, 5 mM EDTA, 1% (v/v) Triton X-100, 0.1 mM Na₃VO₄, 1 mM PMSF, 1 mM phenanthroline, 1 mM benzamidide hydrochloride, 1 mM iodoacetamide]. The total cell lysates were centrifuged at 15000 *g* for 30 min at 4 °C and the protein in the supernatant, measured using the BCA protein assay (Pierce), was adjusted with LB so that each sample contained the same amount of protein. The samples were then incubated for 1 h at 4 °C with the appropriate antibody. Immune complexes were collected after incubation for 1 h at 4 °C with 30 µl of Protein G-

Sepharose beads (Sigma), washed three times with LB and eluted by boiling in 2 × SDS sample buffer [125 mM Tris/HCl (pH 6.8), 10% (v/v) 2-mercaptoethanol, 4% (w/v) SDS, 20% (v/v) glycerol]. When a sequential immunoprecipitation was carried out, the immune complexes from the first immunoprecipitation were brought to a final concentration of 0.75% SDS/100 mM dithiothreitol and then dissociated by boiling for 5 min. The eluted proteins were diluted 10-fold with LB. This preparation was incubated with the new antibody, collected and eluted as above. Samples were then subjected to SDS/PAGE (9% gel) and transferred to PVDF membranes (Immobilon, Millipore) for Western blotting. Filters were blocked with 5% (w/v) fat-free dried milk (Fluka Chemie AG) in TTBS [TBS/0.1% (v/v) Tween 20], incubated with the primary antibody in blocking buffer, washed three times with TTBS, and further incubated with the appropriate horseradish-peroxidase-conjugated anti-species-specific antibody (TAGO). Proteins were revealed by enhanced chemiluminescence (ECL[®]; Amersham). For stripping, the membranes were incubated for 30 min at 50 °C in 62.5 mM Tris/HCl, pH 6.7, containing 2% (w/v) SDS and 100 mM 2-mercaptoethanol, and then extensively washed with TTBS at room temperature. The blots were blocked and reprobed as described above.

In vitro kinase assays

The immune complexes were washed with LB, then with TBS and finally with kinase buffer [20 mM Tris/HCl (pH 7.4), 10 mM MnCl₂], and incubated in 30 µl of kinase buffer containing 2 mM 2-mercaptoethanol, 1 µM ATP, 10 µCi [γ -³²P]ATP (4500 Ci/mmol; ICN) for 4 min at 30 °C. Where indicated, 5 µg of acid-denatured enolase, as an exogenous substrate, was included in kinase buffer. The reaction was stopped by the addition of 2 × sample buffer and the samples were boiled for 5 min. Eluted proteins were resolved by SDS/PAGE (9% gel), the gels were treated with 1 M KOH for 1 h at 55 °C to remove background due to serine phosphorylation, and ³²P-labelled proteins were visualized by autoradiography.

RESULTS

Functional expression of PRLR in CEF

To assess the functional role of the proline-rich sequence (PPVPGP) within the Box 1 region of the long form of the rat PRLR, these four residues were substituted by alanine residues (AAVAGA) by means of site-directed mutagenesis to generate the PRLR_{4P-A}-receptor mutant. As no commercial anti-PRLR antibodies were available when this project was initiated, we tagged the receptor with an HA epitope (YPYDVPDYA), inserted after the first amino acid residue of the mature protein. The experiments were carried out in CEF lacking endogenous PRLR, as determined by Northern-blot hybridization and by immunoprecipitation of metabolically labelled cells with anti-PRLR mAb U6 (results not shown). CEFs were infected with different RCASs, each containing a distinct envelope gene (A or B), which allowed the expression of various foreign genes in a single CEF [33,34]. The receptors were expressed using RCAS-B, and Jak2ΔK, wild-type c-Src and its kinase-inactive mutant (SrcK⁻) were cloned into RCAS-A. Both forms of the receptor were efficiently expressed in CEF, judging from their immunoprecipitation from metabolically labelled cell cultures with either the anti-HA epitope-specific mAb 12CA5 or with the anti-PRLR mAb U6 (Figures 1A and 1B respectively).

Although the PRLR does not have intrinsic tyrosine kinase activity, it became tyrosine phosphorylated upon addition of the

Figure 1 Expression of PRLR and PRLR_{4P-A} in CEF

The PRLR was immunoprecipitated (IP) with either mAb 12CA5 (α HA) (**A**) or mAb U6 (**B**) from [³⁵S]methionine-labelled cultures of control cells infected with empty virus (Vo) (lane 1), or cells expressing either PRLR (lane 2) or PRLR_{4P-A} (lane 3). The immunoprecipitates were analysed by SDS/PAGE (9% gel) followed by fluorography. Molecular-mass markers (kDa) are shown on the left. The position of PRLR (arrow) is shown on the right.

hormone [13,35]. To test the functionality of the HA-tagged PRLR, cells were stimulated with 1 μ g/ml PRL for 5 min, conditions that were found to induce maximal PRLR tyrosine phosphorylation (results not shown). Western-blot analysis of total cell extracts with the anti-phosphotyrosine mAb 4G10 showed receptor phosphorylation in extracts from PRL-stimulated cells (Figure 2A, lane 3 compared with lane 2). This effect was specific, since in extracts from mock-transfected cells the receptor phosphorylated band was absent (Figure 2, lane 1). The identity of this protein band as the PRLR was confirmed by reprobing the blot with the specific antiserum (S46) to the PRLR (Figure 2B [27]). These results indicated that the receptor expressed in CEF was functional.

Deletion or substitution of the proline residues within the Box 1 domain of the PRLR prevents receptor tyrosine phosphorylation and association and activation of Jak2 in response to PRL [23,24]. In agreement with these data, the PRLR_{4P-A} mutant remained unphosphorylated in response to PRL (Figure 2A, lane 7), even when co-expressed with c-Src (Figure 2A, lane 11). This shows that the Box 1 sequence is essential for receptor tyrosine phosphorylation. Interestingly, PRL stimulation of cells expressing the PRLR alone or together with SrcK⁻, in addition to inducing receptor phosphorylation, also induced tyrosine phosphorylation of a protein band of about 130 kDa (p130) (Figure 2, lanes 3 and 5). This p130 band was absent in cells expressing the PRLR_{4P-A} mutant, suggesting that it could be Jak2. Unfortunately, the available polyclonal antibody against murine Jak2 did not recognize chicken Jak2, as judged by Western-blot analysis. Detection of SrcK⁻ (Figure 2A, lanes 4 and 5) and of c-Src in the PRL unstimulated cells (Figure 2A, lanes 8 and 10) must be due to phosphorylation of Tyr-527 by CSK [36,37], as it has been shown previously that both the wild-type and the kinase-inactive mutant of c-Src are similarly recognized by the anti-phosphotyrosine mAb 4G10 [38].

Receptor immunoprecipitation and subsequent *in vitro* kinase reaction in the immune complexes (see the Experimental section) showed that PRL stimulation promoted the phosphorylation of both the receptor and the p130 bands in immunoprecipitates

Figure 2 Analysis of PRL-induced protein tyrosine phosphorylation

Total cell lysates (25 μ g of protein) from unstimulated (—) or PRL (1 μ g/ml) stimulated (+) cultures expressing the PRLR alone (lanes 2 and 3), the PRLR and SrcK⁻ (lanes 4 and 5), the PRLR_{4P-A} alone (lanes 6 and 7), the PRLR and c-Src (lanes 8 and 9) or the PRLR_{4P-A} and c-Src (lanes 10 and 11) were resolved by SDS/PAGE (9% gel). Proteins were transferred to PVDF filters and probed (WB) with anti-phosphotyrosine mAb 4G10 (**A**). The blot was stripped and reprobed with the specific antiserum S46 to the PRLR (**B**). Cells infected with an empty virus (Vo) stimulated with PRL were used as a control (lane 1). Molecular-mass markers (kDa) are shown on the left. Positions of the PRLR, c-Src and p130 (arrows) are shown on the right.

from cells expressing the PRLR, but not in those from cells expressing the PRLR_{4P-A} (Figure 3A, lanes 2 and 4 respectively), as was observed above by Western blotting anti-phosphotyrosine (Figure 2). These data suggest that the p130 phosphorylated band was Jak2. As indicated above, the anti-Jak2 antibody did not recognize the chicken Jak2 in Western blots, but it was able to do it by immunoprecipitation. To confirm the identity of p130 as Jak2, double immunoprecipitation experiments were carried out. First, the PRLR was immunoprecipitated from PRL-stimulated cells, the immune complexes were subjected to *in vitro* kinase reaction and then dissociated by boiling in SDS (see the Experimental section). Proteins extracted from the immune complexes were divided into three identical portions. One was subjected directly to SDS/PAGE for analysis; the other two portions were immunoprecipitated with mAb 12CA5 and a polyclonal antibody against murine Jak2. The immunoprecipitates were also analysed by SDS/PAGE (see the Experimental section). The results showed that the p130 band associated to the receptor immune complex (Figure 3B, lane 1) was, after SDS dissociation, specifically immunoprecipitated by the anti-Jak2 antibody (Figure 3B, lane 3), and, as expected, the mAb 12CA5 immunoprecipitated the PRLR band (Figure 3B, lane 2). We can therefore conclude that p130 is Jak2.

Role of Jak2 in tyrosine phosphorylation of the PRLR

Jak kinases have been implicated in tyrosine phosphorylation of cytokine receptors [5]. To confirm the role of Jak2 kinase activity in the phosphorylation of PRLR, we co-expressed a kinase-

Figure 3 Identification of p130 as Jak2

(A) The PRLR was immunoprecipitated (IP) with mAb 12CA5 (α HA) from extracts (0.5 mg of protein) of unstimulated (–) or PRL (1 μ g/ml) stimulated (+) cultures expressing the PRLR (lanes 1 and 2) or the PRLR_{4P-A} (lanes 3 and 4). Immune complexes were subjected to *in vitro* kinase reaction in presence of [γ -³²P]ATP; ³²P-labelled proteins were resolved by SDS/PAGE (9% gel), the gel was treated with KOH (see the Experimental section) and the bands were revealed by autoradiography. (B) The PRLR was immunoprecipitated with mAb 12CA5 (α HA) from a lysate (1 mg of protein) of PRL (1 μ g/ml)-stimulated cells expressing the PRLR. The immune complex was incubated with [γ -³²P]ATP and then dissociated with SDS and dithiothreitol (see the Experimental section). Proteins extracted from the immune complex were divided into three similar portions. One was directly resolved by SDS/PAGE (lane 1). The others two were diluted with lysis buffer and re-immunoprecipitated with mAb 12CA5 (α HA) (lane 2) or with anti-Jak2 (α Jak2) (lane 3). The three immune complexes were simultaneously resolved by SDS/PAGE and visualized by autoradiography as described for (A). Molecular-mass markers (kDa) are shown on the left; the positions of PRLR, p130 and Jak2 (arrows) are shown on the right.

domain-deleted form of mouse Jak2 (Jak2 Δ K) with the PRLR. Since Jak2 is constitutively associated with PRLR [12,13], we first tested whether Jak2 Δ K could bind the receptor. The receptor was immunoprecipitated from extracts of cells expressing both the PRLR and Jak2 Δ K. PRLR immune complexes were analysed for the presence of Jak2 Δ K by Western blotting with the polyclonal antibody anti-Jak2, which is able to recognize Jak2 Δ K because of its mouse origin. The results showed that Jak2 Δ K was present in PRLR immune complexes from both unstimulated and stimulated PRL cells (Figure 4A, upper panel, lanes 2 and 3). The control for the expression of Jak2 Δ K in the different lysates is shown (Figure 4A, lower panel). Therefore, association between PRLR and Jak2 seems to be constitutive and does not necessarily require the kinase domain of Jak2.

We next analysed the role of Jak2 on receptor tyrosine phosphorylation by co-expressing Jak2 Δ K together with PRLR. In cells expressing the receptor alone, PRL induced receptor phosphorylation on tyrosine residues, as detected by immunoblotting with anti-phosphotyrosine mAb 4G10 (Figure 4B, upper panel, lane 2). However, co-expression of PRLR with Jak2 Δ K suppressed PRL-mediated stimulation of receptor tyrosine

phosphorylation (Figure 4B, upper panel, lane 4). Since co-expression of Jak2 Δ K did not alter the level of immunoprecipitated receptor, as determined by Western blotting with mAb 12CA5 (Figure 4B, lower panel), we concluded that Jak2 is responsible for tyrosine phosphorylation of PRLR.

When the receptor was expressed alone, the kinase reaction of the receptor immune complex from PRL-stimulated cells gave rise to phosphorylation of the endogenous Jak2 and the PRLR (Figure 4C, lane 1), while co-expression of Jak2 Δ K suppressed the phosphorylation of both Jak2 and the PRLR. Similarly, Jak2 Δ K phosphorylation was not detectable (Figure 4C, lane 2). This indicates that the phosphorylation of Jak2 induced by PRL strictly depends on kinase-active Jak2.

Activation of c-Src by PRLR

It has been shown previously in hepatocytes and thymocytes that the PRLR associates and activates the Src family of tyrosine kinases [14,15]. To determine whether PRL stimulates c-Src activity in CEF, we analysed the phosphorylation state of the c-Src fraction bound to the PRLR. The PRLR was immunoprecipitated with mAb 12CA5 from unstimulated or PRL stimulated cultures. The immune complexes were subjected to *in vitro* kinase reaction. After dissociation of the receptor/c-Src complex with SDS, c-Src was immunoprecipitated with mAb 327 and analysed by SDS/PAGE followed by autoradiography. The results showed that PRL induced a two- to three-fold increase in the amount of radioactive phosphate incorporated into the c-Src fraction bound to the PRLR (Figure 5A, lanes 4 and 5). When SrcK– was overexpressed, addition of PRL to cells did not result in an increase in the c-Src band phosphorylation (Figure 5A, lanes 6 and 7), indicating that the increase in the phosphorylation of the wild-type form of c-Src (Figure 5A, lanes 4 and 5) was an autophosphorylation event induced by PRL stimulation. More importantly, the PRL stimulation of cells expressing the receptor mutant resulted also in increased autophosphorylated Src (Figure 5A, lanes 8 and 9), as observed with the wild-type form of the PRLR (Figure 5A, lanes 4 and 5).

It must be observed that, in these experiments, we detected the autophosphorylation of Tyr-416 in the c-Src activation loop. This explains the apparent differences with c-Src tyrosine phosphorylation shown in Figure 2, where anti-phosphotyrosine mAb 4G10 mainly detected phosphorylation by CSK in Tyr-527 [36–38].

These results raised the question of whether the increase in the autophosphorylated c-Src band induced by PRL was due to increased specific activity or to an increase in the amount of c-Src bound to receptor. To this end, the receptor was immunoprecipitated from either unstimulated or PRL-stimulated cells expressing wild-type or mutant receptor together with either c-Src or the SrcK– mutant. After immunoprecipitation, receptor–Src complexes were dissociated and Src was immunoprecipitated with mAb 327. Src was later detected by Western-blot analysis with anti-Src polyclonal antibody SRC-2. As the amount of Src associated with the PRLR was unaltered by PRL stimulation (Figure 5B), the increase in the quantity of [³²P]P_i incorporated into c-Src, previously observed (Figure 5A), resulted from stimulation of c-Src activity and not from increased association with the receptor upon ligand stimulation. Interestingly, the PRLR_{4P-A} mutant was still able to transduce the PRL signal to activate c-Src, suggesting that this event is independent of Jak2. To confirm this, kinase reactions of PRLR immune complexes were carried out in the presence of enolase as an exogenous substrate. PRL stimulated phosphorylation of Jak2, the PRLR and also enolase (Figure 5C, lanes 1 and 2).

Figure 4 Role of Jak2 on PRLR phosphorylation

(A) Association of Jak2ΔK with PRLR. The PRLR was immunoprecipitated (IP) with mAb 12CA5 (αHA) from extracts (1 mg of protein) of PRL (1 μg/ml)-stimulated cells expressing Jak2ΔK alone (lane 1) or from lysates of unstimulated (–) or PRL stimulated (+) cells co-expressing the PRLR and Jak2ΔK (lanes 2 and 3 respectively). The immune complexes were resolved by SDS/PAGE (9% gel) and transferred to PVDF filters (WB) that were probed with anti-Jak2 (αJak2) (upper panel). Vo, cells infected with an empty virus. To compare Jak2ΔK expression

However, co-expression of Jak2ΔK suppressed PRL-induced phosphorylation of both Jak2 and PRLR, while the c-Src kinase-mediated enolase phosphorylation remained unaltered (Figure 5C, lanes 3 and 4). These results demonstrate that PRL stimulates the kinase activity of c-Src associated with the PRLR. This activation seems to be independent of the proline-rich sequence present in the Box 1 region of the receptor and from Jak2.

The activation of c-Src by PRL raises the question of whether this kinase participates in tyrosine phosphorylation of the PRLR. We expressed the receptor, together with c-Src or SrcK –, and asked whether the co-expression of any of these Src forms could affect tyrosine phosphorylation of the receptor upon PRL stimulation. The results from anti-phosphotyrosine Western blots demonstrated that receptor phosphorylation was not altered by the expression of SrcK – (Figure 2, lanes 3 and 5) or of c-Src (Figure 2, lane 9), suggesting that this kinase does not play a role in tyrosine phosphorylation of the PRLR. To confirm this we performed *in vitro* kinase reactions on PRLR or PRLR_{4P-A} receptor immunoprecipitates from unstimulated or PRL stimulated cells that expressed either the receptor alone or the receptor together with c-Src. The results showed that, in cells expressing the receptor alone, PRL promoted the phosphorylation of the PRLR and of Jak2 (Figure 5D, lanes 1 and 2). c-Src had no effect on these phosphorylations (Figure 5D, lanes 3 and 4). When c-Src was co-expressed with PRLR_{4P-A}, PRL was unable to induce the phosphorylation of the receptor and of Jak2 (Figure 5D, lanes 5 and 6 compared with lanes 1 and 2 or lanes 3 and 4). Since we know that, under these experimental conditions, PRL stimulated c-Src activity (Figure 5A), we conclude that c-Src is not involved in PRLR phosphorylation.

DISCUSSION

In the present study we present data which demonstrate that stimulation of c-Src by PRL is independent of both Jak2 kinase activity and PRLR tyrosine phosphorylation. These experiments were carried out in CEF as a system in which no endogenous PRLRs are expressed. CEF were transfected with the long form of the rat PRLR tagged at the N-terminus with the HA epitope. Comparing expression and tyrosine phosphorylation of the normal and the HA-tagged forms of the receptor in CEF, we observed that the HA epitope did not affect receptor function (results not shown). Similarly, it has been reported recently that insertion of a FLAG epitope into the same position on the PRLR does not affect receptor function [39].

The proline-rich sequence of the cytokine receptor family, Box 1, is critical for association and activation of Jak tyrosine kinases [5]. In the present work we studied the role of Jak kinases in

in the different lysates, 25 μg of total protein from these extracts was resolved by SDS/PAGE (9% gel), transferred to PVDF filters and probed with anti-Jak2 (lower panel). (B) Effect of Jak2ΔK expression on PRL-induced PRLR phosphorylation. The PRLR was immunoprecipitated (IP) with mAb 12CA5 (αHA) from lysates (1 mg of protein) of unstimulated (–) or PRL (1 μg/ml)-stimulated (+) cells expressing the PRLR alone (lanes 1 and 2 respectively) or co-expressing the PRLR and Jak2ΔK (lanes 3 and 4 respectively). Proteins were extracted from immune complexes, divided into two identical aliquots that were resolved by SDS/PAGE (9% gel) and transferred to PVDF filters (WB). The filters were separately probed, one with anti-phosphotyrosine mAb 4G10 (αPTyr) and the other with the mAb 12CA5 (αHA). Vo, cells infected with an empty virus. (C) The PRLR was immunoprecipitated (IP) with mAb 12CA5 (αHA) from lysates (0.5 mg of protein) of PRL (1 μg/ml)-stimulated cells expressing the PRLR alone (lane 1) or co-expressing the PRLR and Jak2ΔK (lane 2). Immune complexes were subjected to kinase reactions in the presence of [γ -³²P]ATP, and ³²P-labelled proteins were visualized as described in the legend to Figure 3(A). Molecular-mass markers (kDa) are shown on the left. PRLR and Jak2 (arrows) and the theoretical position of Jak2ΔK (italics) are shown on the right.

PRLR function using a mutant receptor, PRLR_{4P-A}, where the proline residues required for Jak binding were replaced by alanine residues (PPVPGP/AAVAGA). Both the wild-type and the mutated receptor forms were efficiently expressed in CEF by employing RCAS [33,34]. In cells expressing the wild-type receptor, addition of PRL induced tyrosine phosphorylation of

the receptor and of a protein with an apparent molecular mass of 130 kDa. These bands were undetectable when the PRLR_{4P-A} mutant was expressed, in agreement with previous data showing that the presence of the proline-rich sequence in the Box 1 domain is necessary for association and activation of Jak2 and for receptor phosphorylation [23,24]. The anti-Jak2 polyclonal

antibody used in the present study was unable to recognize the endogenous Jak2 present in CEF by Western blot, but it was able to immunoprecipitate the CEF Jak2. This antibody specifically immunoprecipitated the p130 protein from anti-PRLR immune complexes. Together these data demonstrate that p130 is Jak2.

Our initial data indirectly indicated that Jak2 was responsible for receptor phosphorylation. This was confirmed by the use of the Jak2 deletion mutant, Jak2ΔK, which lacked the catalytic domain. PRLR and Jak2ΔK still interacted with each other, indicating that the kinase domain and the enzymic activity of Jak2 could be dispensable for association with the PRLR. Co-expression of the receptor with this kinase-domain-deleted mutant prevented PRLR phosphorylation. No phosphorylation of Jak2ΔK was observed by *in vitro* kinase assays of PRLR immune complexes, indicating that there were no other tyrosine kinases that could phosphorylate Jak2, at least outside of its kinase domain. This is in agreement with data showing that the point mutation, Tyr-1007 → Phe, in Jak2 abolished its kinase activity and phosphorylation [40].

We have shown previously in hepatocytes that PRL stimulation activates c-Src [15]. To study the effect of Box 1 mutations on the association and activation of c-Src by PRL, we analysed the kinase activity of receptor-associated c-Src. The results demonstrated that PRL treatment increased the phosphorylation of the fraction of c-Src associated with either wild-type or mutant PRLR. Stimulation of cells with PRL did not alter the amount of c-Src bound to wild-type or mutant receptor, yet led to increased c-Src kinase activity associated with either receptor form. As the PRLR_{4P-A} was unable to bind and activate Jak2, these results demonstrate that PRL activates c-Src through its receptor independently from Jak2. This was confirmed by using enolase as an exogenous substrate of c-Src in kinase reactions of PRLR immune complexes from cells co-expressing Jak2ΔK.

Lck, a member of the Src family tyrosine kinases, has been implicated in interleukin-2R β phosphorylation [41]. In our system, however, the analysis of PRLR phosphorylation both by anti-phosphotyrosine Western blots and by immunoprecipitate kinase reactions did not show involvement of c-Src activity in the receptor tyrosine phosphorylation. Co-expression of the PRLR with either c-Src or SrcK — did not alter PRLR phosphorylation. Moreover, in cells co-expressing PRLR_{4P-A} and c-Src, addition of PRL, although it induced activation of the proto-oncogene, did not result in receptor phosphorylation.

Our data show that PRL induces activation of c-Src without modifying its level of association with the PRLR. These results are in agreement with those obtained for the interaction between Fyn and the PRLR in the thymoma cell line Nb2 [14]. In contrast, in hepatocytes, PRL induces an increase in both the amount and the specific activity of c-Src bound to the receptor [15]. Other cytokine receptors have also been found to activate different members of the Src family [25,41–45], yet the role of these tyrosine kinases in signal transduction remains to be determined.

The results of the present study demonstrate that activation of c-Src by PRL can be dissociated from stimulation of Jak2 during PRLR signalling and may serve as another, yet undefined, function in signal transduction from the PRLR. The data do not exclude co-operativity among these kinases, which ultimately converge on some common PRL-induced pathways. In fact, Jak activation seems to be required for most, if not all, cytokine-receptor functions [46,47]. It remains to be determined if the downstream events controlled by Src kinases in PRL elicited signal transduction. In this context, it would be possible to speculate that the Src family kinases have a role in cytokine mitogenic pathways, as has been described for growth factors [48–50].

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