Prolactin Receptor Is Associated with c-*src* Kinase in Rat Liver

Juan Jose Berlanga*, Juan Angel Fresno Vara†, Jorge Martín-Pérez, and Josefa P. García-Ruiz

Departamento de Biologia Molecular (J.J.B., J.P.G.-R.)
Centro de Biologia Molecular Severo Ochoa
Campus Cantoblanco
28049 Madrid, Spain

Instituto de Investigaciones Biomédicas C.S.I.C. (J.A.F.V., J.M.P.)
Arturo Dupezier 4
28029 Madrid, Spain

The mechanism of action of the pituitary hormone PRL was studied in hepatocytes of lactating rats. PRL receptor immune complexes obtained from liver lysates have an associated tyrosine kinase activity. The tyrosine kinase has been identified in isolated hepatocytes as pp60$^{c\text{-}src}$. Incubation of hepatocytes with PRL induces the association of PRL receptor with pp60$^{c\text{-}src}$ and the resultant stimulation of its tyrosine kinase activity. Furthermore, PRL stimulates the gene expression of c-*fos*, c-*jun*, and c-*src*. All of these findings support the idea that the pp60$^{c\text{-}src}$ tyrosine kinase participates in the early steps of the PRL intracellular signaling that promotes cell growth in liver cells. (Molecular Endocrinology 9: 1461–1467, 1995)

INTRODUCTION

In vertebrates, the pituitary hormone PRL has been involved in many functions, including reproduction, lactation, growth, differentiation, and immune response. In accordance with PRL pleiotropic functions, PRL receptors (PRLR) are widely distributed in mammalian tissues. PRLR belong to the genetically defined cytokine/hematopoietin receptor family, which includes GH and a number of cytokines [interleukins 2–7 (IL-2 to IL-7), granulocyte/macrophage colony-stimulating factor, granulocyte colony-stimulating factor, and erytropoietin] receptors (1). The members of this large family share common structural elements in their extracellular domain, including four paired cysteine residues and a highly conserved WSXWS motif. Although intracellular domains differ markedly in size and have low sequence homology, a hydrophobic proline-rich segment in the membrane proximal region is highly conserved. This region of homology (box 1) has been shown to be essential for signal transduction in all cases studied (2–7). Although none of these receptors contains any obvious enzymatic activity, it has been shown that they associate with and activate specific tyrosine kinases (8). To date, the Jak and Src tyrosine kinase families have been widely involved with the cytokine/hematopoietin receptors. Jak2 has been shown to participate in the signaling of many receptors, such as the GH receptor in 3T3 fibroblasts (5) or the erytropoietin receptors and the IL-3 receptors in DA3 myeloid cells (9, 10). Jak3 is coupled to the IL-2 receptor in lymphoid and myeloid cells (11, 12), and Jak1, Jak2 and Tyk2 are implicated in the signaling mechanisms of interferons (13, 14). The Src family tyrosine kinases Lck, Lyn, and Fyn, on the other hand, are activated by IL-2, IL-3, and IL-7, respectively (15–17). Thus far, the exact role of these protein tyrosine kinases is not fully understood.

In mammalian cells, two forms of PRLR have been identified and cloned. They are generated by differential splicing of a single gene and differ only in the length of their intracellular domain (1). In addition, a fully functional mutant of the long form that lacks 198 amino acids in its cytoplasmic region has been shown to be predominant in the PRL-dependent Nb-2 11C cell line (18). In this pre-T rat lymphoma cell line, PRLR constitutively associates with Jak2 (19, 20), p59^{fyn} (21), and RAF-1 serine-threonine kinase (22), respectively. In BAF-3 cells transfected with the long form of the PRLR, the receptor also associates with Jak2 (23). In all of these cases, binding of ligand to the PRL receptor activates preassociated kinases. In addition, PRL stimulation triggers the tyrosine phosphorylation of both the kinase and the receptor (20, 23, 24). Jak family activation has been related to the regulation of gene expression through tyrosine phosphorylation of signal transducers and activators of transcription (Stat) transcriptional factors (25). However, the role of PRLR tyrosine phosphorylation is unknown.

To examine the biological function of the PRLR isoforms, Chinese hamster ovary cell lines expressing each form were cotransfected with constructions carrying milk protein promoters. It was thus demonstrated that the long and Nb-2 forms of PRLR are able

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to induce β-lactoglobulin and β-casein promoters (26). In addition, both PRLR isoforms mediate the PRL induction of interferon regulatory factor-1 promoter and cellular proliferation in the IL-3-dependent cell lines (27). However, the function of the short form of the PRLR remains to be established.

The liver is one of the main targets for PRL according to the level of PRLR transcripts, and it is the only tissue in which the short form of the receptor is predominant (28). We previously reported that PRL increases both c-*myc* gene expression (29) and the cytosolic free calcium concentration (30) in isolated hepatocytes. In this work we analyzed the PRL signal transduction pathway in isolated hepatocytes of lactating rats. We demonstrate that PRL stimulation of cells induces the association of PRLR with pp60$^{c\text{-}src}$, pp60$^{c\text{-}src}$ activation and an increase in c-*fos*, c-*jun*, and c-*src* gene expression.

RESULTS

Kinase Activity in PRLR Immune Complexes

We first studied whether the PRLR was associated with a kinase activity that could account for the intracellular signaling of PRL in the liver of lactating rats. For this purpose, PRLR was immunoprecipitated with monoclonal antibody (MAb) T6 from a detergent-solubilized liver extract and assayed for *in vitro* kinase activity (see *Materials and Methods*). An example of these experiments is shown in Fig. 1, lane 1. We consistently detected two phosphorylated proteins, the immunoglobulin G (IgG) heavy chain and a protein of approximately 60 kilodaltons (p60). As some members of the cytokine receptor family associate with protein kinases of the Src family (15, 17, 21, 31–34), an aliquot of the liver extracts was used to immunoprecipitate c-*src* with MAb 327 and assayed for autophosphorylation (see *Materials and Methods*). As indicated in Fig. 1, lane 2, the electrophoretic resolution of the c-*src*-MAb 327 immune complex showed three phosphorylated bands corresponding to an autophosphorylated (pp60$^{c\text{-}src}$ band) product, the IgG, and another unidentified (p45) protein. Interestingly, the p60 detected in PRLR immune complexes comigrated with pp60$^{c\text{-}src}$. Moreover, the ^{32}P incorporated into these proteins was resistant to KOH treatment (data not shown), which means that they were phosphorylated in tyrosine residues. These initial results led us to conclude that a protein tyrosine kinase is associated with PRLR in the liver of lactating rats.

PRLR Association with pp60$^{c\text{-}src}$

Isolated hepatocytes from lactating rats were used to demonstrate a plausible physical association of PRLR with pp60$^{c\text{-}src}$ and to analyze the possible role of PRL in such an interaction. We determined whether c-*src* was coprecipitated with PRLR immune complexes. A representative assay (one of four experiments) is shown in Fig. 2. Four aliquots of 6×10^7 cells were incubated for 30 min in the presence or absence of ovine PRL (oPRL; 1 μg/ml). The cell lysates were used to immunoprecipitate PRLR with MAb U5 (lanes 3 and 4) or pp60$^{c\text{-}src}$ with MAb LA074 (lane 2). A mouse preimmune serum (prel) was used as a control (lane 1). The immune complexes were electrophoretically resolved in a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with anti-pp60$^{c\text{-}src}$ MAb LA074. In the absence of hormone, c-*src* kinase was minimally associated with PRLR (lane 3). However, after a 30-

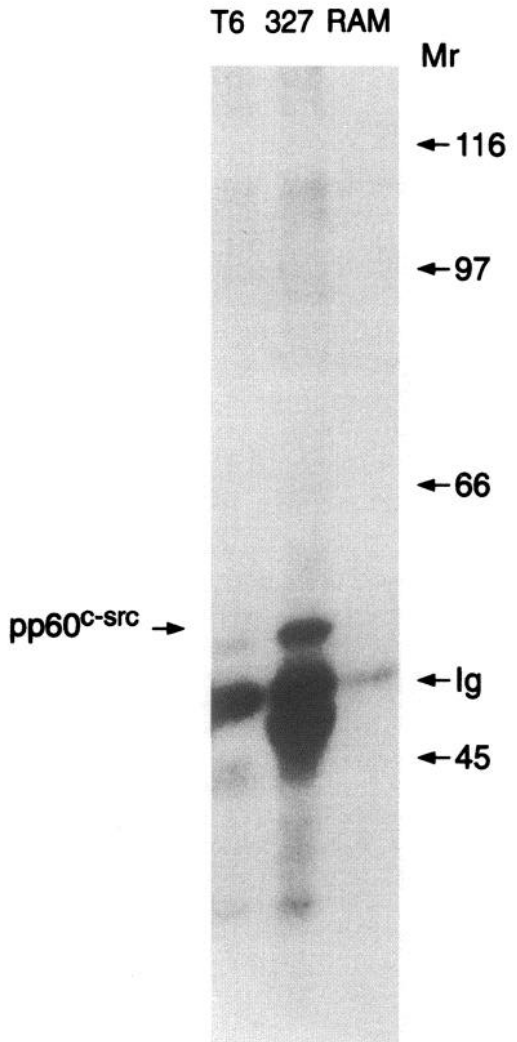

Fig. 1. PRLR Immune Complex Kinase Assay

Proteins from liver of lactating rats were solubilized as described in *Materials and Methods*. Aliquots derived from 1 g tissue were used to immunoprecipitate PRLR with T6 (lane 1) and pp60$^{c\text{-}src}$ with 327 (lane 2). A rabbit antimouse serum was used as a control (lane 3). The immune complexes were subjected to *in vitro* kinase reaction for 10 min at room temperature and resolved in a 7% SDS-polyacrylamide electrophoresis gel. The phosphoproteins were detected by autoradiography. Standard proteins and migration of pp60$^{c\text{-}src}$ are indicated.

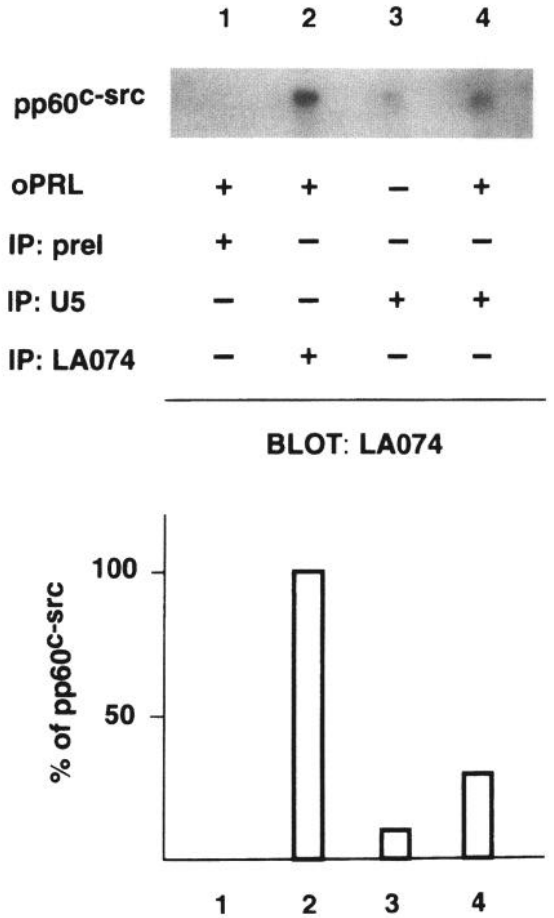

Fig. 2. Association of pp60$^{c\text{-}src}$ with the PRLR

Samples containing 6×10^7 hepatocytes isolated from lactating rats were preincubated for 30 min in the absence of hormone and then incubated for 30 min more with or without oPRL (1 μg/ml). The lysates were used to immunoprecipitate PRLR with U5 (lanes 3 and 4) and pp60$^{c\text{-}src}$ with 327 (lane 2), and preimmune mouse serum was used as a control (lane 1). The immune complexes were dissociated in nonreducing loading buffer, resolved in SDS-10% PAGE, blotted onto nitrocellulose, and probed for pp60$^{c\text{-}src}$ with LA074. Detection was carried out by incubation with [^{125}I]sheep antimouse IgG and autoradiography (see *Materials and Methods*). The *lower panel* shows the densitometric analysis of four different experiments. They are expressed as a percentage of the value obtained with pp60$^{c\text{-}src}$. SEMs were ±3.7 and ±7.6 for the data in lanes 3 and 4, respectively.

min stimulation with PRL, a significant amount of c-*src* coprecipitates with PRLR (lane 4), whereas no c-*src* can be detected in control immunoprecipitates (lane 1). Densitometric analysis of the blots from four different experiments demonstrated that addition of ligand resulted in a 3-fold increase in the amount of pp60$^{c\text{-}src}$ coupled to the receptor in liver cells.

Two different approaches were used to demonstrate that PRL stimulates the tyrosine kinase activity of pp60$^{c\text{-}src}$. As the increase in c-*src* kinase activity correlates with an increase in autophosphorylation (35–37), we first tested whether pp60$^{c\text{-}src}$ was tyrosine phosphorylated in response to PRL. Hepatocytes isolated from lactating rats (aliquots of 6×10^6 cells) were treated for 10 min with different amounts of PRL, and pp60$^{c\text{-}src}$ was immunoprecipitated from the corresponding cell lysates. The content of phosphotyrosine in pp60$^{c\text{-}src}$ was detected by Western blot probing with PY20 antibody. Figure 3 shows that in this system, pp60$^{c\text{-}src}$ is tyrosine phosphorylated in a PRL amount-dependent manner. Densitometric analysis showed that PRL present at a concentration of 0.05 μg/ml increased the phosphotyrosine content of c-*src* by 2-fold, and 1 μg/ml PRL increased the phosphotyrosine content by 3.7-fold.

In a second approach, we performed a time-course stimulation of isolated hepatocytes with PRL and tested the kinase activity of pp60$^{c\text{-}src}$ using acid-denatured enolase as an exogenous substrate. As shown in Fig. 4, the activity of pp60$^{c\text{-}src}$ in response to PRL addition followed a transient stimulation pattern, peaking at 2–10 min and returning to basal levels within 20 min. PRL increased by 1.8-fold the total cellular c-*src* kinase activity within 10 min after stimulation.

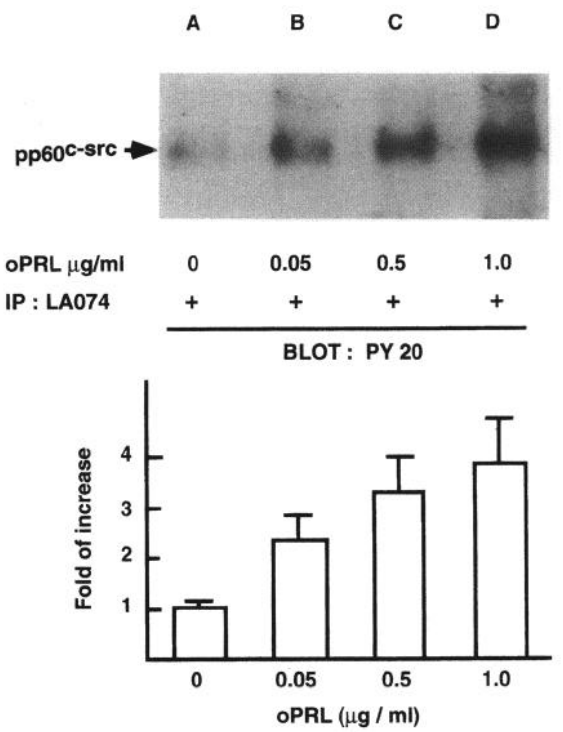

Fig. 3. PRL Induction of pp60$^{c\text{-}src}$ Tyrosine Phosphorylation

Hepatocytes (6×10^6) from lactating rats were incubated with different concentrations of oPRL for 10 min. Then, pp60$^{c\text{-}src}$ was immunoprecipitated from the cell lysates, and the immune complexes were dissociated in nonreducing loading buffer, resolved in SDS-9% PAGE, blotted, probed with the antiphosphotyrosine antibody PY20, and developed with ECL (see *Materials and Methods*). The *lower panel* shows the densitometric analysis of three different experiments. Phosphorylation results were normalized for the amount of pp60$^{c\text{-}src}$ detected by reprobing the blot with LA074, and *error bars* indicate the SEM.

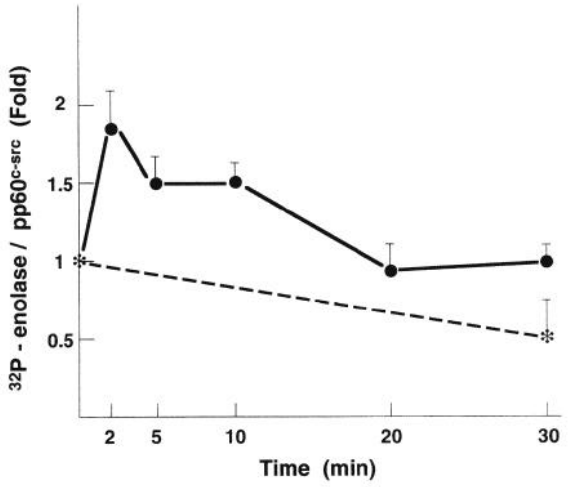

Fig. 4. PRL Induction of pp60$^{c\text{-}src}$ Kinase Activity

Hepatocytes (6×10^6) isolated from lactating rats were incubated with (●) or without (*) oPRL (1 μg/ml) for 0–30 min. pp60$^{c\text{-}src}$ was immunoprecipitated from cell lysates with LA074, and the *in vitro* kinase activity was measured within 3 min using acid-denatured enolase as exogenous substrate in the presence of [γ-^{32}P]ATP. The reaction were stopped by the addition of nonreducing loading buffer, resolved in SDS-9% PAGE, and blotted onto nitrocellulose. Filters were subjected to autoradiography and finally probed with LA074 (see *Materials and Methods*). The phosphorylation results were normalized for the amount of pp60$^{c\text{-}src}$ detected in Western blot and expressed as fold induction with respect to zero time. They are the mean of five separate experiments, and *error bars* indicate the SEM.

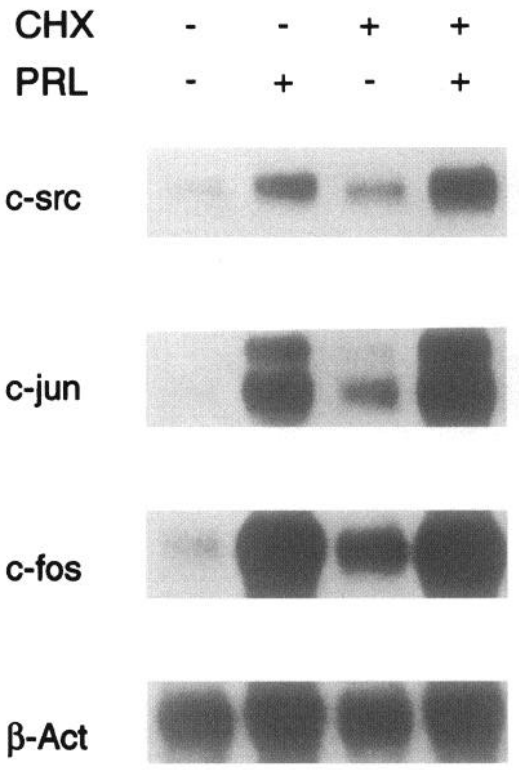

Fig. 5. PRL Activation of Immediate Early Response Genes and c-*src* in Isolated Hepatocytes

Hepatocytes isolated from lactating rats were incubated at 5×10^7 cells/50 ml incubation medium. After 30 min of preincubation, cells were incubated with 1 μg/ml oPRL in the presence or absence of 10 μg/ml cycloheximide for 2 h. Polyadenylated RNA (5 μg) was analyzed by Northern blotting for c-*fos*, c-*jun*, c-*src*, and β-actin mRNAs.

These data are in good agreement with the activation of c-*src* kinase by colony stimulating factor-1 (32). Thus, the results suggest that PRL signal transduction in liver cells is mediated at least in part by PRLR association with pp60$^{c\text{-}src}$ and the stimulation of its tyrosine kinase activity.

PRL Increases the c-*fos*, c-*jun*, and c-*src* Levels of mRNA in Hepatocytes

The changes in cell growth rate and phenotype elicited by growth factors are believed to be initiated by the transcriptional induction of a set of immediate early response genes (38–40). Our previous studies have shown that oPRL stimulates expression of the protooncogene c-*myc* (29). We assessed the possible effect of PRL on the expression of c-*fos*, c-*jun*, and c-*src* in isolated hepatocytes. As shown in Fig. 5, this hormone significantly increases the levels of mRNA for the protooncogenes c-*fos* (9-fold), c-*jun* (13-fold), and c-*src* (3.6-fold). In the absence of protein synthesis, the levels of the above-mentioned genes were increased by factors of 3.1, 7.2, and 3.4, respectively. These data suggest that PRL is involved in the regulation of these important immediate early response genes.

DISCUSSION

It is now well established that cytokines initiate the intracellular signaling pathways by activation of multiple protein tyrosine kinases associated with their receptors (see Ref. 41 for review). The identification of protein tyrosine kinases associated with each receptor is the first step in understanding the biological role of these kinases. In the work presented here we demonstrated that the protein tyrosine kinase pp60$^{c\text{-}src}$ associates with PRLR in liver cells upon PRL stimulation. Another member of the Src family kinases, p56^{fyn}, has been identified as being permanently associated with PRLR isoforms expressed in Nb-2 cells (21). Although pp60$^{c\text{-}src}$ was detected in PRLR immune complexes, we were unable to detect PRLR in Western analysis of PRLR or pp60$^{c\text{-}src}$ immunoprecipitates. A potential explanation for this apparent discrepancy could be that anti-PRLR antibodies (U5 and U6 were used) have a lower affinity for the denatured receptor than for the native form.

In this work we show that PRL promotes both pp60$^{c\text{-}src}$ association with PRLR and activation of this protein tyrosine kinase. As reviewed by Erpel and

Courtneidge (42), activation of Src family kinases could occur in three ways: dephosphorylation of phosphotyrosine 527 at the carboxy-terminal region, competition for the SH2 domain by a high affinity phosphotyrosine-containing ligand, and competition for the SH3 domain by a high affinity proline-rich ligand. The PRLR isoforms have a PPVP motif at the membrane-proximal region that may be involved in the coupling of Jak or Src family kinases. However, activation of the members of the latter family by interaction with the SH3 domain has not yet been reported. The activation of Src by a phosphotyrosine-containing ligand has been demonstrated during ligand activation of platelet-derived growth factor receptor (43). As Jak2 is constitutively associated with PRLR isoforms in Nb-2 (19, 20), BAF-3 (23), mammary gland (44, 45), and 293 cells (46), it is possible that a Jak family member or another protein tyrosine kinase expressed in liver cells mediates a initial tyrosine phosphorylation of PRLR upon PRL stimulation. After the initial tyrosine phosphorylation of PRLR or PRLR-protein tyrosine kinase complex, the activation of $pp60^{c\text{-}src}$ must occur by competition for the SH2 domain. In agreement with this explanation, recently published data have demonstrated that a proline-rich sequence is required for Jak2 association with the PRLR, but is not sufficient for signal transduction (24). In addition, a single phosphotyrosine residue located at the carboxy-terminal region of the PRLR is responsible for PRL-dependent induction of β-casein promoter in 293 cells (46). Interestingly, PRL stimulates tyrosine phosphorylation of different tyrosine residues of PRLR isoforms, but the mutation of the distal tyrosine prevents the receptor from inducing the β-casein promoter. The transducer molecule binding the distal phosphotyrosine of PRLR and involved in the activation of milk protein gene transcription has not yet been identified.

In hepatocytes, we have also shown that PRL stimulates expression of the protooncogenes c-*fos*, c-*jun*, and c-*src*. This suggests that PRL stimulates the expression of genes with AP-1 or SRE sequences in their promoter regions. However, the possible substrates or kinases downstream of $pp60^{c\text{-}src}$ in liver cells to drive PRL messenger to the nucleus need to be established. Src kinases are involved in Ras activation in fibroblasts (47), phosphorylation of cytoskeletal proteins (48, 49), c-*myc* induction (50), or Sam68 phosphorylation (51) among other functions (see Ref. 42 for review). As Raf is activated by PRL (22), it is interesting to speculate that Ras activation may be involved in PRL intracellular signaling in hepatocytes. However, a Src-Ras-independent pathway has been demonstrated in IL-2, granulocyte-macrophage colony-stimulating factor, and epidermal growth factor receptor signaling (52, 53). Which of these two mechanisms controls the expression of PRL-induced expression of c-*myc* in liver cells needs to be determined (29). We hope that future work will clarify our understanding of the interaction among PRLR with $pp60^{c\text{-}src}$, the mechanism of $pp60^{c\text{-}src}$ activation, and the role of this kinase in liver cells.

MATERIALS AND METHODS

Preparation and Culturing of Rat Hepatocytes

Hepatocytes were isolated from lactating Wistar rats, which were maintained on a 12-h light, 12-h dark photoperiod, 2–3 days after delivery. The livers were perfused for 20 min with Krebs-Henseleit (calcium-free) buffer containing 40 mg/ml collagenase (Boehringer Mannheim, Indianapolis, IN) and supplemented with 5 mM glucose, 2 mM glutamine, 100 U/ml penicillin, and 1% BSA (54). Cells were washed and incubated as described previously (29). Cell viability, determined by the trypan blue exclusion test, was approximately 90%. The PRL used (oPRL) was kindly provided by the NIDDK (National Hormone and Pituitary Program).

Antisera and Immunoprecipitations

Isolated hepatocytes were lysed at a concentration of 25×10^6 cells/ml in 25 mM Tris-HCl (pH 7.4), 4 mM EDTA, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride, 0.3 mg/ml bacitracin, 0.3 mg/ml trypsin inhibitor, 1 mg/ml benzamidine, and 2% Triton X-100 for 30 min at 4 C. When entire livers were used, they were rinsed once with ice-cold PBS and homogenized at 4 C in lysis (Triton-free) buffer (1 g liver/5 ml) with a Polytron (Brinkmann Instruments, Westbury, NY) three times for 30 sec each time and then adjusted to 2% Triton X-100. After 30 min of continuous rotation at 4 C, insoluble material was removed by centrifugation at $25,000 \times g$ for 45 min at 4 C. The resulting supernatants were diluted (1:1) with buffer A [20 mM Tris-HCl (pH 7.4), 140 mM NaCl, 5 mM EDTA, and 1% Triton X-100] before immunoprecipitation. The antibodies used in the experiments were U5, U6, or T6 anti-rat PRLR recognizing the extracellular domain of PRLR [characterized by Okamura *et al.* (55)] at 2 μg/ml; 327 anti-$pp60^{c\text{-}src}$ (generously provided by Dr. J. S. Brugge) at 5 μl ascites/ml; LA074 anti-$pp60^{c\text{-}src}$ (Quality Biothech, Camden, NJ) at 5 μl ascites/ml; and PY20 antiphosphotyrosine (ICN, Costa Mesa, CA) at 2 μg/ml. Immunoprecipitations were performed for 2 h at 4 C. Immune complexes were precipitated with rabbit antimouse IgG (RAM) (Nordic Immunological Laboratories, Tilburg, The Netherlands) bound to protein A-Sepharose and washed as follows: three times with buffer B [10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na_3VO_4, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 2 μg/ml leupeptin, and 0.5 mg/ml benzamidine], once with 0.5 M LiCl, and, finally, twice with 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 100 mM NaCl.

In Vitro Kinase Reactions

The clean immune complexes were washed once more with kinase buffer [30 mM Tris-HCl (pH 7.4) and 5 mM $MnCl_2$] and incubated at room temperature in 20 μl kinase buffer containing 10 μCi [γ-^{32}P]ATP (3000 Ci/mmol) and 5 μg acid-denatured enolase for different time intervals. These reactions were stopped at different times by the addition of 20 μl twice concentrated SDS-gel loading buffer and boiled for 10 min. Depending on the experiment, a nonreducing loading buffer (125 mM Tris-HCl, pH 6.8, containing 4% SDS, 20% glycerol, and 18.5 mg/ml iodoacetamide) was alternatively used. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were dried or transferred to nitrocellulose (Schleicher and Schuell, Dassel, Germany) and exposed to Kodak X-Omat AR films (Eastman Kodak, Rochester, NY). The autoradiograms were scanned using a Molecular Dynamic scanner (Sunnyvale, CA) or a PhosphorImager from Bio-Rad (Richmond, CA).

Immunoblotting

The association of PRLR with pp60$^{c\text{-}src}$ in hepatocytes was demonstrated as follows. Aliquots of 6×10^7 cells were incubated with or without oPRL (1 μg/ml) for 30 min at room temperature, pelleted, and disrupted in 3 ml lysis buffer as before. Immunoprecipitations were carried out using mouse preimmune serum (as a control), anti-PRLR (MAb U5), or

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washed three times with buffer B and heated at 80 C for 3 min in 125 mM Tris-HCl, pH 6.8, containing 4% SDS, 20% glycerol, and 18.5 mg/ml iodoacetamide. The proteins were resolved by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose membrane was blocked at 37 C for 2.5 h with Tris-buffered saline containing 5% milk proteins (Fluka BioChemica, Buchs, Switzerland) and 0.05% Tween 20 (TTBS). The blotted proteins were probed for 1 h at room temperature with MAb LAO74 diluted 1:125 in TTBS containing 5% milk protein, then incubated with ^{125}I-labeled sheep antimouse IgG (0.5 μCi/ml; Amersham Corp., Arlington Heights, IL), washed, and exposed to film. Alternatively, nitrocellulose membranes were blocked with either 5% nonfat BSA or 5% milk protein for 3 h and incubated overnight at 4 C with PY20 antibody diluted 1:1000 in TTBS containing 1% BSA or with LAO74 diluted 1:125 in TTBS containing 5% milk protein. The secondary antibody used for detection was labeled with antimouse horseradish peroxidase. The blots were washed and developed using the ECL chemiluminiscence system (Amersham Corp.) according to the manufacturer's instructions.

Isolation and Quantitation of mRNA

Total RNA was isolated from lactating rat hepatocytes using the standard phenol protocol and enriched for polyadenylated RNA by a single passage through an oligo(deoxythymidine)-cellulose column (56). The RNA was fractionated and transferred to Hybond-N nylon membranes as previously described (29). Gel-purified DNA probes were either a 2.6-kb *Pst*I insert of mouse c-*jun*-coding sequence (38) or a 1.2-kb *Pst*I insert of mouse c-*fos*-coding sequence, kindly provided by Dr. Naranjo (Instituto Cajal, Madrid, Spain). The probes for c-*src* and β-actin were purchased from Clontech (Palo Alto, CA). They were labeled by nick translation with [α-^{32}P]deoxy-CTP to a specific activity of $1\text{–}2 \times 10^8$ cpm/μg (57). The hybridization results were expressed as the ratio to β-actin messenger.

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Address requests for reprints to: Dr. P. Garcia-Ruiz, Department de Biologia Molecular, Centro de Biologia Molecular Severo Ochoa, Campus Cantoblanco, 28049 Madrid, Spain.

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