Src Family Kinases Are Required for Prolactin Induction of Cell Proliferation

Juan Ángel Fresno Vara,* Mª Aurora Domínguez Cáceres,* Augusto Silva,† and Jorge Martín-Pérez†‡

*Instituto de Investigaciones Biomédicas and †Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Madrid 28029, Spain

Prolactin (PRL) is a pleiotropic cytokine promoting cellular proliferation and differentiation. Because PRL activates the Src family of tyrosine kinases (SFK), we have studied the role of these kinases in PRL cell proliferation signaling. PRL induced [^{3}H]thymidine incorporation upon transient transfection of BaF-3 cells with the PRL receptor. This effect was inhibited by cotransfection with the dominant negative mutant of c-Src (K>A295/Y>F527, SrcDM). The role of SFK in PRL-induced proliferation was confirmed in the BaF-3 PRL receptor-stable transfectant, W53 cells, where PRL induced Fyn and Lyn activation. The SFK-selective inhibitors PP1/PP2 and herbimycin A blocked PRL-dependent cell proliferation by arresting the W53 cells in G1, with no evident apoptosis. In parallel, PP1/PP2 inhibited PRL induction of cell growth-related genes c-*fos*, c-*jun*, c-*myc*, and *odc*. These inhibitors have no effect on PRL-mediated activation of Ras/Mapk and Jak/Start pathways. In contrast, they inhibited the PRL-dependent stimulation of the SFKs substrate Sam68, the phosphorylation of the tyrosine phosphatase Shp2, and the PI3K-dependent Akt and p70S6k serine kinases. Consistently, transient expression of SrcDM in W53 cells also blocked PRL activation of Akt. These results demonstrate that activation of SFKs is required for cell proliferation induced by PRL.

INTRODUCTION

Prolactin (PRL) is a pleiotropic cytokine promoting cellular proliferation, differentiation, or survival, depending on the physiological and cellular context (Doppler, 1994; Bole-Feysot *et al.*, 1998; Morales *et al.*, 1999). These events are mediated through activation of the PRL receptor (PRLR), a member of the class I superfamily of cytokine receptors (Bazan, 1990; Horseman and Yu-Lee, 1994; Watowich *et al.*, 1996). The PRLR has no inherent enzymatic activity but triggers activation of the associated Jak2 and Src family of tyrosine kinases (SFKs) (Clevenger and Medaglia, 1994; Dusanter-Fourt *et al.*, 1994; Berlanga *et al.*, 1995; Fresno Vara *et al.*, 2000), which phosphorylate PRLR and other signaling molecules involved in the control of cell functions, including PI3K and Shp2 (Ali *et al.*, 1996; Al-Sakkaf *et al.*, 1997; Berlanga *et al.*, 1997).

The Src kinases are modular proteins sharing a high degree of homology in the kinase, SH2 and SH3 domains, whereas the amino-terminal portion confers to each of them some degree of specificity (Thomas and Brugge, 1997; Corey and Anderson, 1999). In addition to c-Src, the SFK prototype, the family has other members: Blk, Fyn, Frg, Hck, Lck, Lyn, and Yes (Thomas and Brugge, 1997). Some of them, Blk, Hck, Fgr, Lck, and Lyn are restricted to hematopoietic tissue, whereas Fyn, c-Src, and Yes are widely expressed (Corey and Anderson, 1999)

Although the association of SFKs to cytokine receptors has been well established (Taniguchi, 1995; Corey and Anderson, 1999), their precise contribution to the signaling mechanisms induced by cytokines remains unclear. The activation of c-Src and Fyn in response to PRL has been previously observed (Clevenger and Medaglia, 1994; Berlanga *et al.*, 1995), and it has been later shown that this event is independent of Jak2 (Fresno Vara *et al.*, 2000). SFK is required for cellular growth induced by a number of growth factors and cytokines, including colony stimulating factor one (CSF-1), gradulocyte-colony stimulating factor (G-CSF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), etc. (Thomas and Brugge, 1997; Corey and Anderson, 1999). Recently, the discovery of the selective SFK inhibitors PP1/PP2 (Hanke *et al.*, 1996; Liu *et al.*, 1999; Schindler *et al.*, 1999) has helped to unravel the role of these kinases in signal transduction (Schlaepfer *et al.*, 1998; Broudy *et al.*, 1999; Conway *et al.*, 1999; Osterhout *et al.*, 1999; Park *et al.*, 1999; Owens *et al.*, 2000).

We recently generated a new cell line by stable expression of PRLR on the IL-3-dependent BaF-3 proB cell line (Palacios

‡ Corresponding author. E-mail address: jmartin@iib.uam.es.

and Steinmetz, 1985). This new cell line, named W53, grows in PRL-enriched media without IL-3 and expresses molecular markers related to B cell differentiation as the λ5 gene (Morales *et al.*, 1999). Here, we have investigated the role of SFK on PRL-induced proliferation. Expression of SrcDM (double mutant c-Src K>A295/Y>F527) efficiently blocked PRL-induced [³H]thymidine incorporation in PRLR transiently transfected BaF-3. The use of the SFK-selective inhibitors, PP1/PP2 and herbimycin A, in W35 cells has helped us to define the role of SFKs in PRL-induced proliferation. Here we show that the SFKs are required for PRL-stimulated DNA synthesis, as well as for expression of growth-related immediate early genes (IEGs), c-*fos*, c-*jun*, and c-*myc*. Moreover, inhibition of SFKs blocks PRL-induced tyrosine phosphorylation of Sam68 and Shp2 and the PI3K-regulated activation of Akt and the p70S6k. Consistently, transient expression of SrcDM in W53 cells also blocked PRL activation of Akt. However, the SFK inhibitors do not affect Jak2 activation and phosphorylation of PRLR and Stat5.

MATERIALS AND METHODS

Reagents

Tissue culture media, sera, and Trizol were purchased from Life Technologies (Renfrewshire, UK). Ovine PRL (PRL, NIDDK-oPRL-20, 31 IU/mg) was kindly provided by the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD). BCA protein assay reagent was from Pierce (Rockford, IL). Anti-p70S6k was a gift of G. Thomas (Friedrich Miescher Institute, Basel, Switzerland). Rabbit polyclonal anti-Sam68 was a gift of S. Fumagalli (Friedrich Miescher Institute). Mouse monoclonal antibody (mAb) U5 to PRLR was purchased from Affinity Bioreagents (Golden, CO). The mAb 327 to c-Src was a kind gift of J.S. Brugge (Harvard University, Cambridge, MA). Anti-phosphotyrosine mAb 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies against Blk (K-23), Fyn (Fyn3), Lyn (H-6), c-Src (SRC2), Jak2 (C-20), Erk2 (C-14), Stat 5 (C-17), Akt1/2 (H-136), Shp2 (SH-PTP2, N-16), Jnk2 (FL), pJnk (G-7), p38 (H-147), HA (Y-11), and c-Abl (K-12) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Lck (L15620) and c-Yes (Y35320) were from Transduction Laboratories (Lexington, KY), and anti-pMek1/2, pErk1/2 (pp42/44), pp70S6k, pAkt, and anti-pp38 antibodies were from New England Biolabs (Beverly, MA). The anti-Src-pY418 (recognizing the autophosphorylation sequence of the SFKs, which is highly conserved) and the secondary antibodies-horseradish peroxidase-conjugated were purchased from Biosource International (Camarillo, CA). The enhanced chemiluminiscence (ECL) kit, radiochemicals, and the Oligolabeling kit were from Amersham Pharmacia Biotech (Buckinghamshire, UK). PP1/PP2, herbimycin A, and LY-294002 were obtained from Alexis Biochemicals (San Diego, CA). Specific probes were used for Northern hybridization for c-*fos*, c-*jun*, c-*myc*, *odc*.

Constructions of Expression Vectors

The PRLR cDNA-coding sequence (P. A. Kelly, Institut National de la Santé et de la Recherche Médicale, Paris, France) was excised from pBlueScript with *Eco*RI (5') and *Sal*I (3') and cloned into the same sites of pEF-Bos-XC (Mizushima and Nagata, 1990) for transient coexpression in BaF-3 cells. The SrcDM (c-Src mutant, K>A295/Y>F527; S. Roche, CRBM-Centre National de la Recherche, France), Csk (J.A. Cooper, Fred Hutchinson Cancer Research Center, Seattle, WA), and the SrcK− (c-Src, K>M295), a kinase-dead mutant of c-Src (K. Ballmer, IMR-PSI, Zurich, Switzerland) were cloned into pCI-neo (Promega, Madison, WI). The Jak2ΔK, a Jak2 form with the C-terminal kinase domain deleted, generated from

the original Jak2 cDNA (J. N. Ihle, St. Jude Children's Research Hospital, Memphis, TN) as described elsewhere (Fresno Vara *et al.*, 2000) was also cloned into pCI-neo. Akt-HA was cloned into pcDNA3 (Luis del Peso, Hospital la Princesa, Madrid; B. Hemmings, Friedrich Miescher Institute, Basel, Switzerland; B. Burgerin, University of Utrecht, the Netherlands).

Cell Lines and Culture Methods

The mouse IL-3–dependent BaF-3 cell line (Palacios and Steinmetz, 1985) was cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 4 mM L-glutamine, penicillin (100 U), and streptomycin (100 µg/ml) and 10% of WEHI-3B supernatant as a source of IL-3. The BaF-3-derived W53 cell line (PRLR transfectants) was cultured in RPMI-1640 containing 10% FCS and 6 ng/ml PRL as previously described (Morales *et al.*, 1999).

Transfection and [³H]Thymidine Incorporation Assays

Transient DNA cotransfection experiments were performed with 20 µg of pEF-Bos-XC-PRLR plasmid and the pCI-neo plasmid empty or containing the cDNA of either Jak2ΔK, SrcDM, Csk, and SrcK− (60 µg each) by electroporation (960 µF and 300 V) into BaF-3 cells (10⁷ cells/sample) with the use of a Gene Pulser (Bio-Rad Laboratories, Hercules, CA). Cells were cultured for 16 h and then seeded into 96-well plates (5 × 10⁴ viable cells/well) with various concentrations of PRL. After 48 h of culture, [³H]thymidine (1 µCi/well) was added, and cells were harvested onto glass fiber filters after 4 h of incubation. Radioactivity incorporation was quantitated in a β-counter (1450 Microbeta Wallac LKB, Turku, Finland).

W53 cell growth was measured by plating 5 × 10⁴ cells/well were plated on 96-well flat-bottom plates and cultured for 24 h on RPMI-1640 supplemented with 10% FCS and 6 ng/ml PRL, in the presence of different concentrations of SFK inhibitors, LY-294002, or equivalent amounts of solvent (dimethyl sulfoxide [DMSO], dilution 1:1000) as a control. Each well was pulsed for 4 h with 0.5 µCi [³H]thymidine, cells were harvested, and incorporated radioactivity was quantified as above.

Transient coexpression of Atk-HA tagged and SrcDM in W53 cells was carried out by electroporation as above: 4 × 10⁷ cells/sample were electroporated (960 µF and 300 V) with Akt-HA (40 µg) and either pCI-neo empty or pCI-neo-SrcDM (80 µg each). After culture for 24 h, cells were maintained 16 h in RPMI-1640 containing 1% horse serum to make them quiescent and subsequently stimulated with 100 mg/ml PRL for 1 h. Cells were then lysed and analyzed.

BrdU Pulse-Label Experiments and Flow Cytometry Analysis

Cell cycle kinetics were carried out by the bromodeoxyuridine (BrdU)/anti-bromodeoxyuridine method as previously described (Silva *et al.*, 1997). Briefly, cultures of 5 × 10⁵ cells/ml were incubated with 10 µM PP1, and 90 min later, cells were pulse-labeled for 30 min with 10 µM of BrdU. At the end of the labeling period, cells were washed twice with prewarmed culture media and resuspended at 5 × 10⁵ cells/ml in culture media containing 6 ng/ml PRL and 10 µM PP1. At given times, aliquots of 2 × 10⁶ cells were collected from the cultures, centrifuged at 500 × *g* for 5 min at room temperature, and then fixed in 1 ml of phosphate-buffered saline (PBS)-70% ethanol for at least 1 h at 4°C. Fixed cells were resuspended in 2 ml of 2 M HCl containing 10 µl of pepsin buffer (0.4 mg/ml pepsin in 0.1 M HCl) and incubated for 20 min at 37°C, washed three times in PBS and incubated for 1 h at 25°C in PBS-Tween buffer (PBS, with 0.5% Tween-20, 0.5% FCS) and 10 µl of fluorescein isothiocyanate (FITC)-labeled anti-BrdU (Becton Dickinson, San Diego, CA). Cells were then washed twice in PBS and resuspended in 1 ml of PBS containing 20 µl of propidium iodide (10 mg/ml) just before the flow cytometry analysis, which was

Figure 1. Role of SFK and Jak2 in PRLR-transduced proliferative signals. BaF-3 cells were transiently cotransfected with the pEF-Bos-XC-PRLR in combination with pCI-neo or pCI-neo containing the cDNA of Csk, SrcK−, SrcDM, or Jak2ΔK; 16 h later cells were stimulated with increasing concentrations of PRL and then pulsed with [^{3}H]thymidine. The incorporation of radioactivity into DNA was quantified as described in MATERIALS AND METHODS. The results are expressed as a fold of [^{3}H]thymidine incorporation referred to BaF-3 cells transfected with the empty vector (pCI-neo). The results represent the averagea ± SD of three independent experiments carried out in triplicate.
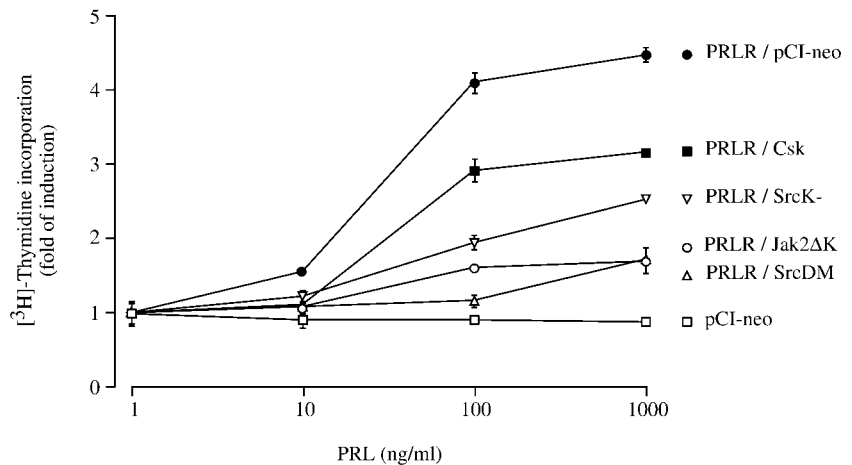
performed on an EPICS-XL flow cytometer (Coulter, Hialeah, FL). Background signals were set by incubating BrdU-unlabeled cells with 10 μl FITC-anti-BrdU. Because PP1 was dissolved in DMSO, control cultures were incubated with the equivalent volume of DMSO (dilution 1:1000) instead of PP1.

Cell Stimulation, Immunoprecipitation, and Western Blot Analysis

W53 cultures were washed with RPMI-1640 to remove PRL and cultured overnight with medium containing 1% horse serum. The next day cells were pretreated for 2 h with 10 μM PP1/PP2, or the equivalent volume of DMSO (dilution 1:1000), as a control. For herbimycin A (0.7 μM), cells were pretreated overnight. Cells were then left unstimulated or stimulated with 100 ng/ml PRL and harvested after 10 min of incubation. Stimulation was stopped by washing the cells once in ice-cold PBS; cells were subsequently lysed with 1 ml per 2×10^7 cells of lysis buffer [LB: 1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na$_3$VO$_4$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM phenantroline, 1 mM benzamidine hydrochloride, 1 mM iodoacetamide]. The total cell lysates, the supernatants from a centrifugation of 15,000 × g for 30 min at 4°C, were compensated with LB for the same protein concentration after being determined by the BCA protein assay. An aliquot was boiled in 1× SDS sample buffer (62.5 mM Tris-HCl [pH 6.8], 5% β-mercaptoethanol, 2% SDS, 10% glycerol) and stored at −80°C until further use. The remainder of the cell lysates were incubated for 1 h at 4°C with the appropriate antibody. Immune complexes were collected by incubation for 1 h at 4°C with 30 μl of protein G-Sepharose beads (Sigma, St. Louis, MO), washed several times with LB and eluted by boiling in 2× SDS sample buffer. The immunoprecipitates of SFKs or Akt to be blotted were dissociated with freshly prepared 2× SDS sample buffer (containing 18.3 mg/ml iodoacetamide, without β-mercaptoethanol) at 60°C for 3 min.

For Western blotting analysis, samples were subjected to SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Filters were blocked with 5% fat-free dried milk (Fluka BioChemika, Neu-Ulm, Switzerland) in TTBS (10 mM Tris-HCl, pH 7.4, 0.1% Tween 20), or 5% bovine serum albumen in TTBS for anti-phosphotyrosine immunodetections. The blocked membranes were incubated with the primary antibody in blocking buffer, washed three times with TTBS, and further incubated with the suitable horseradish peroxidase-conjugated anti-species–specific antibody. Proteins were visualized by ECL (Amersham Pharmacia Biotech, Buckinghamshire, UK).

In Vitro AutoKinase Assay

The in vitro autokinase assays, were carried out as described previously (Fresno Vara *et al.*, 2000). Briefly, the immune complexes were washed with LB, then with TBS and finally with kinase buffer [20 mM Tris-HCl (pH 7.4), 10 mM MnCl$_2$], and then incubated for 4 min at 30°C in 30 μl of kinase buffer containing 2 mM β-mercaptoethanol, 1 μM ATP, 10 μCi [γ-^{32}P]ATP (4500 Ci/mmol, ICN). The reaction was stopped by the addition of 2× sample buffer and boiled for 5 min. Eluted proteins were resolved by SDS-7% PAGE, the gels were treated with 1 M KOH for 1 h at 55°C to remove background due to serine phosphorylation, and ^{32}P-labeled proteins were visualized by autoradiography.

Northern Blot Analysis

W53 cultures at 5×10^5 cells/ml were maintained overnight in RPMI-1640 medium supplemented with 1% horse serum. Then cells were pretreated for 2 h with 10 μM PP1/PP2 or the equivalent volume of DMSO (dilution 1:1000) as a control. At 0 h cells were stimulated with 100 ng/ml PRL. Aliquots of 5×10^6 cells were taken at different times and total RNA was isolated from them with the use of Tryzol. RNA was fractionated by electrophoresis through a 1% agarose gel containing 6% formaldehyde and transferred onto Nytran membranes (Schleicher & Schuell, Dassel, Germany) by capillary blotting. Blots were hybridized with cDNA probes labeled with the Oligolabeling kit with the use of [α-^{32}P]dCTP (3000 Ci/mmol; Amersham Pharmacia Biotech). After several washes, the hybridization signals on the blotted membrane were visualized by autoradiography.

RESULTS

The SFK is required for cellular growth induced by a number of growth factors and cytokines, including CSF-1, G-CSF, EGF, PDGF, etc. (Thomas and Brugge, 1997; Corey and Anderson, 1999). Because PRL induces the activation of SFKs and Jak-2 kinases (Clevenger and Medaglia, 1994; Berlanga *et al.*, 1995), independently of one another (Fresno Vara *et al.*, 2000), we analyzed the involvement of these enzymes in the PRL-induced proliferative response. The first observation was that, in BaF-3 cells transiently cotransfected with the PRLR and the empty pCI-neo plasmid, PRL stimulated [^{3}H]thymidine incorporation in a dose-dependent manner, reaching a plateau at ~1000 ng/ml PRL (Figure 1,

A

B
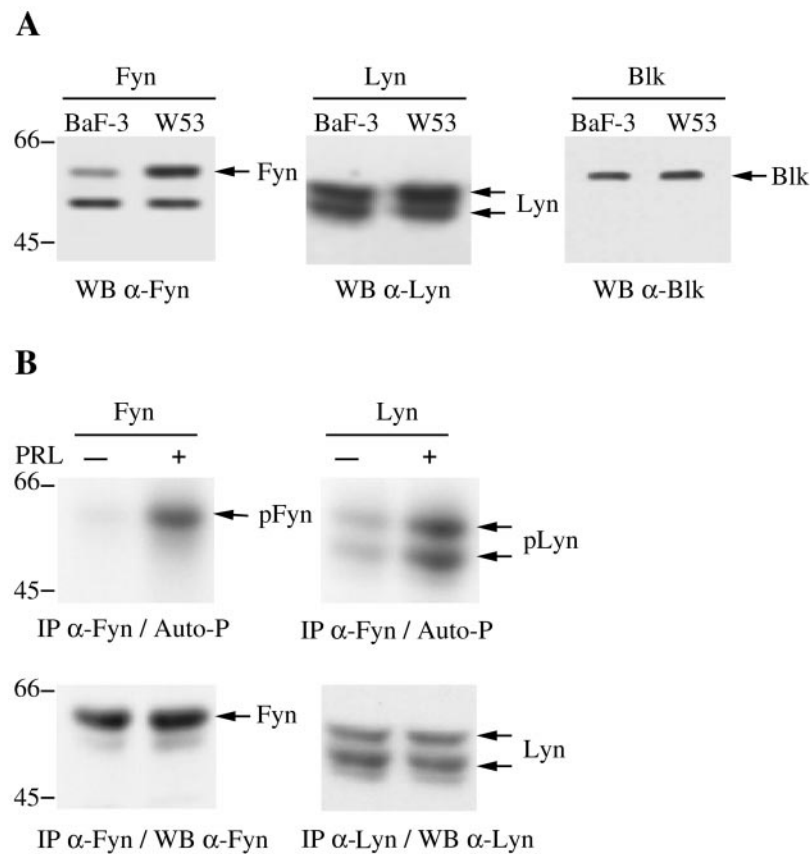
Figure 2. Expression of SFKs in BaF-3 and W53 and its activation by PRL. (A) Exponentially growing BaF-3 and W53 cells were collected and lyzed. Total cell extracts from both cell types, normalized for protein concentration, were separated by SDS-7% PAGE and immunoblotted with specific antibodies against the members of the SFK. (B) Quiescent and 10-min PRL-stimulated (100 ng/ml) W53 cells were lyzed and Blk, Fyn, and Lyn were immunoprecipitated with their specific antibodies. One-third of the immune complexes was subjected to autophosphorylation reactions and analyzed by SDS-7% PAGE and autoradiography (Auto-P). The other two-thirds of immune complexes were separated by SDS-7% PAGE and blotted against their specific antibodies (see MATERIALS AND METHODS).

PRLR/pCI-neo).

SFK activity is abolished by mutation at the ATP-binding site (K>M295 in the chicken c-Src kinase-dead mutant, SrcK−) or through phosphorylation of the tyrosine residue at the C-terminal tail (Y527 in the chicken c-Src) catalyzed by Csk, which facilitates an inactive enzyme conformation (Brown and Cooper, 1996). With the use of BaF-3 cells we analyzed the effect of transient coexpression of PRLR with pCI-neo containing the cDNAs of SrcK−, Csk, or SrcDM, a dominant negative form of c-Src combining the mutation both at the ATP-binding site (K>A295) and at the Csk tyrosine phosphorylation site (Y>F527; Mukhopadhyay *et al.*, 1995), which confers an open conformation to this mutant. As shown in Figure 1, coexpression of the receptor with either SrcK− (PRLR/SrcK−) or Csk (PRLR/Csk) partially inhibited PRL-induced DNA replication, as compared with cells that expressed only PRLR (PRLR/pCI-neo). When SrcDM (K>A295/Y>F527) was cotransfected with PRLR (PRLR/SrcDM), a 78% inhibition on PRL stimulation of DNA synthesis was observed, as compared with cells cotransfected with the receptor together with the empty pCI-neo plasmid (Figure 1). The role of Jak2 on PRL-dependent cell proliferation was also analyzed by cotransfection of BaF-3 cells with PRLR and Jak2ΔK (PRLR/Jak2ΔK). This dominant negative mutant of Jak2, with the kinase domain deleted (Fresno Vara *et al.*, 2000), blocked PRL-stimulated [³H]thymidine incorporation to the same extent as SrcDM (Figure 1). These data with the use of different mutants in

transient DNA cotransfection experiments strongly favor the requirement of SFK and Jak2 for cellular proliferation induced by PRL.

To further investigate the role of SFK on cell proliferation, we used W53 cells, a PRLR-stable transfectant BaF-3-derived cell line that depends on PRL for proliferation (Morales *et al.*, 1999). Because W53 cells showed changes in the gene expression pattern associated with B cell differentiation program (Morales *et al.*, 1999), we first determined the expression of SFK members in BaF-3 and in W53 cells by Western blotting of total cell extracts. From the seven members of this family of kinases, only Fyn, Lyn, and Blk were expressed both in BaF-3 and in W53 cells (Figure 2A). It should be noted that the levels of Fyn were higher in W53 than in BaF-3. Next, we determined which of these Src kinases were activated upon PRL stimulation of W53 cells. To this end, cultures of W53 cells were maintained overnight in RPMI-1640 containing 1% horse serum to make them quiescent. A set of cultures were then stimulated with 100 ng/ml PRL for 10 min. From extracts of quiescent and PRL-stimulated cells, normalyzed for protein concentration, Fyn, Lyn, and Blk were immunoprecipitated. One-third of each of the immune complexes was submitted to autokinase reaction for 4 min at 30°C in the presence of [γ-³²P]ATP, to separation by SDS-PAGE, and to subsequent autoradiography (see MATERIALS AND METHODS). As shown in Figure 2B, PRL stimulated only autophosphorylation/activation of Fyn (pp59) and Lyn (pp53/pp56) (Figure 2B, top). No autophosphory-

lation signal could be observed for Blk. The other two-thirds of the immune complexes were blotted against their specific antibodies to determine the amounts of Fyn (p59) and Lyn (p53/p56) (Figure 2B, bottom).

To study the role of SFKs in PRL induction of W53 proliferation, we used selective inhibitors of the SFK, such as the pyrazolopyridine derivatives PP1 and PP2 (Hanke *et al.*, 1996; Liu *et al.*, 1999; Schindler *et al.*, 1999) or the ansamycin antibiotic herbimycin A (Schlaepfer *et al.*, 1998; Abe *et al.*, 2000; Bosco *et al.*, 2000; Langlais *et al.*, 2000). Addition of PP1, PP2, or herbimycin A to W53 cells inhibited the PRL stimulation of thymidine incorporation in a dose-dependent manner, with an IC_{50} of ~5 μM for PP1 and PP2 (Figure 3A) and of ~0.4 μM for herbimycin A (Figure 3A). To prove the efficacy of these SFK inhibitors, we monitored the tyrosine phosphorylation of the SFK activation loops by Western blot analysis with the anti-Src-pY418 polyclonal antibody. Because the sequence around the autophosphorylation site is highly conserved among the SFKs, this antibody should recognize autophosphorylated Fyn (pp59) and Lyn (pp53/pp56). As observed in Figure 3B, PRL induced autophosphorylation of Fyn and Lyn, detected as a doublet. After 10 min, phosphorylation increased by 1.7-fold; results alike were observed in PRL-stimulated hepatocytes (Berlanga *et al.*, 1995). After 30 min phosphorylation decreased toward basal levels. Treatment of W53 cells with PP1 abolished both basal and PRL-induced activation of Fyn and Lyn autophosphorylation (Figure 3B). Similarly, herbimycin A (0.7 μM) inhibited activation of these SFKs (Fresno Vara, Cáceres, Silva, and Martín-Pérez, unpublished results).

The phosphorylation of Sam68, a specific SFK cellular substrate in G1 and mitosis (Fumagalli *et al.*, 1994; Lock *et al.*, 1996; Fusaki *et al.*, 1997; Lang *et al.*, 1999) was also determined. Stimulation of W53 cells with 100 ng/ml PRL for 10 min caused an increase in the phosphotyrosine content of Sam68 (Figure 3C), as detected by Western blot with the anti-phosphotyrosine mAb 4G10 on the Sam68 immunoprecipitates. As observed above for the activation loop of the SFKs, some basal tyrosine phosphorylation was observed in Sam68 (Figure 3C). Treatment of W53 with PP1 before addition of PRL abolished Sam68 phosphorylation (Figure 3C). Together with the results of Figure 2B, we concluded that addition of PRL to W53 cells induced activation of Fyn and Lyn and, as a consequence, brought about an increase in the phosphotyrosine content of Sam68, which was eliminated by PP1 treatment. Furthermore, SFKs also phosphorylate the SH2 domain containing tyrosine phosphatase Shp2 (Feng *et al.*, 1993; Liu *et al.*, 1997), which is activated by PRL on 293 PRLR transfected cells (Ali *et al.*, 1996). We observed that PRL caused tyrosine phosphorylation of Shp2 when added to unstimulated W53 cells. This PRL action was mediated by SFKs as it was blocked by PP1 (Figure 3D).

To analyze the involvement of SFKs in Jak2-mediated effects on PRLR activation, PRL-stimulated W53 cells were treated with PP1, a selective SFK inhibitor. As shown in Figure 4A, no effect was observed on Jak2 autophosphorylation, which has been described as one of the earliest intracellular events after PRLR activation (Bole-Feysot *et al.*, 1998). Moreover, PRLR tyrosine phosphorylation was not inhibited by PP1 treatment (Figure 4B), an event reported to be mediated by Jak2 activity (Lebrun *et al.*, 1994; Fresno Vara *et al.*, 2000). Finally, we evaluated tyrosine phosphorylation

of Stat5 by Jak2 after recruitment by phosphotyrosine residues of the intracellular domain of PRLR (Gouilleux *et al.*, 1994; DaSilva *et al.*, 1996). As shown in Figure 4C, PRL-induced tyrosine phosphorylation of Stat5 in W53 cells was not modified by addition of PP1. Herbimycin A was also unable to inhibit Jak2-dependent phosphorylation of Stat5 (Fresno Vara, Cáceres, Silva, and Martín-Pérez, unpublished results). Therefore, we conclude that the Jak2/PRLR/Stat5 pathway is independent of PRL stimulation of Fyn and Lyn in W53 cells.
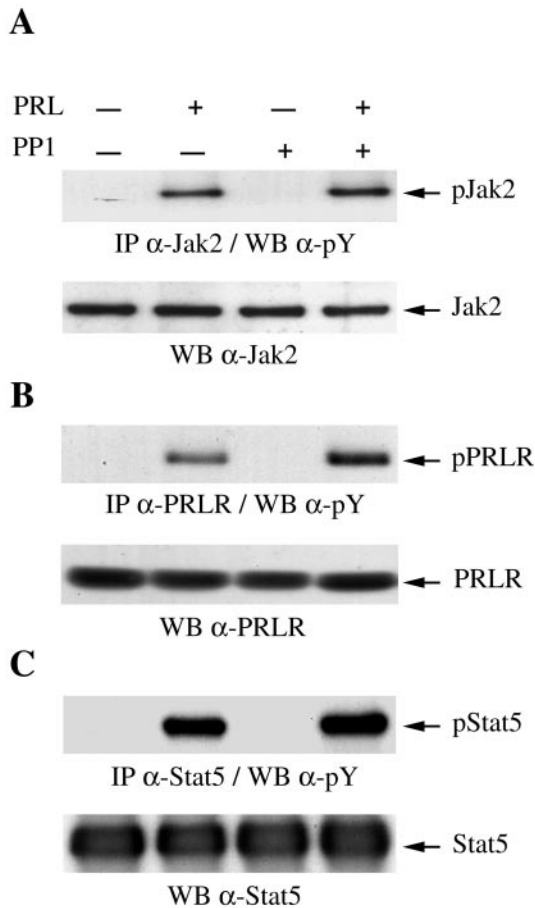
To define more precisely the role of these kinases on cell cycle progression, BrdU pulse-label experiments on W53 cells with or without 10 μM PP1 were carried out. PRL-stimulated cells, pulse-labeled during S-phase, progressed through G2-, M-, and G1-phases. Therefore, 24 h after the BrdU pulse, a homogeneous distribution of the labeled cell population was observed in each of the three cell cycle compartments G1, S and G2+M in DMSO-treated cells (Figure 5A). In contrast, addition of 10 μM PP1 to the cultures caused accumulation of cells in the G1-phase (Figure 5B). It should be noted that no signs of apoptotic cells were detected during the course of these experiments. These results indicate that SFKs were required for the G1/S transition of PRL-stimulated W53 cells.

Induction of cell proliferation by growth factors and cytokines is associated with transcriptional stimulation of growth related genes such as c-*fos*, c-*jun*, c-*myc*, etc., which are required for G1/S transition (Karin *et al.*, 1997), and we have previously shown in rat liver hepatocytes that PRL induces c-*fos* and c-*jun* expression (Berlanga *et al.*, 1995). Therefore we studied the role of SFKs on PRL-mediated induction of these genes by analyzing the effect of PP1 and PP2 on their expression by Northern blot. PRL stimulation of W53 cells induced the expression of c-*fos*, c-*jun*, c-*myc* and *odc*, a c-*myc*-dependent cell growth-related gene (Bello-Fernandez *et al.*, 1993), at later times (Figure 6, Control). The c-*fos* and c-*jun* expression was transient, reaching maximal expression 0.5 h poststimulation and was no longer detected after 3 h. Interestingly, a second peak of c-*jun* was observed after 9 h of PRL stimulation. In contrast, c-*myc* expression increased up to 1 h poststimulation, was maintained for at least 6 h and diminished by 9 h poststimulation. Finally, *odc* expression was observed 3 h after PRL stimulation and reached a plateau between 6 and 9 h poststimulation. Inhibition of SFKs activities by PP1 caused a strong decrease in the levels of all these growth-related genes, although it did not alter their temporal pattern of expression (Figure 6, PP1). Similar amounts of total RNA were loaded for each sample as it is shown by membrane staining with methylene blue (Figure 6, lower panel). These results are in agreement with the requirement of SFKs for PRL-induced cell proliferation, although it is surprising the inhibitory effect of PP1 on all these genes. However, a general inhibitory effect of PP1 on PRL-induced gene transcription was excluded because PP1 did not inhibit the *bcl*-2 increase induced by PRL. The same effect on the expression of these genes was observed with PP2 (Fresno Vara, Cáceres, Silva, and Martín-Pérez, unpublished results).

PP1 caused accumulation of cells in the G1-phase and a significant decrease in the PRL induction of c-*fos*. Expression of c-*fos* is mediated by the Mapk pathway (Karin *et al.*, 1997), and previous data have shown that PRL induces Erk1/2
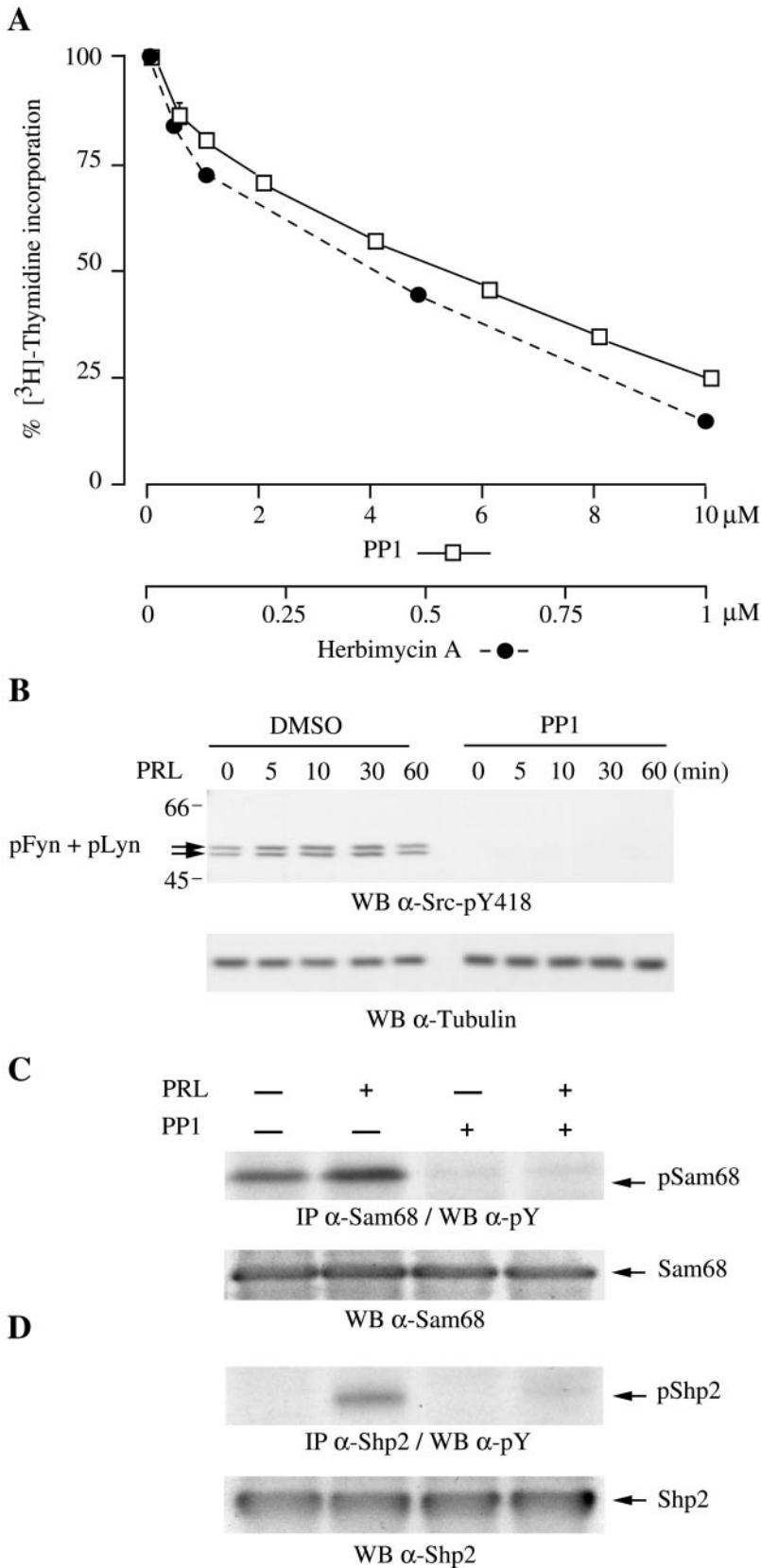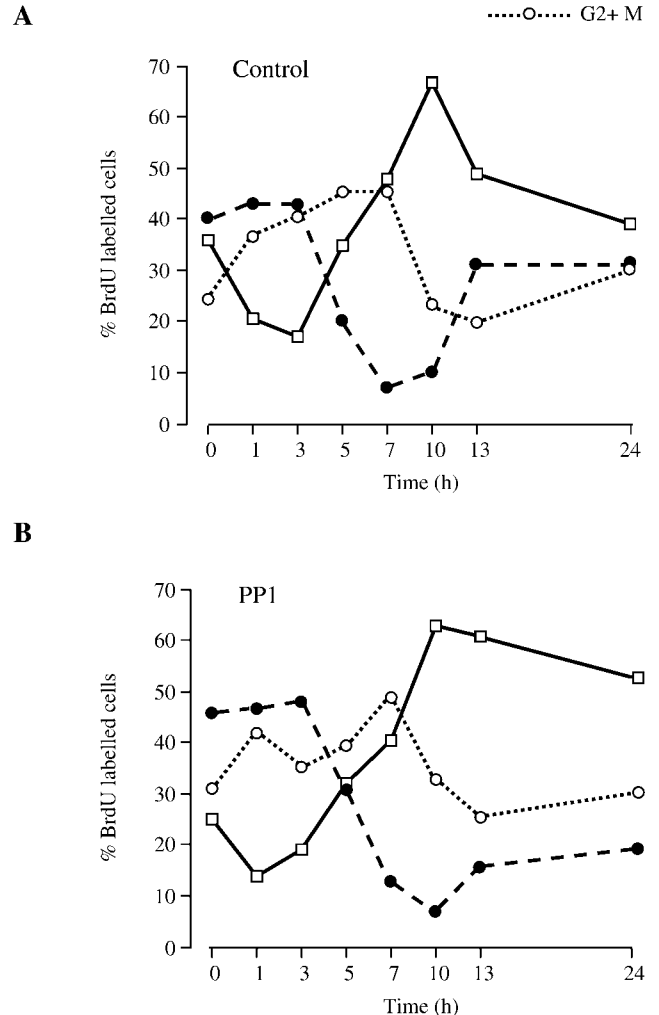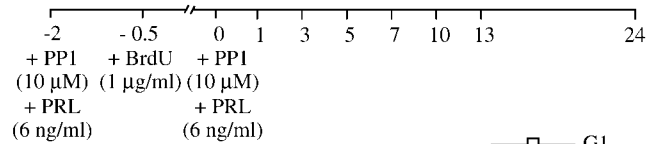
Figure 3. Effect of selective SFK inhibitors on PRL induction of W53 proliferation. (A) W53 cells exponentially growing in RPMI-10% FCS and 6 ng/ml PRL were cultured for 24 h in the presence of increasing concentrations of PP1, herbimycin A, or DMSO (as a control) and then pulsed with [³H]thymidine, and the incorporation of radioactivity into DNA was quantitated as described in MATERIALS AND METHODS. (B) W53 serum-starved cells were incubated with 10 μM PP1 or DMSO (as a control) for 2 h before PRL stimulation (100 ng/ml) for the indicated times. Cell extract protein (20 μg) was subjected to SDS-7% PAGE and the activation of SFKs was measured by Western blot with the use of anti-phospho-specific c-Src-PY418 polyclonal antibody. The doublet (labeled with arrows) represents pFyn (pp59) plus pLyn (pp53 + pp56). Sam68 (C) or Shp2 (D) were immunoprecipitated from cell extracts (0.5 mg of protein). Immunoprecipitates were subjected to SDS-7% PAGE and Western blot analysis with anti-phosphotyrosine mAb 4G10 (top). As a loading control, membranes were stripped and reprobed with anti-α-tubulin, anti-Sam68, or anti-Shp2 antibodies, respectively (bottom), as described in MATERIALS AND METHODS.

A

PRL   —   +   —   +
PP1   —   —   +   +

← pJak2

IP α-Jak2 / WB α-pY

← Jak2

WB α-Jak2

B

← pPRLR

IP α-PRLR / WB α-pY

← PRLR

WB α-PRLR

C

← pStat5

IP α-Stat5 / WB α-pY

← Stat5

WB α-Stat5

Figure 4. PRL-induced activation of Jak2, PRLR, and Stat5. W53 serum-starved cells were incubated with 10 μM PP1 or DMSO for 2 h before stimulation with 100 ng/ml PRL for 10 min. Cell extracts were prepared and Jak2 (A), the PRLR (B), and Stat5 (C) were immunoprecipitated with their specific antibodies from 0.5 mg of protein. Immunoprecipitates were subjected to SDS-7% PAGE and Western blot analysis with anti-phosphotyrosine mAb 4G10 (top). As a loading control, membranes were stripped and reprobed with the corresponding specific antibody (bottom), as described in MATERIALS AND METHODS.

activation (Piccoletti *et al.*, 1994). Here, we observed that Mek1/2 and Erk1/2 (p42/p44), members of Mapk signaling cascade, were stimulated in W53 cells upon PRL stimulation (Figure 7); a little effect was observed in Jnk-activation, whereas no stimulation was detected in p38 (Fresno Vara, Cáceres, Silva, and Martín-Pérez, unpublished results). Phosphorylation/activation of Mek1/2 and Erk1/2 by PRL is SFKs-independent because its inhibition did not alter this kinase cascade.

Activation of PI3K by growth factors and cytokines takes a central stage in cell signaling (Leevers *et al.*, 1999) and could be mediated by SFK (Pleiman *et al.*, 1994). Because PRL activates both SFKs (Fresno Vara *et al.*, 2000) and PI3K (Al-Sakkaf *et al.*, 1997; Berlanga *et al.*, 1997), we determined whether the PRL mitogenic activity required PI3K activity in W53 cells. The PI3K-selective inhibitor LY-294002 blocked [³H]thymidine incorporation in PRL-stimulated W53 cells

-2	- 0.5	0	1	3	5	7	10	13	24
+ PP1	+ BrdU	+ PP1							
(10 μM)	(1 μg/ml)	(10 μM)							
+ PRL		+ PRL							
(6 ng/ml)		(6 ng/ml)							

—□— G1
--●-- S
·······○······· G2+ M

A

B

Figure 5. Role of SFKs in W53 cell cycle progression. W53 cells were pretreated with 10 μM PP1 (B) or DMSO (A, as a control) for 2 h in the presence of PRL (6 ng/ml) and labeled for the last 30 min with BrdU. Cultures were then washed to eliminate BrdU and stimulated with PRL (6 ng/ml) in the presence of 10 μM PP1 or DMSO. Aliquots of cells were collected and fixed at the indicated times, labeled with FITC-anti-BrdU and PI and then analyzed by flow cytometry as described in MATERIALS AND METHODS. The results are expressed as percentages of BrdU-labeled cells in the three cell cycle compartments, G1, S, and G2+M, as defined by the PI label. This is one representative experiment of four independent experiments.

(Figure 8A). It has been established that PI3K mediates activation of Akt and p70S6k via the Pdks (Brennan *et al.*, 1999; Paradis *et al.*, 1999). So, we next assessed the ability of PRL to stimulate these kinases and the role of SFKs in this
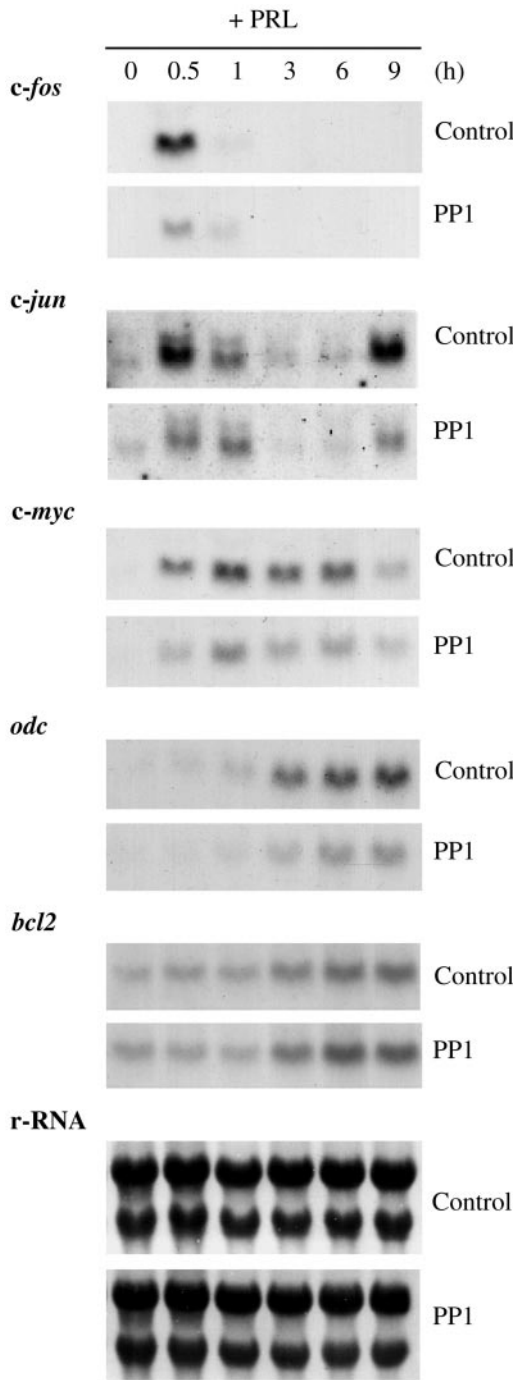
Figure 6. Effect of the SFK inhibition on PRL-induced expression of growth-related IEGs. W53 serum-starved cultures were pre-treated with PP1 or DMSO (as a control) for 2 h and then stimulated with PRL, aliquots of cells were collected at the indicated times, total RNA was extracted, and the expression of c-*fos,* c-*jun,* c-*myc, odc,* and *bcl2* was analyzed by Northern blot as described in MA-TERIALS AND METHODS. As a loading control the rRNAs were visualized by methylene blue staining (bottom).
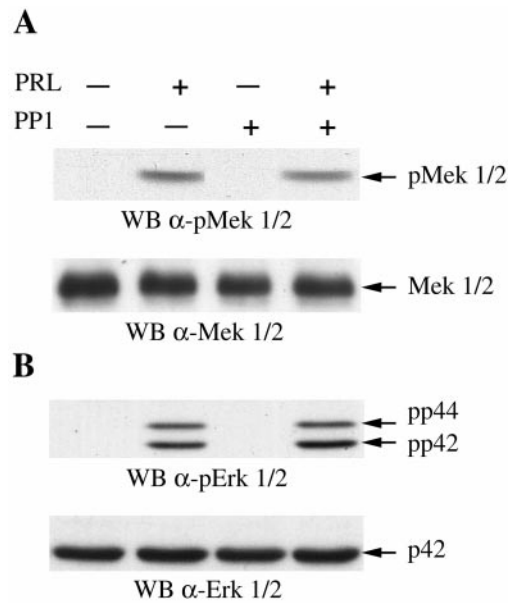

Figure 7. PRL stimulation of Mapk was independent of SFKs. W53 serum-starved cells were incubated with 10 μM PP1 or DMSO for 2 h before stimulation with 100 ng/ml PRL for 10 min. Cell extract protein (20 μg) was subjected to SDS-9% PAGE and the activation of Mek1 and Erk1/2 was measured by Western blot with the use of anti-phospho-specific antibodies (top). Membranes were stripped and reprobed with the corresponding specific antibody for loading controls (bottom), as described in MATERIALS AND METHODS.

signaling pathway. As observed in Figure 8B, addition of PRL to W53 cells activated both p70S6k and Akt. While Akt phosphorylation was rapidly stimulated, within 5 min of the cytokine addition, the activation of the p70S6k occurred at much later times. Treatment of cells with PP1 blocked PRL stimulation of both p70S6k and Akt (Figure 8B), indicating that the PRL activation of this PI3K-regulated pathway is, at least in part, modulated by SFKs. Consistent with these observations, inhibition of SFKs with herbimycin A also blocked PRL activation of both p70S6k and Akt (Fresno Vara, Cáceres, Silva, and Martín-Pérez, unpublished results). To reinforce the evidences that SFKs control PRL-mediated activation of the PI3K pathway, W53 cells were transiently cotransfected with Akt HA-tagged and either pCI-neo or pCI-neo-SrcDM, and 40 h later cells were stimulated with PRL (100 ng/ml) for 1h. The transfected Akt-HA was recovered by immunoprecipitation with the anti-HA mAb 12CA5 and its phosphorylation was determined by Western blotting with anti-pAkt. As expected, after 1 h of PRL stimulation, Akt was phosphorylated, however coexpression of SrcDM blocked PRL stimulation/phosphorylation of Akt (Figure 8C), demonstrating that the SFKs are directly involved in PRL mediation of Akt activation.

DISCUSSION

Biochemical evidences suggest that Src kinases are essential components of the growth factor/cytokines receptor signaling (Taniguchi, 1995; Thomas and Brugge, 1997; Corey and

A

B

C
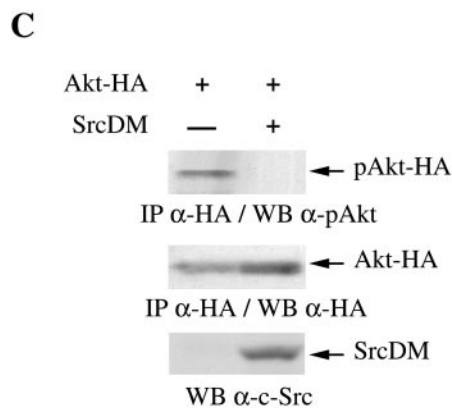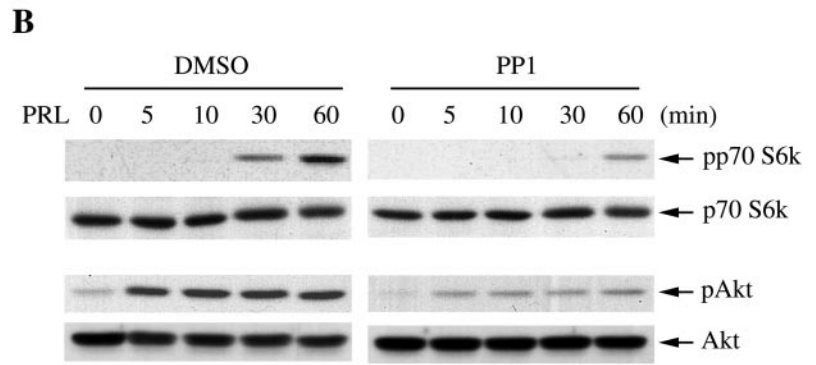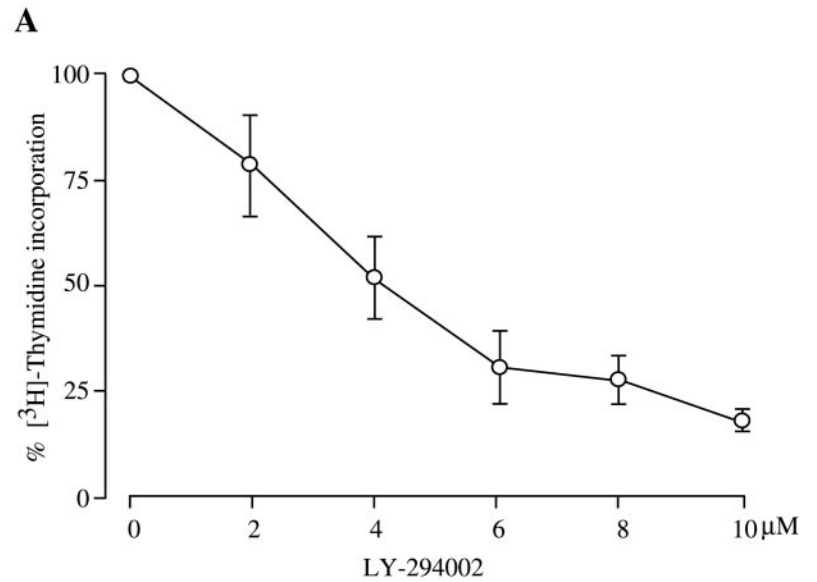
Figure 8. PRL-stimulated W53 cell proliferation required PI3K-dependent pathways. (A) W53 cells exponentially growing in RPMI-10% FCS and 6 ng/ml PRL were cultured for 24 h in the presence of increasing concentrations of LY294002 or DMSO (as a control) and then pulsed with [³H]thymidine. The incorporation of radioactivity into the newly synthesized DNA was quantitated. The results represent the averages ± SD of three independent experiments carried out in triplicate. (B) W53 serum-starved cells were incubated with 10 μM PP1 or DMSO for 2 h before addition of PRL (100 ng/ml) for the indicated times. Cell extract protein (20 μg) was subjected to SDS-7% PAGE and the activation of p70S6k and Akt was measured by Western blot with the use of anti-phospho-specific antibodies (top). The stripped membranes reprobed with the corresponding specific antibody for loading controls are shown at the bottom. (C) W53 cells were transiently transfected with Akt-HA alone or with SrcDM, 24 h later cells were made quiescent in 1% horse serum without PRL for 16 h, and then stimulated with 100 ng/ml for 1 h. Akt-HA was immunoprecipitated with anti-HA mAb 12CA5 and the phosphorylated Akt-HA was detected by Western blot with anti-pAkt. The membrane was later reblotted with anti-HA Y11 polyclonal antibody for control of Akt-HA expression. In parallel, SrcDM expression was determined by Western blot with 327 mAb.

Anderson, 1999). Because PRL stimulation of SFKs occurs independently of Jak2 (Fresno Vara *et al.*, 2000), we explored the signaling pathways activated by PRL in which SFKs could be implicated. For this purpose we used dominant negative mutants of Src and SFK-selective inhibitors of different natures, such as the pyrazolopyridine derivatives PP1

and PP2 (Hanke *et al.*, 1996; Liu *et al.*, 1999; Schindler *et al.*, 1999) or the ansamycin antibiotic herbimycin A (Schlaepfer *et al.*, 1998; Abe *et al.*, 2000; Bosco *et al.*, 2000; Langlais *et al.*, 2000). We found that SFK was required for PRL-induced proliferation, because the transient expression of PRLR with SrcK−, Csk, or SrcDM inhibited [³H]thymidine incorpora-

tion, in BaF-3 cells. SrcK− and Csk partially blocked PRL-induced cell growth, and the SrcDM caused the strongest inhibition. SrcK− contains the mutation K>M295 at the ATP-binding site, whereas Csk phosphorylates a tyrosine residue at the C terminus of SFKs, which facilitates an inactive enzyme conformation (Brown and Cooper, 1996). The SrcDM is a dominant negative form of c-Src, combining both the mutation at the ATP-binding site (K>A295) and at the Csk tyrosine phosphorylation site (Y>F527) (Mukhopadhyay *et al.*, 1995). The greater inhibitory effect of SrcDM could be explained by the fact that, in addition to being a kinase-dead mutant, it also has an open conformation that exposes its SH2 and SH3 domains (Pawson, 1997), which suggests that they could be implicated in the modulation of SFK functions. The relevance of the Src molecule as an adaptor protein has been previously described; in transgenic mice, expression of chicken SrcK− could rescue osteoclast functions in src−/− mice and complement adhesion defects in src−/− mouse fibroblasts (Kaplan *et al.*, 1995; Schwartzberg *et al.*, 1997). Several members of SFK, Fyn, Blk, and Lyn are expressed in both BaF-3 and W53 cells. Noticeably, the levels of Fyn are slightly higher in W53 than in BaF-3 cells. However, PRL induces activation of only Fyn and Lyn. Although c-Src is not detected in W53 cells, SrcDM inhibits PRL-induced cell proliferation. Perhaps, the high degree of structural homology among the SFK members and their functional redundancy (Lowell and Soriano, 1996; Thomas and Brugge, 1997) could explain the effects of the chicken C-Src mutants, SrcK− and SrcDM, in W53 cells.

We also investigated the role of Jak2 in PRL signaling. Coexpression of Jak2ΔK with the receptor blocked PRL induction of [³H]thymidine incorporation to the same extent as SrcDM. This result is in agreement with those indicating that Jak2 is required for PRL induction of cell proliferation (DaSilva *et al.*, 1994; Lebrun *et al.*, 1995; Parganas *et al.*, 1998).

Consistent with the above described findings, the SFK-selective inhibitors PP1/PP2 and herbimycin A inhibited PRL-induced cell growth. In fact, PP1 caused cell cycle arrest and accumulation in G1, suggesting that SFKs are required by PRL-stimulated W53 cells for G1/S transition, as it has been previously shown for some growth factors in fibroblasts (Twamley *et al.*, 1993; Barone and Courtneidge, 1995; Roche *et al.*, 1995). These data together with those obtained with CSK, SrcK−, and SrcDM substantiate the requirement of SFKs for PRL induction of cell proliferation.

Cytokines and growth factors activate signal transduction cascades leading to the induction of a large number of IEGs, which in turn initiate processes driving cells to DNA synthesis and mitosis. Because SFK inhibition altered the normal G1/S transition, we analyzed the role of SFKs on IEGs expression induced by PRL. We found that inhibition of SFKs resulted in a strong decrease in the levels of c-*fos*, c-*jun*, and c-*myc* and of the delayed c-*myc*-responsive gene *odc* but did not alter their temporal expression pattern. However, this inhibitory effect seems specific for cell cycle-related genes because no changes were observed in cell cycle-unrelated genes, such as *bcl2*.

It has been proposed that, after activation of PDGF-receptor in fibroblasts, c-*myc* expression is dependent on SFK, whereas c-*fos* transcription relies on the Ras/Mapk pathway (Barone and Courtneidge, 1995). Indeed, the c-*fos* promoter contains responsive elements activated by Ras/Mapk and Jak/Stat pathways (Rajotte *et al.*, 1996; Karin *et al.*, 1997). It is also well established that PRL also activates the Jak/Stat pathways (Bole-Feysot *et al.*, 1998). Here we observed that PRL-induced phosphorylation of the PRLR and activation of Mek1/2, Erk1/2, and Jak2/Stat5 was independent of SFKs. Therefore, we conclude that inhibition of cell proliferation by SrcDM or by SFK inhibitors was concomitant with the blockage of IEGs c-*fos*, c-*jun*, and c-*myc* expression, independently of PRL-mediated activation of the Jak/Stat, Ras/Mapk pathways. Whether these two pathways are interconnected in this model system remains to be determined. In 293 cells expressing growth hormone receptors, Jak2 was involved in the activation of the Erk1/2- and Stat-signaling pathways by growth hormone (Winston and Hunter, 1995).

The phosphorylation/activation of Shp2 upon PRL stimulation of W53 cells was mediated, at least in part, by SFKs, considering its inhibition by PP1. However, in 293 PRLR-transfected cells, PRL stimulation of Shp2 phosphorylation seems to be mediated by Jak2, because a mutant PRLR unable to stimulate this tyrosine kinase fails to transmit signals for Shp2 activation (Ali *et al.*, 1996). Both results are not necessarily contradictory, because activation/phosphorylation of Shp2 may require Jak2 and SFKs. The inhibition of Shp2 tyrosine phosphorylation by PP1 was concomitant with a slight increase in the tyrosine phosphorylation of Jak2, PRLR, and Stat5, suggesting that Shp2 could negatively regulate the phosphorylation of PRLR/Jak2/Stat5. In this context, it has been recently shown that mutation of a tyrosine residue of the growth hormone receptor, which prevents binding of Shp2 to the receptor, prolongs growth hormone receptor /Jak2/Stat5b phosphorylation induced by growth hormone (Stofega *et al.*, 2000).

PRL induction of W53 proliferation required PI3K activity, as demonstrated by its blocking effect by LY-294002. Moreover, transient expression of SrcDM as well as the SFK inhibitors PP1 and herbimycin A blocked PRL stimulation of PI3K-mediated pathways leading to activation of Akt and p70S6k. The IL-3 activation of PI3K has been linked to Shp2 (Welham *et al.*, 1994; Craddock and Welham, 1997; Gadina *et al.*, 1998; Gu *et al.*, 2000). Shp2 has been described as an adaptor protein mediating interaction between the cytokine receptors and the PI3K/Akt pathway via Shp2/Grb2/Gab2 (Gu *et al.*, 2000). Shp2 also has been found associated with Grb2-Sos, leading to activation of the Ras/Mapk pathway (Li *et al.*, 1994; Pazdrak *et al.*, 1997; Gadina *et al.*, 1998). In W53 cells, inhibition of PRL induction of Shp2 tyrosine phosphorylation by PP1 did not modify the PRL activation of either Mek1/2-Erk1/2 or Jak2/Stat5 pathways but paralleled inhibition of the PI3K/Akt as well as cell proliferation. Together, these data suggest that Shp2 could mediate a variety of signaling pathways, depending on the cellular context and the specific stimulatory cytokine. Whether Shp2 mediated SFK activation of the PI3K pathways in PRL-stimulated W53 remains to be determined.

The efficacy of PP1 on the SFKs was shown by its inhibitory effect on the PRL-stimulated tyrosine autophosphorylation of Fyn and Lyn activation loops. This inhibition was also observed with herbimycin A (Fresno Vara, Cáceres, Silva, and Martín-Pérez, unpublished results). Consistently, the phosphorylation of Sam68, initially described as an SFK mitotic substrate (Fumagalli *et al.*, 1994) and also associated
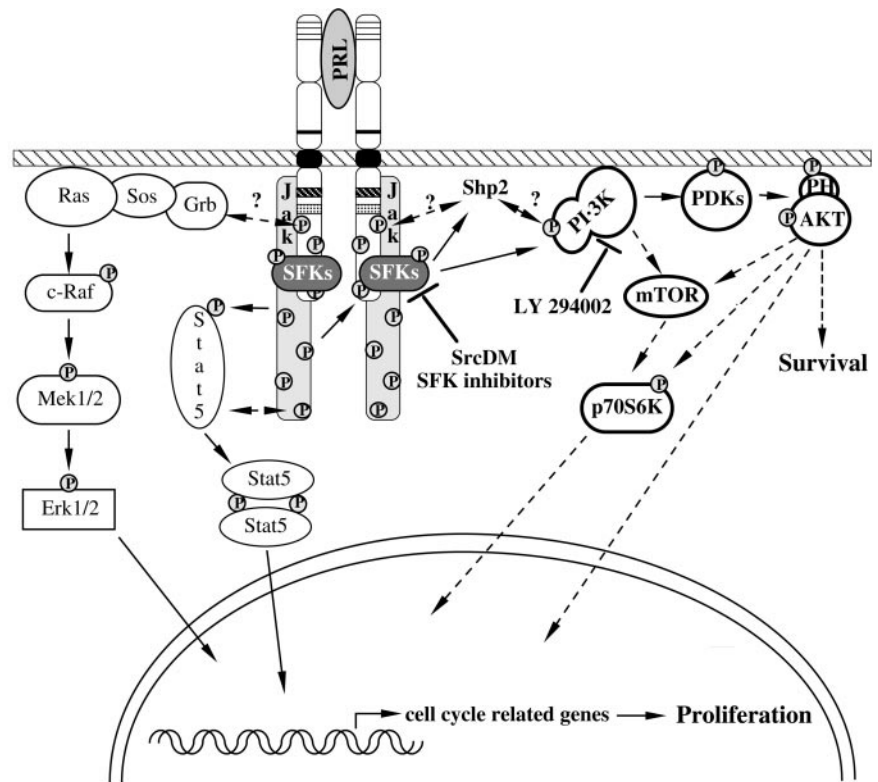
Figure 9. Role of SFKs in the control of PRL induction of W53 cell proliferation. Our model places Src kinases associated with PRLR and activated by PRL induction of PRLR dimerization. The SFKs control stimulation of the PI3K-dependent p70S6k and Akt, which in turn should provide signals leading to G1/S-phase progression. Our results show that PRL activation of Jak2/Stat and Ras/Mapk pathways is independent of SFK-stimulated signals.

with G1/S transition in lymphocytes (Barlat *et al.*, 1997), was abolished by treatment with PP1.

It has been reported that c-Abl and p38 are both sensitive to PP1 in vitro (Liu *et al.*, 1999). We therefore analyzed the stimulation/phosphorylation of p38 and c-Abl by PRL in W53 cells but were unable to detect their activation (Fresno Vara, Cáceres, Silva, and Martín-Pérez, unpublished results), indicating that the effects of PP1 in W53 cells are mediated by the inhibition of Src kinases.

Jak2 activation by PRL seems to be required for most cytokine responses (Lebrun *et al.*, 1994; Goupille *et al.*, 1997; Pezet *et al.*, 1997). However, PRL can independently stimulate Src kinases, as observed with a PRLR mutant unable to bind Jak2 but capable of activating c-Src (Fresno Vara *et al.*, 2000), and the findings described here support this conclusion. Our data also demonstrate that Src kinases control PRL-mediated activation of Shp2 and the PI3K pathway in W53 cells, which are also implicated in modulating the expression of cell cycle-regulating genes. Our results, together with those obtained by others (Brennan *et al.*, 1999; Dufner *et al.*, 1999; Gu *et al.*, 2000), allows us to implicate SFKs in PRL-induced proliferation of W53 cells (Figure 9). Future experiments with the use of inducible expression of SrcDM and dominant negative forms of the PI3K subunits and Akt will help to clearly define the SFK-signaling pathways. In addition, expression different mutant forms of the PRLR, in tyrosine residues, and in box I, as well as inducible expression of Jak2 inactive forms, will help us to further determine the role of the SFKs and Jak2 on PRL signaling.

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