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Src Mediates Prolactin-Dependent Proliferation of T47D and MCF7 Cells via the Activation of Focal Adhesion Kinase/Erk1/2 and Phosphatidylinositol 3-Kinase Pathways

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Prolactin (PRL) stimulates breast cancer cell proliferation; however, the involvement of PRL-activated signaling molecules in cell proliferation is not fully established. Here we studied the role of c-Src on PRL-stimulated proliferation of T47D and MCF7 breast cancer cells. We initially observed that PRL-dependent activation of focal adhesion kinase (Fak), Erk1/2, and cell proliferation was mediated by c-Src in T47D cells, because expression of a dominant-negative form of c-Src (SrcDM, K295A/Y527F) blocked the PRL-dependent effects. The Src inhibitor PP1 abrogated PRL-dependent *in vivo* activation of Fak, Erk1/2, p70S6K, and Akt and the proliferation of T47D and MCF7 cells; Janus kinase 2 (Jak2) activation was not affected. However, *in vitro*, Fak and Jak2 kinases were not directly inhibited by PP1, demonstrating the effect of PP1 on c-Src kinase as an upstream activator of Fak. Expression of Fak mutant Y397F abrogated PRL-dependent activation of Fak, Erk1/2, and thymidine incorporation, but had no effect on p70S6K and Akt kinases. MAPK kinase 1/2 (Mek1/2) inhibitor PD184352 blocked PRL-induced stimulation of Erk1/2 and cell proliferation; however, p70S6K and Akt activation were unaffected. The phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 abolished cell proliferation and activation of p70S6K and Akt; however, PRL-dependent activation of Erk1/2 was not modified. Moreover, we show that both c-Src/PI3K and c-Src/Fak/Erk1/2 pathways are involved in the up-regulation of c-*myc* and cyclin *d1* expression mediated by PRL. The previous findings suggest the existence of two PRL-dependent signaling cascades, initiated by the c-Src-mediated activation of Fak/Erk1/2 and PI3K pathways that, subsequently, control the expression of c-Myc and cyclin D1 and the proliferation of T47D and MCF7 breast cancer cells. (*Molecular Endocrinology* 17: 2268–2282, 2003)

THE INTERACTION OF PROLACTIN (PRL) with its receptor controls a variety of physiological functions, including the proliferation of normal and tumor mammary gland epithelium (for a review see Ref. 1). The PRL receptor (PRLR) belongs to the cytokine/hematopoietic receptor superfamily (2) and is devoid of intrinsic enzymatic activity. Binding of PRL to the PRLR activates the associated nonreceptor tyrosine kinase Janus kinase 2 (Jak2) (3), which, in turn, phosphorylates the PRLR, generating docking sites for Src homology domain 2 (SH2)-containing molecules and stimulating signaling cascades such as Stats (Jak2-signal transducers and activators of transcription) and MAPK (Grb2/Sos-Ras-Raf-MAPK) (4–7). Activation of Src kinases by PRL has been observed in different experimental models including hepatocytes, PRLR-transfected chicken embryo fibroblasts, and Nb2, HC11, and W53 cell lines (8–12). More recently, a critical role for Src kinases in PRL-dependent cell proliferation (12) has been demonstrated in W53 cells (13).

Increasing evidence supports the involvement of PRL in breast cancer. The PRLR is detected in 80% of human breast cancer (14), and, in fact, is overexpressed in tumor cells of breast cancer samples (15). Because normal and tumor mammary epithelial cells synthesize PRL and its receptor (16, 17), the hormone could behave as an autocrine growth factor for human breast cancer cells. Consistent with the fact that PRL is mitogenic in the majority of breast cancer cell lines (18), PRL was shown to activate MAPK pathways in T47D cells (19). Moreover, the expression of cyclin D1,

Abbreviations: DMSO, Dimethylsulfoxide; ECL, enhanced chemiluminescence; Fak, focal adhesion kinase; FCS, fetal calf serum; HA, hemagglutinin; Jak2, Janus kinase 2; mAb, monoclonal antibody; Mek, MAPK kinase; PI3K, phosphatidylinositol 3-kinase; PRL, prolactin; PRLR, PRL receptor; SDS, sodium dodecyl sulfate; SH2, Src homology domain 2; Stat, signal transducer and activator of transcription; TBS, Tris-buffered saline; TTBS, TBS with 0.05% Tween 20; WB, Western blot.

required for G_1/S cell cycle transition, was shown to be regulated by PRL in MCF7 cells (20).

Recent findings demonstrate that migration of breast cancer cells is enhanced by PRL (21). Focal adhesion kinase (Fak), localized at focal contacts, is involved in the control of cellular responses, such as cell spreading, migration, survival, and proliferation (22–24). Integrin-induced autophosphorylation of Fak at Y397 generates a SH2 binding site for c-Src and Fyn Src kinases (25, 26). Phosphorylation of additional sites in Fak by the Src kinases (27) regulates its catalytic activity and enables the association of docking proteins such as Cas, paxillin, and the Grb2-Sos complex, and consequently links the Fak and Ras/MAPK pathways (28, 29). This could be the mechanism underlying the control of cyclin D1 expression and cell cycle progression by Fak (30). Moreover, Fak is activated by the v-Src oncogene (31) and cell surface receptors, such as PRLR (32). Interestingly, some biological effects of PRL in mammary gland epithelium are mediated by interactions with the extracellular matrix (33). These data suggest that phosphorylated Fak integrates multiple transduction pathways in response to external signals provided by the extracellular matrix and growth factors.

Additional pathways, such as phosphatidylinositol 3-kinase (PI3K) (34), required for proliferation of W53 cells (12), have also been implicated in PRL-induced cell proliferation.

Involvement of c-Src in malignant transformation of mammary gland epithelium is suggested by the increased c-Src kinase activity found in breast carcinomas (35), and because functional c-Src is required for the induction of mammary tumors by polyomavirus middle T antigen (36). Furthermore, transgenic mice expressing constitutively activated c-Src (Y527F), under the control of the mouse mammary tumor virus promoter/enhancer, show increased hyperplasia of mammary gland epithelium, which eventually generates tumors (37). Similarly, expression of oncogenic Src in human breast cancer cell MCF7 activates cyclin D1 expression (38).

Notwithstanding available data implicating PRL in breast cancer pathogenesis and progression, however, the timely regulation of the downstream targets that mediate these effects has not been clearly established. In this study we have investigated the functional role of Src on PRL-dependent stimulation of T47D and MCF7 cell proliferation. Our results show that activation of c-Src by PRL leads to the independent stimulation of Fak-Erk1/2 and PI3K signaling pathways, which are required for the induction of c-*myc* and cyclin *d1* expression and the proliferation of T47D and MCF7 human breast cancer cells.

RESULTS

To confirm the mitogenic activity of PRL, cells were maintained for 3 d in serum-free, phenol red-free DMEM. Addition of PRL to nonstimulated cultures of T74D (5×10^4 cells) increased thymidine incorporation in a concentration-dependent manner. Maximal stimulation, 2.2-fold over basal levels (12,280 $\pm$ 1,283 cpm), was observed at 100 ng/ml, reaching a plateau (Fig. 1A). This concentration was subsequently used for further experiments. Under these conditions, no apoptosis was detected by flow cytometry analysis of propidium iodide-labeled cells (data not shown).

Considering that Src kinases are activated by PRL in a variety of cell types (8–12), we analyzed the role of these tyrosine kinases in the induction of T47D cell proliferation by PRL. First, we analyzed the expression pattern of Src kinases activated by PRL in T47D cells. We observed that c-Src, Fyn, Yes, and Blk were expressed in T47D cells, as determined by Western blot (WB) analysis of cell extracts with monospecific antibodies (data not shown). Kinase activity was subsequently studied, at different time points, by immunoprecipitation of c-Src, Fyn, Yes, and Blk from PRL-stimulated (100 ng/ml) cells. Next, the immune complexes were subjected to *in vitro* ^{32}P autophosphorylation assays. We observed that only c-Src autophosphorylation/activation was induced by PRL. The 1.8-fold activation above basal levels was transient and peaked at 5 min after stimulation of T47D cells with PRL (Fig. 1B).

Src kinases have been previously reported to control Fak activation (39–41). To determine the role of Src kinases on PRL-dependent stimulation of T47D cell proliferation and Fak activation, we initially performed *in vitro* ^{32}P autophosphorylation assays with Fak immunoprecipitates from cells stimulated with PRL (100 ng/ml) at different times. PRL induced a 2-fold transient activation of Fak above basal levels at 10 min (Fig. 1B). Moreover, to evaluate the effect of PRL stimulation on Fak tyrosine phosphorylation in T47D cells, nonstimulated cells were treated with increasing amounts of PRL for 15 min. Fak phosphorylation was determined in anti-Fak immunoprecipitates by WB with antiphosphotyrosine 4G10 monoclonal antibody (mAb). The results show that maximal phosphorylation of Fak was reached with 50–100 ng/ml of PRL (Fig. 1C). Then, we determined the kinetics of Fak tyrosine phosphorylation after stimulation with PRL (100 ng/ml). Fak phosphorylation was detected at 5 min after PRL addition, reached maximal levels after 15 min of stimulation, and decreased at 45 min (Fig. 1D). We subsequently studied the effect of PRL stimulation (100 ng/ml for 15 min) on tyrosine phosphorylation of paxillin in T47D cells, currently considered a substrate of Fak. Thus, paxillin was immunoprecipitated, and its phosphotyrosine (pY) content was determined by WB with mAb 4G10. As shown in Fig. 1E, PRL stimulated the phosphorylation of paxillin (Fig. 1E).

Stimulation by PRL is known to activate Erk1/2 in many cell types (1). Addition of PRL (100 ng/ml, 15 min) to nonstimulated T47D cultures resulted in Erk1/2 activation, as determined by WB with phospho-

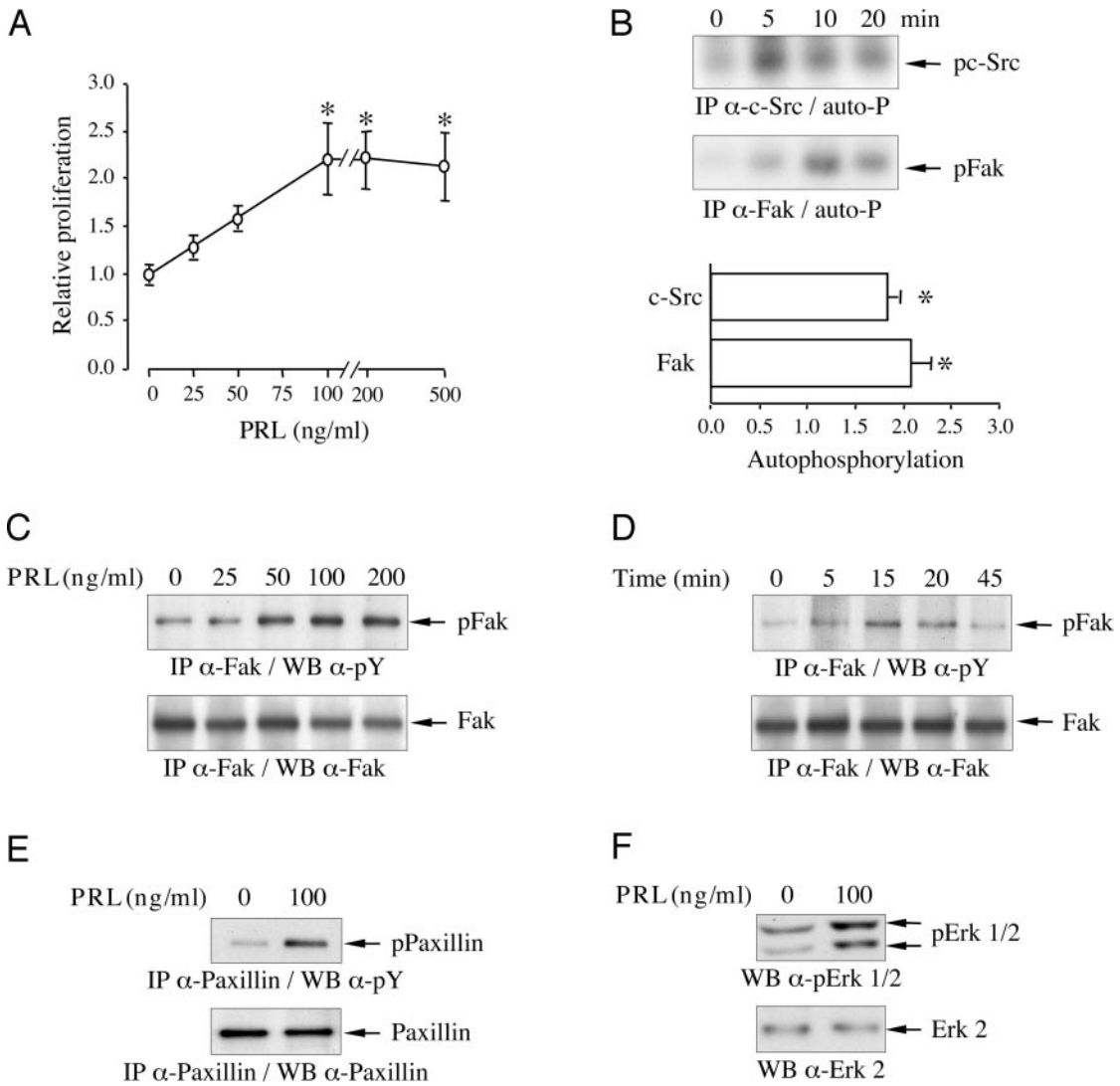

Fig. 1. Effect of PRL on Proliferation of T47D Cells: Modulation of Fak, c-Src, and Erk1/2 Kinase Activities and Tyrosine Phosphorylation of Fak and Paxillin by PRL

A, T47D cells were seeded in 24-well plates (5×10^4 cells per well) in DMEM-phenol red-free-10% FCS. After 24 h, they were transferred to serum-free media for 48 h, cultured in the presence of increasing concentrations of PRL for 72 h, and pulsed with 0.5 μCi [^{3}H]thymidine/well for the final 4 h. Thymidine incorporation was measured as described in *Materials and Methods*. B, c-Src and Fak were immunoprecipitated using 500 μg of protein extract from cells stimulated with PRL (100 ng/ml) for 0, 5, 10, and 20 min. The immune complexes were subjected to autophosphorylation reactions and separated by 7% SDS-PAGE, and the radioactivity in the c-Src and Fak proteins was measured using an Instantimager, as described in *Materials and Methods*. The results are expressed as fold increase above basal levels. Resting cells were treated with increasing concentrations of PRL (0, 25, 50, 100, and 200 ng/ml) for 15 min (panel C) or for different time periods (0, 5, 15, 20, and 45 min) with PRL (100 ng/ml) (panel D). Fak was immunoprecipitated (IP) from 500 μg of protein extract and tyrosine phosphorylation was determined by WB with 4G10 antiphosphotyrosine mAb. E, Paxillin IP was performed with 500 μg protein extract from resting or PRL-stimulated (100 ng/ml, 15 min) cells, and the phosphotyrosine (pY) content was determined by WB with mAb 4G10. F, Erk1/2 activation was measured with 25 μg of protein extract from resting or PRL-stimulated (100 ng/ml, 15 min) cells by WB with anti-phospho-Erk1/2 antibodies. *Lower panels* show the stripped membranes reprobed with the antibody used for immunoprecipitation (C, D, and E) or with anti-Erk2 (F). The data are representative of three separate experiments. In panel A, control cell (nonstimulated) values are given as the unit for cell proliferation. The data presented are the mean ± SE (*bars*) of three independent experiments carried out in triplicate. The *asterisk* (*) represents $P < 0.05$ compared with the control group in the Student's *t* test.

specific antibodies defining Erk1/2 activation status (Fig. 1F). As previously observed for Fak and paxillin, basal levels of Erk1/2 activation were detected in T47D nonstimulated cells.

To evaluate the role of c-Src in PRL-mediated signaling events leading to the proliferation of T47D cells, we performed transient transfection with the dominant-negative c-Src kinase (SrcDM). As shown in Fig.

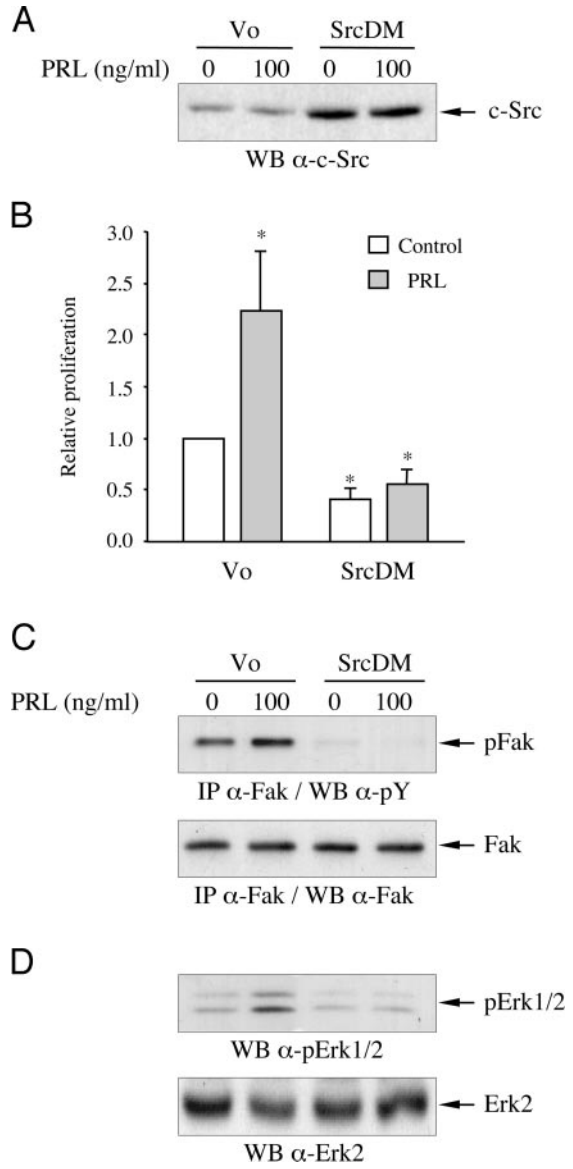

Fig. 2. Effect of SrcDM Expression on PRL-Induced Cell Proliferation and Fak and Erk1/2 Activation in T47D Cells

T47D cells were transiently transfected with either pSGT-SrcDM or the empty vector (Vo) and stimulated with PRL (100 ng/ml, 15 min). A, Protein extracts (25 μg) were separated on 9% SDS-PAGE, and WB analysis was performed with anti-c-Src antibody. B, Thymidine incorporation was calculated as in Fig. 1. C, Fak was immunoprecipitated from 500 μg of protein extract with anti-Fak and its phosphotyrosine (pY) content determined by WB with mAb 4G10. D, Activation of Erk1/2 was evaluated as in Fig. 1F. In panel B, values are presented as the mean ± SE (*bars*) of three independent experiments carried out in triplicate. The *asterisk* (*) represents $P < 0.05$ compared with the control group (Vo) in Student's *t* test (n = 3). The data in panels C and D are representative of three separate experiments.

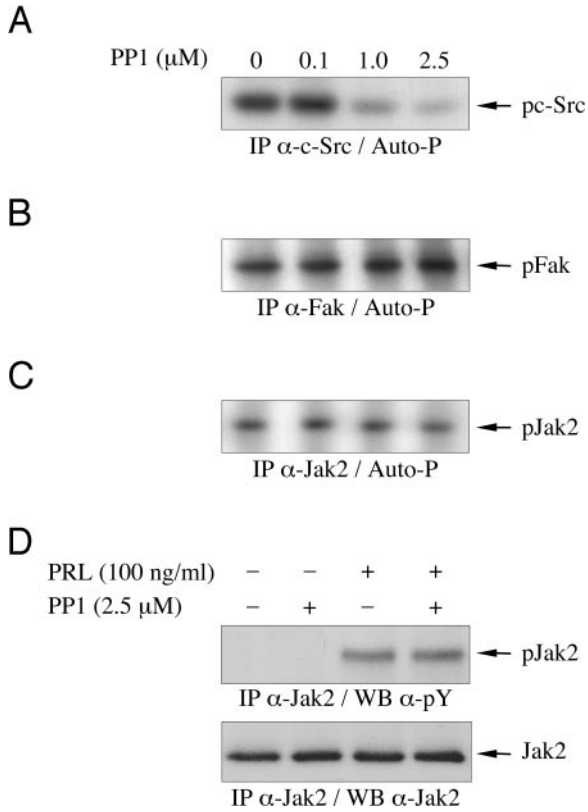

Fig. 3. Effect of Src Inhibitor PP1 on PRL-Induced Activation of c-Src, Fak, and Jak2

c-Src (panel A), Fak (panel B), and Jak2 (panel C) were immunoprecipitated (IP) from 500 μg of protein extract of T47D cells stimulated with PRL (100 ng/ml) for 5 min, and 10 min for Fak, and then subjected to autophosphorylation (Auto-P) reactions for 5 min in the presence of increasing concentrations of PP1 (0, 0.1, 1, and 2.5 μM). Reaction mixtures were subjected to 7% SDS-PAGE, and dried gels exposed to x-ray film to detect ^{32}P-labeled proteins. Results are representative of four separate experiments. D, T47D cells were incubated in the presence of DMSO (control) or PP1 (2.5 μM, 2 h) before the addition of PRL (100 ng/ml, 15 min). Jak2 was immunoprecipitated from 500 μg of protein extract with specific antibodies, and tyrosine phosphorylation was detected by WB with mAb 4G10. *Lower panel* shows the stripped membrane reprobed with anti-Jak2 antibody. The results are representative of two independent experiments.

2A, transfection of T47D cells with SrcDM resulted in high expression of the mutant protein, inhibiting PRL-induced [^{3}H]thymidine incorporation below basal levels (Fig. 2B) and reducing the phosphorylation of Fak and Erk1/2 induced by PRL (Fig. 2, C and D). The selective inhibitor of Src kinases PP1 (42, 43) blocked the *in vitro* kinase activity associated with Src immunoprecipitates (Fig. 3A); 1 μM PP1 inhibited the autophosphorylation of c-Src induced by PRL. However, Fak autocatalytic activity was not affected, at concentrations as high as 2.5 μM (Fig. 3B). Because Jak2 activation is considered a key element in PRL cell signaling (1) and is required for PRL mitogenic activity in mouse mammary gland epithelial HC11 cells (44), we tested the effect of Src inhibition on the activation of Jak2 by PRL. The *in vitro* autophosphorylation assay for Jak2 was performed in the presence of increasing PP1 concentration. As shown in Fig. 3C, PP1 con-

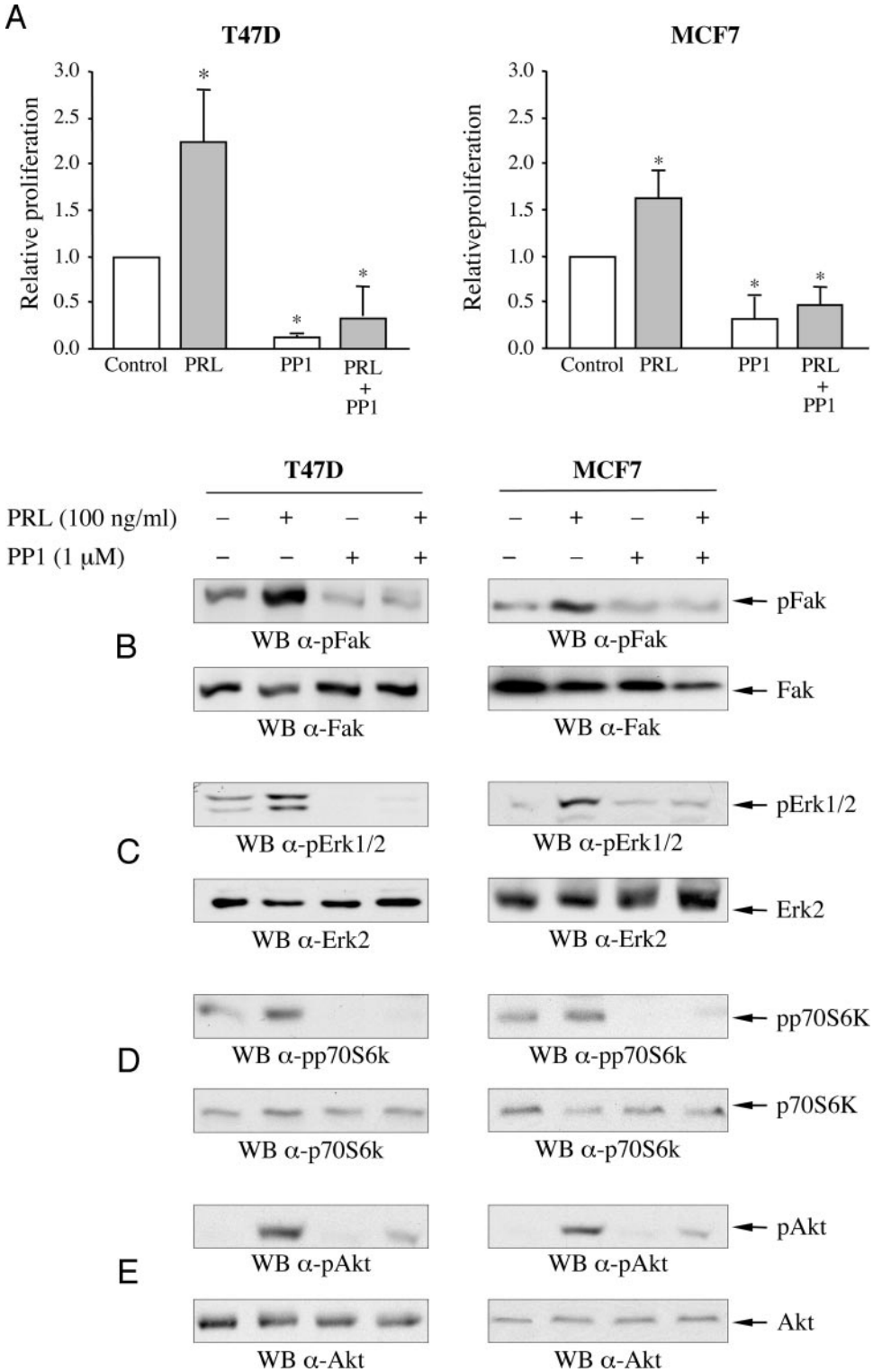

Fig. 4. Effect of the Src Inhibitor PP1 on PRL-Induced Cell Proliferation and Activation of Fak, Erk1/2, p70S6K, and Akt in T47D and MCF7 Cells

A, T47D and MCF7 cells were seeded in 24-well plates (5×10^4 cells per well) in DMEM-10% FCS and 24 h later transferred to serum-free media for 48 h. Subsequently, cells were incubated in the absence or presence of PRL (100 ng/ml) and/or PP1 (1 μM) for 72 h, and pulsed with 0.5 μCi [^{3}H]thymidine/well for the final 4 h. Thymidine incorporation was measured as described in *Materials and Methods*. Src inhibition by PP1 on PRL-mediated activation of Fak, Erk1/2, p70S6K, and Akt was analyzed by incubating resting cells in the presence of DMSO (control) or PP1 (1 μM) for 2 h before the addition of PRL (100 ng/ml, 15 min). B, Fak was immunoprecipitated from cell extracts and immune complexes were subjected to 7% SDS-PAGE and WB analysis

centrations up to 2.5 μM, which abrogated c-Src activity, were unable to modify the auto-phosphorylation of Jak2. We next determined the involvement of c-Src on PRL-dependent activation of Jak2 in T47D cells. Addition of PRL (100 ng/ml for 15 min) increased tyrosine phosphorylation of Jak2, and PRL-dependent activation of Jak2 was unaffected by pretreatment of cells with PP1 (2.5 μM) (Fig. 3D). Similar results were obtained by transient expression of SrcDM in T47D cells (data not shown).

To investigate the involvement of c-Src in PRL-dependent mitogenic signaling in breast cancer cells, T47D and MCF7 cell lines were treated with 1 μM PP1. As shown in Fig. 4A, PP1 inhibited both basal and PRL-induced proliferation in these cells. Subsequently, we analyzed the role of c-Src in the control of PRL-dependent signaling pathways in the presence of 1 μM PP1. We observed that addition of PRL (100 ng/ml for 15 min) to nonstimulated T47D and MCF7 cells increased the phosphorylation/activation of Fak, Erk1/2, and PI3K-dependent serine/threonine kinases p70S6K and Akt (Fig. 4, B–E) and was inhibited by treatment of T47D and MCF7 cells with PP1 before PRL stimulation, demonstrating that c-Src mediates the activation of these kinases by PRL (Fig. 4, B–E).

Direct phosphorylation of Fak by c-Src (27) facilitates the activation of the MAPK pathway (40, 45). Therefore, to investigate the role of Fak in PRL-stimulated cell proliferation, we obtained T47D and MCF7 cell lines expressing Fak Y397F mutant tagged with an hemagglutinin (HA) epitope (Fak-F397) (Fig. 5A). As shown in Fig. 5B, expression of the mutant blocked PRL-induced [^{3}H]thymidine incorporation in both cell lines.

The functional effect of Fak-F397 expression on the activation of Fak phosphorylation by PRL was further studied by WB detection with mAb 4G10 in Fak immunoprecipitates from resting and PRL-stimulated T47D and MCF7 cells. We show that PRL increases Fak phosphotyrosine content (Fig. 4B) and that the expression of Fak-F397 abrogates this effect in both cell lines (Fig. 5C). Moreover, expression of the Fak mutant abolished the previously observed activation of Erk1/2 by PRL (Fig. 5D). In contrast, Fak-F397 had no effect on the stimulation of the PI3K pathway by PRL, because phosphorylation of both p70S6K and Akt was increased upon stimulation of Fak-F397 T47D- and MCF7-expressing cells by PRL (Fig. 5, E and F, respectively). These results evidence the significance of Fak activation in mediating Erk1/2 activation and T47D and MCF7 cell proliferation induced by PRL and demonstrate that the PRL-stimulated PI3K pathway is independent of Fak activation.

The previous data suggest the involvement of MAPKs in the control of T47D and MCF7 proliferation induced by PRL. Thus, to evaluate their contribution to PRL-mitogenic signaling, both cell lines were treated with PD184352 (1 μM), a selective inhibitor of Mek1/2 (MAPK kinase) activity (46). PD184352 decreased both basal and PRL-stimulated levels of [^{3}H]thymidine incorporation (Fig. 6A). Moreover, PD184352 treatment abolished basal and PRL-induced Erk1/2 activation levels in both T47D and MCF7 cells (Fig. 6B). However, interference of the MAPK signaling pathway did not affect PRL-induced p70S6K and Akt phosphorylation (Fig. 6, C and D).

To evaluate the relevance of PI3K-dependent signaling pathways in the induction of T47D and MCF7 cell proliferation by PRL, the PI3K-selective inhibitor LY294002 (10 μM) was used. The results show that inhibition of PI3K activity with LY294002 abolished basal and PRL-stimulated T47D and MCF7 cell proliferation (Fig. 7A). In addition, stimulation of p70S6K and Akt phosphorylation by PRL was inhibited by LY294002 below basal levels (Fig. 7, B and C). In contrast, Erk1/2 phosphorylation induced by PRL remained unaltered when PI3K activity was inhibited (Fig. 7D). We observed no inhibitory effect of PD184352 and LY294002 on the activation of c-Src and Fak by PRL (data not shown).

The aforementioned PRL-stimulated mitogenic signaling cascades should ultimately induce the expression of c-*myc* and cyclin *d1*, critical molecules for G_1/S cell cycle progression. We therefore evaluated whether PRL activation of c-Src and the subsequent stimulation of the PI3K and/or the Erk1/2 pathways were required for the control of c-*myc* and cyclin *d1* expression in T47D and MCF7 cells. Thus, cells were stimulated with 100 ng/ml PRL for different time periods in the presence or absence of PP1, PD184352, or LY294002, and mRNA expression of c-*myc* and cyclin *d1* was determined by Northern analyses from 16 μg of total RNA with specific probes. As shown in Fig. 8A, c-*myc* and cyclin *d1* mRNA levels were increased by PRL in T47D and MCF7 cells. Interestingly, we showed that c-Src and the c-Src-dependent PI3K and Erk1/2 pathways are critical mediators of PRL in the up-regulation of mRNA expression of c-*myc* and cyclin *d1*, as demonstrated by the down-regulation in gene expression due to selective inhibition of these kinases (Fig. 8A). Similar results were obtained when the ex-

with mAb 4G10. Activation of Erk1/2 (panel C), p70S6K (panel D), and Akt (panel E) was analyzed by WB with anti-phospho-specific antibodies in 25 μg protein extracts from resting or PRL-stimulated (100 ng/ml, 15 min) cells. *Lower panels* show the stripped membranes reprobed with the corresponding antibodies (panels B, C, D, and E) for total specific protein. The data are representative of three separate experiments. For thymidine incorporation (panel A), values are presented as the mean ± SE (*bars*) of three independent experiments carried out in triplicate. The *asterisk* (*) represents $P < 0.05$ compared with the control group in Student's *t* test.

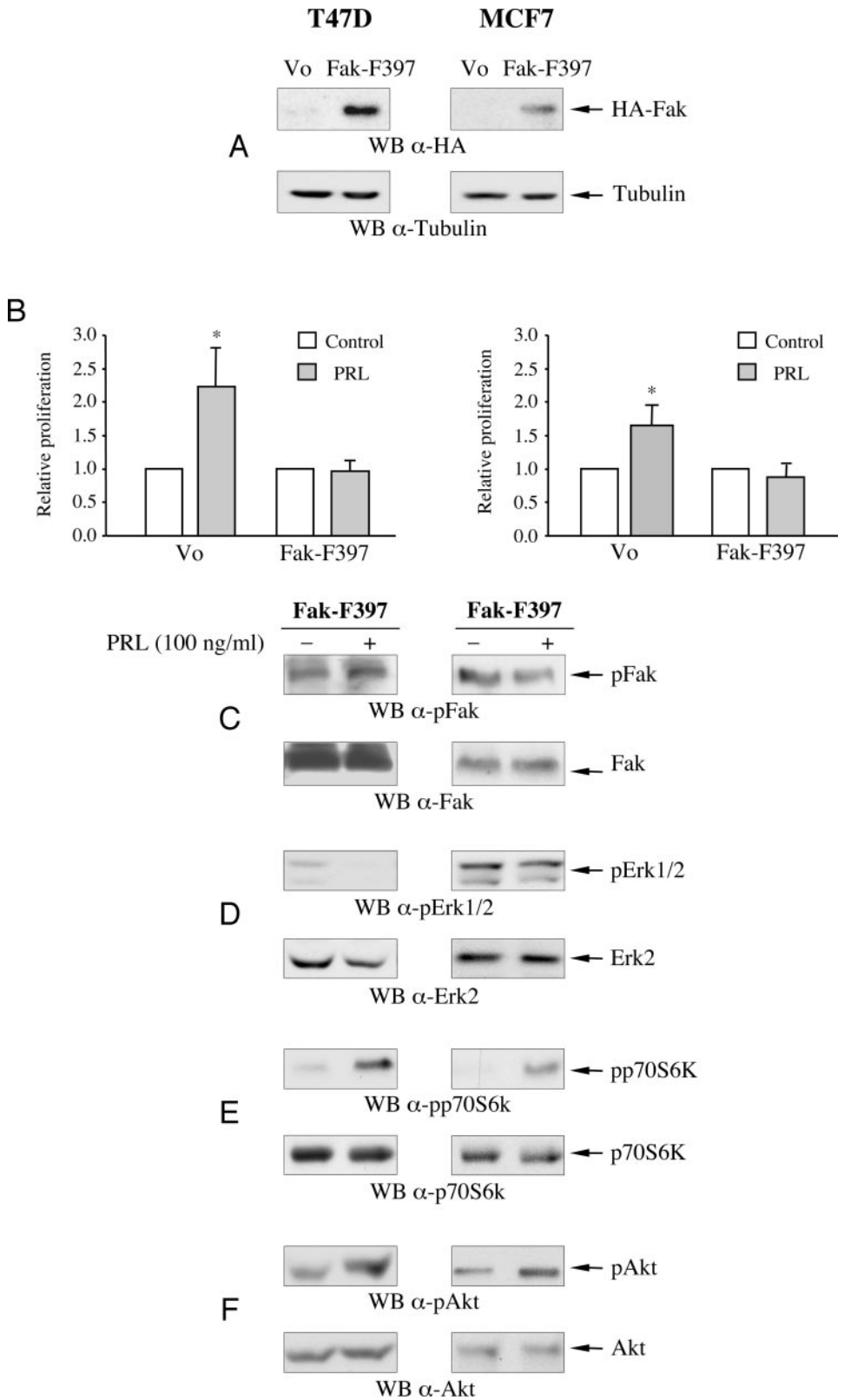

Fig. 5. Effect of Fak-F397 Expression on PRL-Induced Cell Proliferation and the Activation of Fak, Erk1/2, p70S6K, and Akt Induced by PRL in T47D and MCF7 Cells

T47D (*left panels*) and MCF7 (*right panels*) cells were transfected with pcDNA3 (Vo) or HA-tagged pcDNA3-FakF397, selected with G418 (2 mg/ml), and tested for expression of the Fak mutant. A, Protein extracts (25 μg) were separated by 7% SDS-PAGE and WB was performed with anti-HA. Tubulin was detected for loading control. B, The effect of Fak-F397 expression in

pression of c-*myc* and cyclin *d*1 was analyzed by WB (Fig. 8B).

DISCUSSION

PRL involvement in breast cancer has been clearly established (20); however, the sequential stimulation of elements involved in signaling cascades that lead to cell proliferation has not been fully demonstrated. Here we describe that addition of PRL to T47D and MCF7 epithelial human breast cancer cells determines the activation of Src kinases, which, independently, stimulate: 1) the activation of Fak/Erk1/2 and the PI3K-dependent p70S6K and Akt kinases, 2) the up-regulation of c-*myc* and cyclin *d1* mRNA expression and 3) cell proliferation.

The moderate activation of signaling molecules and cell proliferation over basal levels induced in T47D and MCF7 by PRL may be due to the tumor status of these cells. Breast cancer cells normally produce PRL and PRLR (16, 47), among other growth factors, which by an autocrine/paracrine feedback loop allow their growth in serum-free media. In this context, it has been recently shown that addition of PRL to MCF7 cells induced a slight increase in proliferation (20). The differences in the basal levels of activation of the signaling molecules analyzed in this study should be considered as experimental variability among the different experiments performed. However, we consider that increased phosphorylation induced by PRL is clearly detected even with high background levels (*e.g*. Figs. 1F, 4C, 5F, 6B, and 7D). As previously considered for basal phosphorylation levels, the different effect of PI3K, Erk1/2, and Src inhibitors on basal cell proliferation may be explained by the endogenous production of growth factors by tumor cells that initiate diverse signaling pathways involving these kinases.

Activation of Src kinases by PRL occurs in a variety of cells (8–12). c-Src, Fyn, Yes, and Blk are the Src kinases expressed in T47D cells (data not shown); however, c-Src is the only member activated by PRL. The degree of c-Src activation (1.8 fold) induced in T47D cells by PRL is similar to that observed in other experimental models (8–12). In addition, abrogation of c-Src function, by overexpression of SrcDM or its inhibitor PP1 (42, 43), blocked PRL-dependent cell proliferation and down-regulated the activity of growth-related signaling molecules, such as Fak, Erk1/2, and the PI3K-dependent p70S6K and Akt kinases below basal levels. In contrast, inhibition of c-Src function did not affect the activation of Jak2 by PRL. The fact that Src kinases are stimulated by a variety of growth factors and cytokines (48–50), and that breast cancer cells synthesize their own growth factors, may help to maintain a certain level of c-Src activation, which, in turn, could account for the residual activity of signaling molecules and the proliferation rate encountered in the absence of exogenous PRL.

Here we observed that PP1 abolished c-Src activity both *in vivo* and *in vitro*, as was previously reported (42, 43). However, PP1 was unable to block PRL-stimulated Fak autophosphorylation *in vitro*. Therefore, the inhibition of Fak phosphorylation mediated by PP1 observed in T47D and MCF7 PRL-stimulated cells is not a direct effect on Fak, but a consequence of c-Src inhibition, which phosphorylates Fak. Phosphorylation of Fak on Y925 by c-Src recruits SH2-Grb2, resulting in the activation of the Sos-Ras-Raf-Mek-Erk pathway (28, 29). Our results demonstrate that overexpression of Fak-F397 in T47D and MCF7 cells abolishes the activating effect of PRL on Fak, Erk1/2 phosphorylation and cell proliferation, but does not affect PRL stimulation of the PI3K-dependent p70S6K and Akt kinases. These data are in agreement with reports showing that Y397F mutation disrupts c-Src binding and reduces Fak phosphotyrosine content (29). Breast cancer tumors have increased c-Src and MAPK activities (35, 51); moreover, the participation of the c-Src/Ras/MAPK pathway in T47D cell growth has been proposed (52). Our results show that activation of Erk1/2 by PRL is correlated with increased proliferation of T47D and MCF7 cells, considering that the inhibition of PRL-activated Erk1/2 by the Mek1 inhibitor PD184352 reduces T47D and MCF7 proliferation. Similar results were obtained with other MAPK pathway inhibitors (PD98059, U0126) (data not shown). These observations are consistent with the correlation between PRL stimulation of Erk1/2 and enhanced proliferation of T47D cells (19). Furthermore, our data suggest that Erk1/2 activation by PRL in these cell lines is mediated by c-Src/Fak because it is abrogated by c-Src inhibition and by expression of the Fak-F397 mutant. It is noteworthy that, whereas PRL activation of Erk1/2 is mediated by c-Src in epithelial human breast cancer cells (T47D and MCF7), in the mouse hematopoietic W53 cell line the activation of Erk1/2 by PRL is independent of Src kinases, because PP1 has no effect (12). Therefore, the previous data imply that PRL mitogenic signaling cascades are cell type dependent.

PRL-induced thymidine incorporation in T47D and MCF7 cells was carried out as in Fig. 4. C, Fak was immunoprecipitated from 500 μg of protein extract with anti-Fak antibodies, and tyrosine phosphorylation was determined by WB with mAb 4G10. Activation of Erk1/2 (D), p70S6K (E), and Akt (F) was measured by WB with anti-phospho-specific antibody using 25 μg of protein extracts from resting or PRL-stimulated (100 ng/ml, 15 min) cells. *Lower panels* show the stripped membranes reprobed with the corresponding antibodies (C, D, E, and F) for total specific protein. The data are representative of three separate experiments.

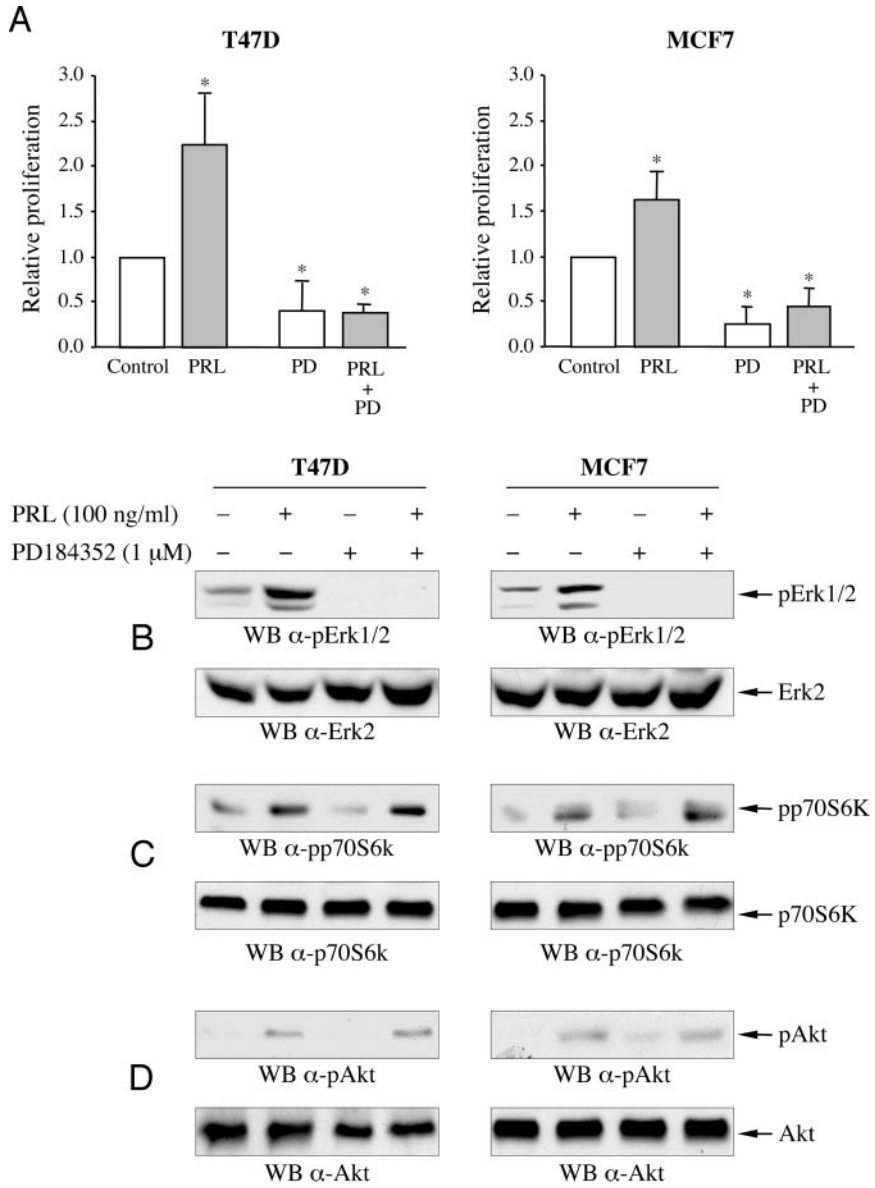

Fig. 6. Effect of the Mek1/2 Inhibitor PD184352 on Cell Proliferation and Activation of Fak, Erk1/2, p70S6K, and Akt Induced by PRL in T47D and MCF7 Cells

A, The effect of PD184352 (1 μM) on PRL-induced thymidine incorporation in T47D and MCF7 cells was carried out as in Fig. 4. The effect of Mek1/2 inhibition by PD184352 (1 μM) in the activation of Fak, Erk1/2, p70S6K, and Akt mediated by PRL was carried out as in Fig. 4. Activation of Erk1/2 (B), p70S6K (C), and Akt (D) was measured by WB with anti-phospho-specific antibodies using 25 μg of protein extract from resting or PRL-stimulated (100 ng/ml, 15 min) cells. *Lower panels* show the stripped membranes reprobed with their corresponding antibodies (B, C, and D) for total specific protein. The data are representative of three separate experiments. For thymidine incorporation (A), values are presented as the mean ± SE (*bars*) of three independent experiments carried out in triplicate. The *asterisk* (*) represents $P < 0.05$ compared with the control group in Student's *t* test.

Jak2 is a relevant mediator of PRL mitogenic signaling in T47D cells (32). In this work we show that PP1, at concentrations that block Src kinase activity, was unable to alter activation of Jak2 by PRL *in vivo* and *in vitro*; similar results were obtained in W53 cells (12). These findings suggest that Src kinases could be engaged in PRL signal transduction either downstream of PRLR dimerization and Jak2 activation or in a Jak2-independent pathway. In this context, we have previously reported that, in chicken embryo fibroblasts, c-Src activation by PRL is independent of Jak2 because a PRLR mutant, unable to interact and activate Jak2, could still mediate the activation of c-Src (11). Moreover, the participation of Fak in the activation of Jak2 by PRL could be excluded, considering that PP1 inhibits PRL stimulation of Fak activity without affecting Jak2 phosphorylation. Although in Chinese hamster ovary cells transfected with the GH re-

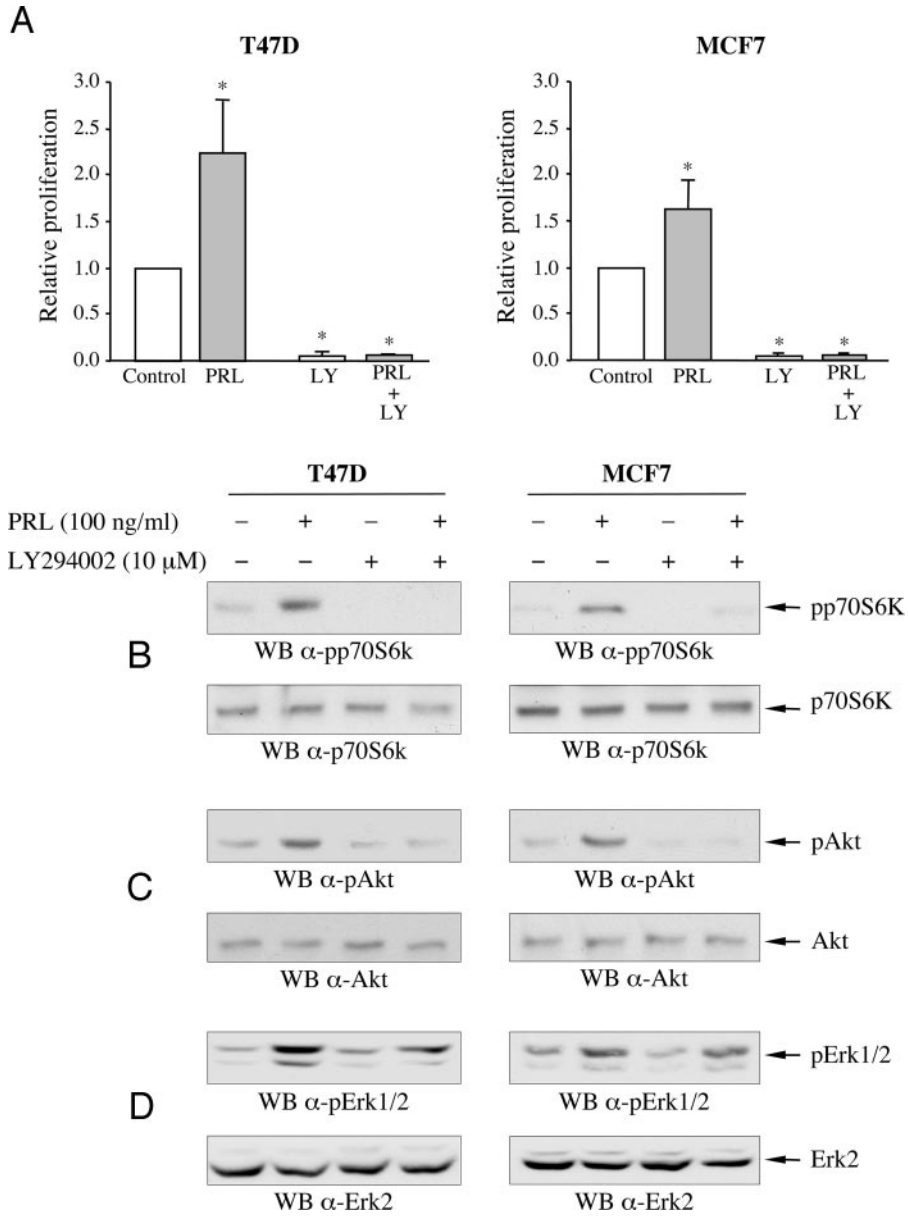

Fig. 7. Effect of the PI3K Inhibitor LY294002 on Cell Proliferation and Activation of Fak, Erk1/2, p70S6K, and Akt induced by PRL in T47D and MCF7 Cells

A, The effect of LY294002 (LY) (10 μM) on PRL-induced thymidine incorporation in T47D and MCF7 cells was carried out as in Fig. 4. The effect of PI3K inhibition by LY294002 (10 μM) on the activation of Fak, Erk1/2, p70S6K, and Akt by PRL was carried out as in Fig. 4. Activation of p70S6K (B), Akt (C), and Erk1/2 (D) was determined by WB with anti-phospho-specific antibodies using 25 μg of protein extracts from resting or PRL-stimulated (100 ng/ml, 15 min) cells. *Lower panels* show the stripped membranes reprobed with the corresponding antibodies (B, C, and D) for specific total protein. The data are representative of three separate experiments. For thymidine incorporation (A), values are presented as the mean ± SE (*bars*) of three independent experiments carried out in triplicate. The *asterisk* (*) represents $P < 0.05$ compared with the control group in Student's *t* test.

ceptor, Fak coimmunoprecipitates with Jak2 upon hormone stimulation (53), our data indicate that in T47D and MCF7 cells, Src and Fak activities are not required for the activation of Jak2 by PRL.

It has been previously demonstrated that expression of c-*myc* and cyclin *d1* is required for cell proliferation. In this work we observed that proliferation of T47D and MCF7 cells stimulated by PRL is associated with the up-regulation of c-*myc* and cyclin *d1* expression. Moreover, the data we present support the fact that c-Src, by regulating both Fak/Erk1/2 and PI3K PRL-stimulated pathways, controls the expression of c-*myc* and cyclin *d1* (Fig. 8). Consistently, we have previously shown that PRL-stimulated c-*myc* expression was regulated by Src kinases in W53 cells (12). The involvement of Src in cyclin *d1* expression was

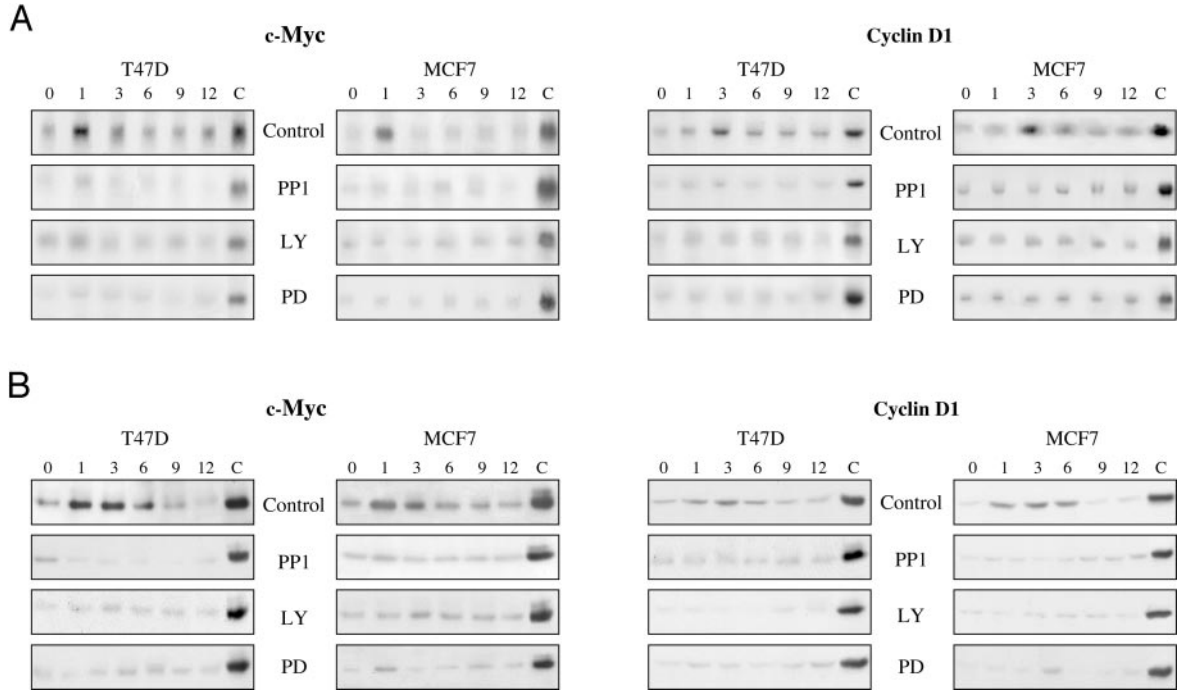

Fig. 8. Effect of the Src Inhibitor PP1, the Mek 1/2 Inhibitor PD184352 (PD), and the PI3K Inhibitor LY294002 (LY), on PRL-Mediated Expression of c-*myc* and cyclin *d1* in T47D and MCF7 Cells

T47D (*left panels*) and MCF7 (*right panels*) cells were seeded in DMEM-10% FCS and 24 h later transferred to serum-free medium for 72 h. Resting cells were incubated in the absence or presence of PP1 (1 μM), PD 184352 (1 μM), or LY294002 (10 μM) for 2 h before stimulation with PRL (100 ng/ml) for the indicated time periods (h). The expression of c-*myc* and cyclin *d1* was determined by Northern blotting using 16 μg of RNA extracts (panel A) and WB using 25 μg of protein extracts (panel B), as described in *Materials and Methods*. A positive control of RNA (16 μg) and protein cell extracts (25 μg) was included on each blot (C). Positive controls for c-myc Northern and Western blots consist of total RNA and protein extracts from T47D and MCF7 cells stimulated with 10% serum for 1 h. The positive controls for cyclin D1 Northern and Western blots are total RNA and protein extracts from T47D and MCF7 cells stimulated with 10% serum for 3 h. The data are representative of three separate experiments.

demonstrated in breast cancer cells, because expression of the oncogenic form of Src (v-Src) activated the promoter cyclin *d1* in MCF7 cells (38). Regarding the control of cyclin *d1* expression due to signal transduction via activation of Fak/Erk1/2 by Src, it was reported that activation of Fak by integrins resulted in increased transcription of cyclin *d1* via the activation of the Erk1/2 pathway (30). Further results obtained with MCF7 cells implicate the Jak2/Stat5 pathway in the increase of cyclin *d1* expression observed upon stimulation with PRL (54). The previous data indicate that, in addition to the γ-interferon-activated sequence sites regulated by the Jak2/Stat5 pathway (54), the cyclin *d1* promoter also contains Fak/Erk1/2 pathway-responsive EtsB binding sites (30). Our results demonstrate that PRL, via c-Src, activates PI3K-dependent Akt and p70S6K serine/threonine kinases and cyclin *d1*. Akt is involved in regulating cyclin D1 expression in mammary tumors developed by transgenic mice for middle T antigen (55). Consistent with this report, ectopic expression of phosphatase and tensin homolog on chromosome 10 (PTEN) in MCF7 cells blocks Akt activation and cyclin *d1* expression (56). Moreover, PI3K-p70S6K kinase has been similarly implicated in the control of cyclin *d1* expression because rapamycin was shown to inhibit p70S6K and cyclin *d1* expression in vascular endothelial cells (57).

The data presented here show that addition of PRL to T47D and MCF7 cells results in the activation of c-Src, Fak/Erk1/2, PI3K-dependent p70S6K and Akt pathways, c-*myc* and cyclin *d1* expression, and increased cell proliferation, and abrogation of c-Src activity inhibits all the events. Fak-F397 mutation or inhibition of MAPKs with PD184352 was unable to modify the activation of PI3K; furthermore, inhibition of PI3K by LY294002 did not affect the Fak/Erk1/2 pathway. However, both pathways are required for c-*myc* and cyclin *d1* expression and enhanced cell proliferation. In conclusion, our results report that regulation of c-Src/Fak/Erk1/2 and c-Src/PI3K cell-signaling pathways is mediated by c-Src when T47D and MCF7 breast cancer cells are stimulated with PRL, and, in turn, they regulate c-*myc* and cyclin *d1* expression and cell proliferation (Fig. 9). It is clearly established that Src kinases are mediators of multiple factors involved in proliferation, angiogenesis, and migration of breast cancer cells. Thus, Src kinases may represent relevant targets in breast cancer therapy.

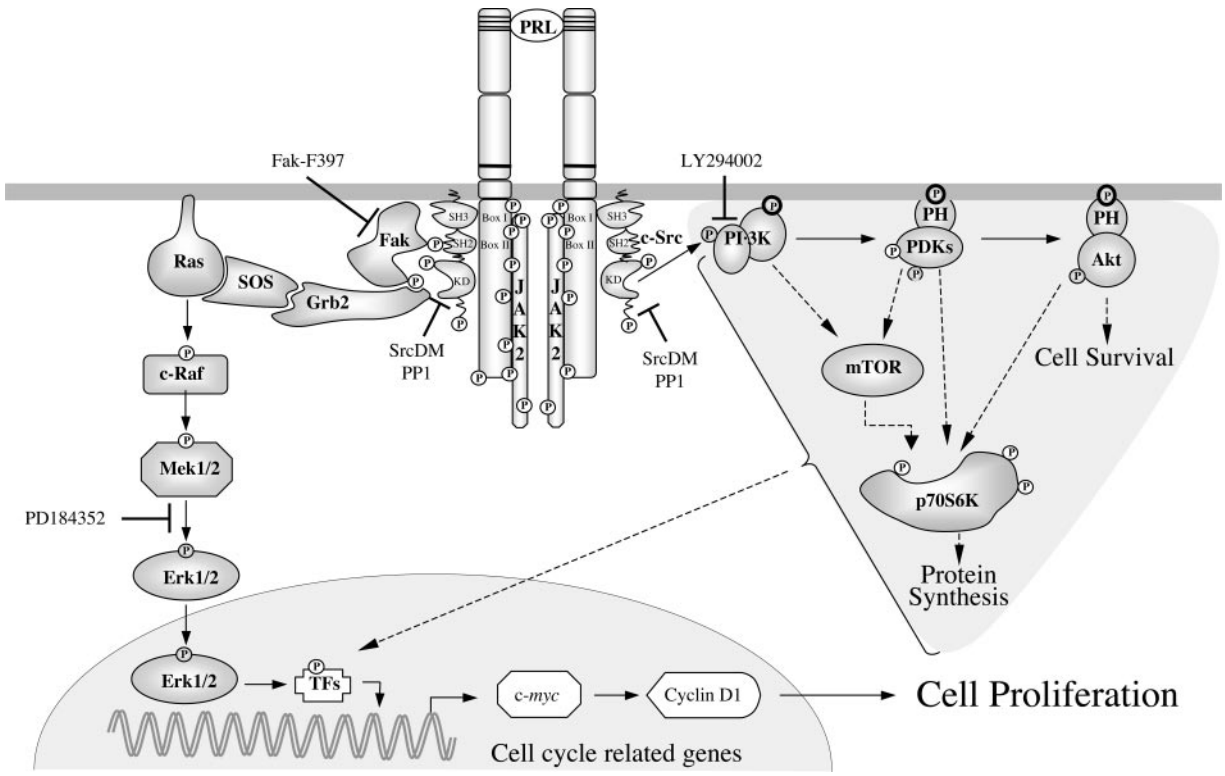

Fig. 9. Role of c-Src in PRL-Induced Proliferation of T47D and MCF7 Cells

PRL induces dimerization of PRLR and activation of c-Src, which mediates the activation of PI3K/Akt/p70S6K and Fak/Erk1/2 signaling cascades, providing signals for c-Myc and cyclin D1 expression, required for cell proliferation. Our results show that PRL activation of Jak2 is independent of c-Src. Grb2, Adaptor protein with SH2 and SH3 domains; mTOR, mammalian target of rapamycin; PDKs, phospholipid-dependent kinase; PH, pleckstrin domain; SOS, Son of Sevenless.

MATERIALS AND METHODS

Materials

Ovine 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). Mouse mAb 327 against c-Src was a gift from J. S. Brugge (Harvard University, Boston, MA). Antiphosphotyrosine mAb 4G10 was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Affinity-purified rabbit polyclonal antibodies to Src kinases (SRC-2), Erk2 (C14), Fak (A17), Akt (H-136), and Jak2 (C20) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antipaxillin was purchased from Transduction Laboratories, Inc. (Lexington, KY). Anti-p70S6K was obtained from George Thomas (Friedrich Mistier Institute, Basel, Switzerland). Antiphospho antibodies to pErk1/2, pp70S6K, and pAkt are from New England Biolabs (Beverly, MA). Secondary horseradish peroxidase-conjugated antibodies were purchased from Biosource International (Camarillo, CA). The enhanced chemiluminescence (ECL) kit, radiochemicals, and the oligolabeling kit were from Amersham Pharmacia Biotech (Buckinghamshire, UK). PP1/PP2 were obtained from Alexis Biochemicals (San Diego, CA). PD98059 and U0146 were obtained from Calbiochem (San Diego, CA). PD184352 was a gift from Philip Cohen (MRC Protein Phosphorylation Unit, University of Dundee, Dundee, Scotland, United Kingdom). Protein G-Sepharose and protein A-Sepharose, Tri Reagent, and other reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO).

Cell Culture and Transfection

T47D cells were routinely grown in DMEM supplemented with 10% fetal calf serum, 0.1 IU/ml insulin, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2 and 95% air at 37 C. The pSGT plasmid containing the cDNA for the dominant-negative mutant of c-Src, SrcDM (K295A/Y527F), was obtained from S. Roche (Centre de Recherche de Biochimie Macromoléculaire-Centre National de la Recherche Scientifique, Unité Propre de Recherche 1086 France; Ref. 58). Exponentially growing T47D cells (5×10^6 cells) were transiently transfected using the calcium phosphate precipitation method with 60 μg of pSGT-SrcDM. As a control, T47D cultures were transfected with 60 μg of the pSGT empty vector. After 6 h incubation with the DNA precipitates, cells were washed with DMEM and incubation proceeded for an additional 24-h period. T47D-Fak-F397 and MCF7-Fak-F397 cell lines were generated by transfection using Transfast reagent (Promega Corp., Madison, WI). The Fak mutant Y397F cloned into pcDNA3-Fak-F397 (5 μg) (D. Schlaepfer, Scripps Research Institute, La Jolla, CA) was mixed with 5 μl of Transfast in 2 ml of culture media for 10 min at room temperature and added to the cell plate (5×10^6 cells). After incubation at 37 C for 1 h, 8 ml of complete medium were added and incubation proceeded for an additional 12 h. Cultures were then washed twice with DMEM containing 10% fetal calf serum (FCS), and cultured for 48 h before selection with G418 (2 mg/ml). Two weeks later, the concentration of G418 was reduced to 1 mg/ml and fixed as the concentration for cell culture.

Immunoprecipitation and WB Analysis

Cells (1×10^6 cells per dish) for immunoprecipitation and WB analysis were maintained in phenol red-free and serum-free DMEM 72 h before stimulation with PRL. Subsequently, cells were washed twice in ice-cold Tris-buffered saline (TBS: 20 mM Tris-HCl, pH 7.6; 140 mM NaCl) with 0.1 mM Na_3VO_4 and lysed at 4 C in 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.6; 50 mM NaCl; 30 mM sodium pyrophosphate; 5 mM EDTA; 0.5% Nonidet P-40; 1% Triton X-100; 50 mM NaF; 0.1 mM Na_3VO_4; 1 mM phenylmethylsulfonylfluoride; 1 mM benzamidine; 1 mM iodoacetamide; 1 mM phenantroline). Cell lysates were obtained by centrifugation at 17,000 $\times$ *g* for 15 min at 4 C, protein concentration in the supernatant was determined by BCA protein assay (Pierce Chemical Co., Rockford, IL), and lysates were adjusted to equivalent concentrations with lysis buffer. Immunoprecipitation was performed by incubating lysates (500 μg/ml of protein) with 1–2 μg of the appropriate specific antibody for 2 h at 4 C. Immune complexes were recovered with protein G-Sepharose or protein A-Sepharose beads. They were washed three times with ice-cold lysis buffer, boiled in 2$\times$ sodium dodecyl sulfate (SDS) sample buffer (125 mM Tris-HCl, pH 6.8; 10% 2-mercaptoethanol; 4% SDS; 20% glycerol; 0.01% bromophenol blue). Supernatants were collected and subjected to SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane, which was blocked overnight at 4 C with 5% nonfat milk in TTBS (TBS with 0.05% Tween 20). Blocking solution for phosphotyrosine immunoblotting was 5% BSA in TTBS. Incubation with primary specific antibodies and horseradish peroxidase-conjugated secondary antibodies was performed in blocking solution for 1 h at room temperature. ECL detected immunoreactive bands. Membrane stripping was performed by incubating the membrane for 30 min at 50 C in 62.5 mM Tris-HCl (pH 6.7) containing 2% SDS and 100 mM 2-mercaptoethanol and extensively washing with TTBS at room temperature. Stripping was checked by reexposure to ECL, and membranes were subsequently blocked and reproved as described above.

Immune Complex Kinase Assay

Immunoprecipitates were first washed with 50 mM LiCl, 10 mM Tris-HCl (pH 7.4), 0.1 mM Na_3VO_4, then with TBS, 0.1 mM Na_3VO_4, and finally with kinase buffer (20 mM Tris-HCl, pH 7.2, 10 mM $MnCl_2$, 2 mM 2-mercaptoethanol). The immune complexes of Fak, Src kinases, and Jak2 were resuspended in 30 μl of kinase buffer containing 5 μCi of [^{32}P]-γ-ATP (5000 Ci/mmol, 10 mCi/ml, Amersham) and incubated at 30 C for 5 min. Reactions were stopped by addition of 2$\times$ SDS sample buffer and boiled for 3 min. The reaction mixtures were centrifuged and the supernatants analyzed by SDS-PAGE. The gels were subsequently dried and exposed to x-ray film to visualize the ^{32}P-labeled protein bands, or counted in an Instantimager (Packard Instruments, Downers Grove, IL).

Proliferation Assays

Cell proliferation assays were determined by thymidine incorporation. Exponentially growing cells were plated in phenol red-free DMEM-10% FCS into 24-well plates (5×10^4 cells per well), rinsed twice with serum-free media 24 h later, and cultured in this medium for 48 h. Cultures were then incubated for 72 h in the absence or presence of PRL and/or PP1, PD184352, or LY294002 dissolved in dimethylsulfoxide (DMSO); control cultures were incubated with an equivalent volume of DMSO (1:500 dilution). Each well was pulsed with 0.5 μCi of [^{3}H]thymidine (48 Ci/mmol; Amersham Pharmacia Biotech) for the final 4 h. Cells were trypsinized and harvested onto glass fiber filter paper; the filters were dried and counted in a solid scintillation counter (1450 Microbeta Wallac LKB, Turku, Finland). All samples were assayed in triplicate. [^{3}H]Thymidine uptake of T47D and MCF7 unstimulated cells (5×10^4 cells per well) was 12,280 $\pm$ 1,283 cpm and 10,212 $\pm$ 807 cpm (n = 6), respectively.

Northern Blot Analysis

Total RNA was isolated using Tri Reagent. RNA was fractionated by electrophoresis on 1% agarose gel containing 6% formaldehyde and transferred to Nytran membranes (Schleicher & Schuell, Dassel, Germany) by capillary blotting. Blots were hybridized with cDNA probes labeled with the Oligolabeling kit using 25 μCi α-[^{32}P]dCTP (3000 Ci/mmol; Amersham Pharmacia Biotech). After several washes, the hybridization signals were visualized by autoradiography.

Statistical Analysis

Results were analyzed by Student's *t* test. Differences between two means with a $P < 0.05$ were regarded as significant. All values were expressed as means $\pm$ SE of at least three experiments.

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