Lyn is activated during late G_1 of stem-cell-factor-induced cell cycle progression in haemopoietic cells

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Stem cell factor (SCF) binds the receptor tyrosine kinase c-Kit and is critical in haemopoiesis. Recently we found that the Src family member Lyn is highly expressed in SCF-responsive cells, associates with c-Kit and is activated within minutes of the addition of SCF. Here we show that SCF activates Lyn a second time, hours later, during SCF-induced cell cycle progression. In cells arrested at specific phases of the cell cycle with the drugs mimosine, aphidicolin and nocodazole, maximal Lyn kinase activity occurred in late G_1 and through the G_1/S transition. Similarly, kinetic studies of SCF-induced cell cycle progression found that activation of Lyn preceded the G_1/S transition and was maintained into early S-phase. Activation of Lyn was paralleled by two events critical for the G_1/S transition, increases in cyclin-dependent kinase 2 (Cdk2) activity and phosphorylation of the retinoblastoma gene product (Rb). Lyn was associated with Cdk2; Cdk2-associated Lyn was heavily phosphorylated on serine and threonine residues both *in vitro* and *in situ* during S-phase. Inhibition of Lyn activity with PP1 disrupted association with Cdk2 and decreased the numbers of cells entering S-phase. The degree of phosphorylation of Rb in PP1-treated cells suggested an increased number of cells arrested in the middle of G_1. These findings demonstrate that SCF activates the Src family member Lyn before the G_1/S transition of the cell cycle and suggest that Lyn is involved in SCF-induced cell cycle progression.

Key words: growth factors, haematopoiesis, receptor tyrosine kinase, signal transduction, Src family members.

INTRODUCTION

Stem cell factor (SCF) promotes viability and induces the proliferation of haemopoietic progenitor cells. In addition, SCF is potently synergistic when combined with growth factors such as erythropoietin, interleukin 3 and granulocyte/macrophage colony-stimulating factor (GM-CSF) (reviewed in [1,2]). The receptor for SCF is a product of the *kit* proto-oncogene. c-Kit has intrinsic protein tyrosine kinase activity and is related to the receptors for platelet-derived growth factor (PDGF) and colony-stimulating factor 1 (CSF-1) [2]. The critical nature of SCF in stem cell physiology is illustrated by the marked aberrations in haemopoiesis, pigmentation and reproduction in mice expressing either defective SCF (steel mice) or c-Kit (white spotting mice) [1].

As with other receptor tyrosine kinases (RTKs), SCF induces the dimerization of c-Kit as well as increases in autophosphorylation [3]. Tyrosine phosphorylation of c-Kit leads to the recruitment of proteins containing Src homology region 2 ('SH2') domains to the receptor complex. These include phosphoinositol 3-kinase, Grb2, Chk, Shp1, Shp2, Stat1, RasGAP and phospholipase Cγ [4–12]. Downstream of c-Kit activation are multiple pathways leading to increases in gene expression. Included among these are the Ras–Raf–mitogen-activated protein kinase ('MAPK') pathway and the Janus kinase ('JAK')–signal transduction and activators of transcription ('STAT') pathway [6,9,12–15].

Previous work has demonstrated that Src family members are activated during mitosis of fibroblasts [16]. Further, Courtneidge and co-workers have shown that Src, Fyn and Yes are required for fibroblast cell division in response to PDGF, CSF-1 and epidermal growth factor (EGF), ligands that interact with RTKs [17,18]. We have recently found that the Src family member Lyn is highly expressed in SCF-responsive cells, associates with c-Kit and is phosphorylated within minutes of the addition of SCF [19]. Because little is known about the role of Src family members in the cell cycle progression of haemopoietic cells, we examined Lyn activity during the later events of SCF-induced cell cycle progression of the factor-dependent megakaryoblastic cell line Mo7e. We have found that stimulation with SCF induced increases in Lyn kinase activity that peaked in late G_1 and were maintained into early S-phase. Lyn was associated with cyclin-dependent kinase 2 (Cdk2); increases in serine and threonine phosphorylation of Cdk2-associated Lyn were observed in cells arrested in early S-phase. Inhibition of Lyn activity with PP1, an inhibitor of Src family members, decreased the number of cells that entered S-phase after stimulation with SCF. These results suggest that Lyn has a role in cell cycle progression induced by SCF in haemopoietic cells.

EXPERIMENTAL

Cells, growth factors and antibodies

Mo7e cells were maintained in growth medium consisting of RPMI 1640, 10% (v/v) fetal calf serum (FCS) (Atlanta Biologics, Norcross, GA, U.S.A.), 2 mM L-glutamine and 1% (w/v)

Abbreviations used: Cdk2, cyclin-dependent kinase 2; CSF-1, colony-stimulating factor 1; EGF, epidermal growth factor; FCS, fetal calf serum; GM-CSF, granulocyte/macrophage colony-stimulating factor; PDGF, platelet-derived growth factor; Rb, retinoblastoma gene product; RTK, receptor tyrosine kinase; SCF, stem cell factor.

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penicillin/streptomycin (50 μg/ml) supplemented with recombinant human GM-CSF (1 ng/ml) and human SCF (30 ng/ml). Human GM-CSF and SCF were purchased from PeproTech (Rocky Hill, NJ, U.S.A.). Polyclonal antibody used to immunoprecipitate Lyn was kindly provided by Dr. Joe Bolen (DNAX, Palo Alto, CA, U.S.A.) and has been described previously [20]. Monoclonal antibody used to immunoblot Lyn was purchased from Transduction Labs (Lexington, KY, U.S.A.). Immunoprecipitation and immunoblotting of Cdk2 and the retinoblastoma gene product (Rb) was performed with antisera purchased from Santa Cruz (Santa Cruz, CA, U.S.A.) and PharMingen (San Diego, CA, U.S.A.) respectively.

Cell cycle studies

The study of SCF-induced cell cycle progression was performed as follows. Mo7e cells were cultured in growth medium for 18 h. Cells were then washed twice with RPMI 1640, and resuspended at 5×10^5/ml in RPMI 1640/10 % (v/v) FCS without SCF. Cells were cultured for 18 h to synchronize in G_0/G_1, then either harvested or stimulated with SCF (100 ng/ml) for the number of hours indicated. To harvest samples for cell cycle analysis, 5×10^5 cells were washed twice in PBS and resuspended in 1 ml of KI buffer, which consisted of 0.37 % (v/v) Nonidet P40, 0.1 % sodium citrate, 0.2 mg/ml RNase and 0.05 mg/ml propidium iodine, pH 7.4. Cells were also harvested for immune complex kinase assays as described in the next section. DNA staining was assessed with the use of FACS and results were analysed with the Multiplus AV program (Phoenix Flow Systems, San Diego, CA, U.S.A.). Studies with drugs to arrest cells at different phases of the cell cycle were performed by incubating Mo7e cells in RPMI 1640/10 % (w/v) FCS/100 ng/ml SCF for 18 h and then culturing for 16 h with SCF in combination with the appropriate drug (100 μM mimosine, 0.5 μM aphidicolin or 50 μg/ml nocodazole). Cells were then harvested for cell cycle analysis as described above. To examine the effect of PP1 (Calbiochem, San Diego, CA, U.S.A.) on SCF-induced cell cycle progression, Mo7e cells were cultured for 12 h with SCF. Cells were then incubated for 18 h with either medium in the absence of growth factor or medium supplemented with 100 ng/ml SCF in the presence or absence of 5 μM PP1. Samples were then harvested for cell cycle analysis or lysates were prepared to examine Lyn activity, c-Kit activity or Rb phosphorylation.

Immune complex kinase assays

Cells to be assayed for kinase activity were prepared and harvested in parallel with cells collected for cell cycle analysis. Cells were washed twice in PBS and lysed in 1 ml of lysis buffer [1 % (v/v) Triton X-100/50 mM NaCl/10 mM Tris/HCl (pH 7.5)/5 mM EDTA/30 mM sodium pyrophosphate/5 mM NaF/25 mM β-glycerol phosphate/5 mM sodium orthovanadate/0.1 % *p*-nitrophenol phosphate/1 mM PMSF] at 2×10^7 cells/ml. Cell lysates were clarified by centrifugation (5000 ***g*** for 20 min at 4 °C) and protein concentration was determined with the bicinchoninic acid ('BCA') protein detection kit (Pierce, Rockford, IL, U.S.A.). For Lyn kinase assays, 500 μg of protein from clarified cell lysates was immunoprecipitated with anti-Lyn; the antigen–antibody complexes were captured with Protein A–Sepharose. Immunoprecipitates were washed six times with lysis buffer, then incubated for 15 min at 30 °C in kinase buffer [25 mM Hepes/10 mM $MnCl_2$ (pH 7.5)] containing 50 μCi/ml [γ-^{32}P]ATP. Samples were then washed twice with lysis buffer and eluted from the Protein A–Sepharose with SDS sample buffer. Kinase assays performed with a tyrosine kinase substrate included 1 μg of enolase and were terminated with SDS sample buffer. Cdk2 kinase assays were performed by incubating Cdk2 immunoprecipitates in kinase buffer containing 10 mM Hepes, pH 7.2, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 50 μCi/ml [γ-^{32}P]ATP and 3 μg of histone H1, a substrate for cyclin-dependent kinases.

Metabolic radiolabelling with [^{32}P]P_i

Cells were incubated for 16 h in the presence or absence of SCF. The cells were washed twice in phosphate-free RPMI 1640 and resuspended in phosphate-free RPMI 1640/1 % phosphate-free FCS/0.5 mCi/ml [^{32}P]P_i. Cells were incubated for 90 min at 37 °C, centrifuged and resuspended in 1 ml of lysis buffer. Lysates were incubated for 20 min on ice, clarified by high-speed centrifugation and immunoprecipitated with antisera specific for Cdk2 as described above.

Electrophoresis and immunoblotting

Proteins were resolved by SDS/PAGE, and transferred to Immobilon as described previously [19]. Autoradiography was performed to detect proteins radiolabelled in immune complex kinase assays. Immunoblotting was performed with the indicated antibodies as described previously [19]. In brief, blots were blocked and incubated with the unconjugated primary antibody indicated. After rigorous washing, blots were incubated first with biotinylated secondary antibody (rabbit or mouse, as appropriate), then with peroxidase-conjugated streptavidin. Proteins were detected with the Renaissance Reagent for enhanced chemiluminescence (NEN, Boston, MA, U.S.A.).

Phosphoamino acid analysis

Either Lyn or Cdk2 immunoprecipitates were resolved with SDS/PAGE and transferred to Immobilon. Phosphorylated Lyn was identified by autoradiography, then excised and incubated for 90 min at 110 °C in 5.7 M HCl. The HCl was evaporated under vacuum in a SpeedVac; pellets were then dissolved in 5 μl of distilled water containing 2 mg/ml each of phosphotyrosine, phosphoserine and phosphothreonine and subjected to one-dimensional high-voltage thin-layer electrophoresis. Samples were applied to cellulose (100 μm thickness)-coated glass plates (E. Merck) and separated at 1.5 kV for 40 min with the use of pyridine/acetic acid/water (10:100:1890, by vol.). Phosphoamino acid standards were identified with ninhydrin and the experimental samples were detected by autoradiography.

RESULTS

To examine the activation of Src family members during SCF-induced cell cycle progression we first examined the suitability of Mo7e cells for these studies. This factor-dependent megakaryoblastic cell line was derived from a human leukaemia patient and expresses high levels of c-Kit [21,22]. We have shown previously that SCF induces increases in [^{3}H]thymidine incorporation of Mo7e cells [19]. In addition, culture in SCF results in increases in cell numbers (results not shown). Because SCF induces both DNA synthesis and mitosis of these cells, they were used to study SCF-induced cell cycle progression.

We next examined the kinetics of SCF-induced cell cycle progression. Mo7e cells were arrested in G_0/G_1 through overnight culture in the absence of exogenous growth factors, then stimulated with SCF and harvested for cell cycle analysis after 8, 10, 12 and 14 h. Increases in the percentage of cells in S-phase were observed 10 h after the addition of SCF; maximal per-

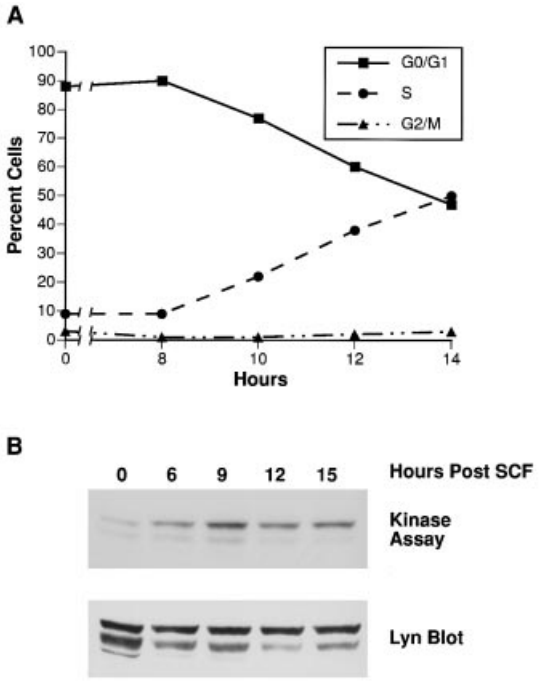

Figure 1 Time course of Lyn activity during SCF-induced cell cycle progression

Mo7e cells were arrested in G_0/G_1 through culture overnight in the absence of growth factors. The cells were then supplemented with SCF and harvested at the times indicated for (**A**) cell cycle analysis and (**B**) kinase assays. Immune complex kinase assays were performed as described in the Experimental section with Lyn immunoprecipitated from equivalent protein amounts. Samples were resolved with SDS/PAGE and transferred to Immobilon. The upper portion shows an autoradiogram of immune complex kinase assays; the lower portion shows a Lyn immunoblot of immune complex kinase assays.

centages of cells in S-phase were found 14 h after the addition of SCF (Figure 1A).

Although ligands interacting with RTKs are known to activate Src family members during cell cycle progression of fibroblasts, little is known about the role of Src family members in SCF-induced cell cycle progression of haemopoietic cells. Because our previous studies have shown that the Src family member Lyn is highly expressed in Mo7e cells, we examined the effect of SCF-induced cell cycle progression on Lyn kinase activity. Interestingly, Lyn autophosphorylation increased 6 h after SCF addition and was maintained for 15 h afterwards (Figure 1B). Importantly, the expression of Lyn protein levels remained relatively constant during the course of the study (Figure 1B, lower panel).

Progression of the cell cycle from G_1 to S-phase is mediated by a number of well-defined biochemical events. Two events known to occur late in G_1 and through the G_1/S transition are the activation of Cdk2 and the phosphorylation of Rb [23–26]. Figure 2 compares the effect of SCF-induced cell cycle progression on Lyn activity, Cdk2 activity and Rb phosphorylation. Cell cycle analysis, shown in Figure 2(A, lower panel), demonstrated that in this experiment 46% of the cells were in S-phase after a 12 h treatment with SCF. In contrast, only 6% of the factor-deprived cells were in S-phase. Immune complex kinase assays of Lyn and Cdk2 demonstrated that SCF induced increases in Lyn autophosphorylation activity as well as increases in the capacity of Cdk2 to phosphorylate histone H1 (Figures 2A and 2B, upper panels). Equivalent amounts of both Lyn and Cdk2 were present in both experiments (Figure 2A, middle panel, and Figure 2B, lower panel). Alteration in the migration of Rb, an event associated with increases in Rb phosphorylation, was also noted after a 12 h stimulation with SCF (Figure 2C). Taken together, these results demonstrate that the activation of Lyn during SCF-induced cell cycle progression is accompanied by the activation of Cdk2 and Rb phosphorylation.

To examine further the relationship of Lyn activity to SCF-induced cell cycle progression we used drugs (mimosine, aphidicolin and nocodazole) that arrest cells in G_1, early S-phase or early mitosis respectively [27–29]. After treatment of the cells with the indicated drug overnight, samples were collected for cell cycle analysis and kinase assays. Treatment with mimosine resulted in 77% of the cells being in G_1, whereas 48% of the cells were in S-phase after culture in aphidicolin. Treatment with nocodazole resulted in 52% of the cells being in G_2/M (Figure 3B, lower panel). The results of the kinase studies are shown in Figure 3(A). Increases in both the autophosphorylation of Lyn and the phosphorylation of enolase were noted in aphidicolin-treated cells in comparison with those treated with either mimosine or nocodazole. In addition, Lyn autophosphorylation activity in nocodazole-treated cells was greater than that observed after treatment with mimosine. Immunoblotting studies with Lyn-specific antisera demonstrated that equivalent amounts of the 56 and 53 kDa Lyn isoforms were present after each of the drug treatments (Figure 3B). These results demonstrate that Lyn activity is maximal in cells arrested in early S-phase with aphidicolin.

To explore the relationship between Lyn activation and the G_1/S transition, we examined Lyn autophosphorylation activity after release from a G_1 block. Cells were treated overnight with mimosine, washed extensively, cultured in SCF and harvested at the times indicated in Figure 4 for either Lyn kinase assays or cell cycle analysis. Interestingly, Lyn autophosphorylation activity increased 1 h after removal of mimosine and preceded the entry of cells into S-phase by 1 h (Figure 4A, and Figure 4B, lower panel). Immunoblotting with antisera specific for Lyn demonstrated that Lyn protein was not increased during the course of the experiment (Figure 4B). In summary, studies with drugs that arrest cells at defined stages of the cell cycle, as well as time-course studies of SCF-induced cell cycle progression, indicate that Lyn activity increases late in G_1 and remains elevated through the G_1/S transition into early S-phase.

Previous work has shown that a fraction of total cellular Lyn is induced to associate with, and phosphorylate, Cdk2 after treatment of factor-independent cells with chemotherapeutic agents [30]. These studies also suggested that Lyn-mediated phosphorylation of Cdk2 had a role in cell cycle arrest induced by these genotoxic agents [30]. To gain insight into the potential role of Lyn in the normal events of cell cycle progression, we examined whether Lyn and Cdk2 were associated during late G_1 or early S-phase. Figure 5(A) shows that Lyn was co-immunoprecipitated with Cdk2 isolated from cells treated with either mimosine or aphidicolin. Reciprocal studies also demonstrated that Cdk2 could be co-immunoprecipitated with Lyn (results not shown). We next assessed the effect of cell cycle progression on phosphorylation of Lyn in the Cdk2 complex. As expected, the capacity of Cdk2 to phosphorylate histone H1 was increased in cells arrested in early S-phase (Figure 5B). Figure 5(D) demonstrates that this was not the result of increases in Cdk2 protein expression. In addition, a heavily phosphorylated doublet of 56 and 53 kDa co-precipitated with Cdk2 after treatment with aphidicolin (Figure 5B). Immunoblotting studies with Lyn-specific antisera demonstrated that this protein was Lyn (Figure

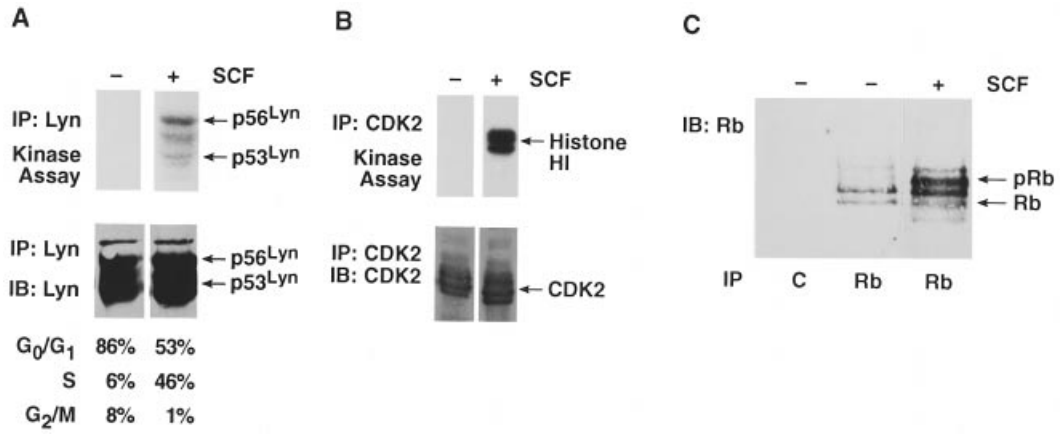

Figure 2 SCF-induced activation of Lyn is accompanied by activation of Cdk2 and phosphorylation of Rb

Mo7e cells were arrested in G_0/G_1 through culture overnight in the absence of growth factors. The cells were then stimulated with SCF for 12 h and harvested for cell cycle analysis as well as the Lyn, Cdk2 and Rb studies. (**A**) Activation of Lyn kinase activity during SCF-induced cell cycle progression. Lyn was immunoprecipitated (IP) from equivalent protein amounts and samples were resolved with SDS/PAGE, transferred to Immobilon and radiolabelled proteins were detected with autoradiography. Immunoblotting (IB) with antisera specific for Lyn is shown in the middle panel. The lower portion shows the results of the cell cycle analysis. (**B**) Activation of Cdk2 during cell cycle progression. Immune complex kinase assays were performed with Cdk2 immunoprecipitated (IP) from equivalent protein amounts. Histone H1 was included as a substrate for Cdk2. Samples were resolved with SDS/PAGE, transferred to Immobilon and radiolabelled proteins were detected with autoradiography. Immunoblotting (IB) with Cdk2 specific antisera is shown in the lower panel. (**C**) Phosphorylation of Rb during cell cycle progression. Equivalent protein amounts were immunoprecipitated (IP) with antibody specific for Rb or an isotype-matched control (C), resolved with SDS/PAGE and transferred to Immobilon. Immunoblotting (IB) was performed with antisera specific for Rb. Phosphorylated Rb (indicated with pRb) migrates more slowly than the unphosphorylated form (indicated with Rb).

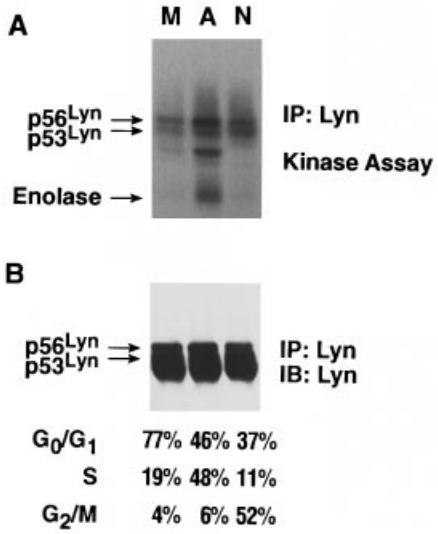

Figure 3 Lyn activity is maximal in cells arrested in early S-phase with aphidicolin

Mo7e cells were grown in SCF overnight and then treated for 16 h with SCF in the presence of mimosine (100 μM, indicated with M), aphidicolin (0.5 μM, indicated with A) or nocodazole (50 μg/ml, indicated with N). Cells were harvested for cell cycle analysis or immune complex kinase assays. Lyn was immunoprecipitated (IP) from equivalent protein amounts, immune complex kinase assays were performed and samples were resolved with SDS/PAGE and transferred to Immobilon. (**A**) Autoradiography of immune complex kinase assays. (**B**) Lyn immunoblot (IB) of immune complex kinase assays. The lower portion shows the results of the cell cycle analysis.

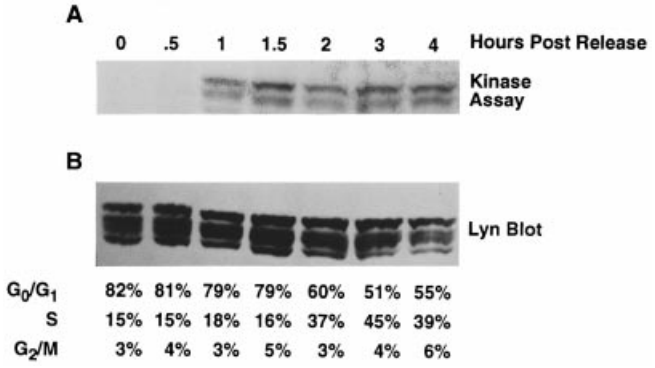

Figure 4 Lyn activation precedes the G_1/S transition

Mo7e cells were grown in SCF overnight and then treated for 16 h with SCF and 100 μM mimosine. To release them from the mimosine block, cells were washed twice and resuspended in culture medium supplemented with SCF (100 ng/ml). At the times indicated, samples were harvested for either cell cycle analysis or immune complex kinase assays. Kinase assays were performed with Lyn immunoprecipitated from equivalent protein amounts; samples were resolved with SDS/PAGE and transferred to Immobilon. (**A**) Autoradiography of immune complex kinase assays. (**B**) Lyn immunoblot of immune complex kinase assays. The lower portion shows the results of the cell cycle analysis.

5C). Equivalent amounts of Lyn were also observed in both Cdk2 immunoprecipitates shown in Figure 5(C). These findings demonstrate that Cdk2-associated Lyn is phosphorylated *in vitro* during early S-phase. To determine whether Lyn was a substrate for Cdk2 (a serine kinase), phosphoamino acid analysis was performed. Figure 5(E) demonstrates that Cdk2-associated Lyn was phosphorylated on serine and threonine residues. In contrast, autophosphorylated Lyn was phosphorylated predominantly on tyrosine residues (Figure 5E). These studies demonstrate that Lyn is associated with Cdk2 during late G_1 and early S-phase and that, *in vitro*, Cdk2-associated Lyn is phosphorylated on serine and threonine residues in early S-phase.

Next, we were interested in determining whether Cdk2-associated Lyn was phosphorylated on serine residues *in situ*

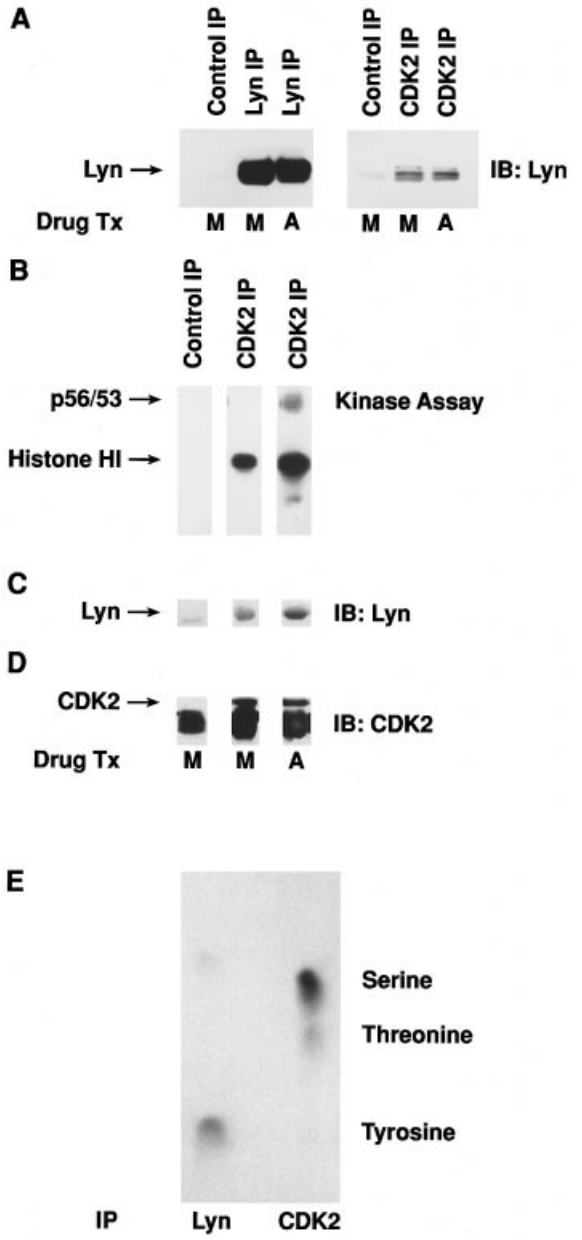

Figure 5 Lyn co-immunoprecipitates with Cdk2 and is phosphorylated during early S-phase

Mo7e cells were grown in SCF overnight, treated for 16 h with SCF and either mimosine (100 μM) or aphidicolin (0.5 μM) and then lysed. (**A**) Lyn co-immunoprecipitates with Cdk2. Immunoprecipitates (IP) were performed with equivalent protein amounts from lysates prepared from cells treated with either aphidicolin (A) or mimosine (M). Samples were resolved with SDS/PAGE, then transferred to Immobilon and immunoblotted (IB) with antibody specific for Lyn. (**B**) A phosphorylated doublet of 53 and 56 kDa co-precipitates with Cdk2 in cells treated with aphidicolin. Mo7e cells were treated with either mimosine or aphidicolin overnight. Cells were lysed and equivalent amounts of protein were immunoprecipitated with antisera specific for Cdk2 or control antisera. Immune complex kinase assays were performed; samples were resolved with SDS/PAGE and transferred to Immobilon. Radiolabelled phosphoproteins were detected by autoradiography. (**C**) Lyn-specific antibody recognizes the 56/53 kDa doublet. Antiserum specific for Lyn was used to immunoblot the immune complex kinase assay from (**B**). (**D**) Cdk2 immunoblot of kinase assays from (**B**). (**E**) Cdk2-associated Lyn is phosphorylated on serine and threonine residues. Phosphoamino acid analysis was performed on either autophosphorylated Lyn or Cdk2-associated Lyn as described in the Experimental section.

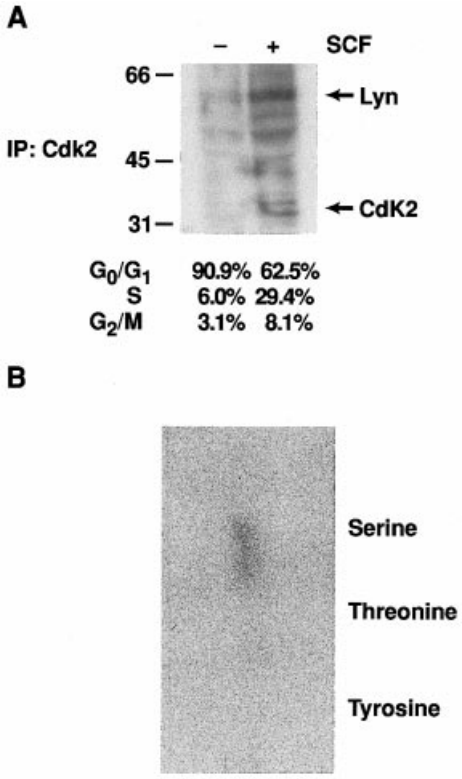

Figure 6 Cdk2-associated Lyn is phosphorylated on serine residues *in situ* during SCF-induced cell cycle progression

(**A**) Lyn is phosphorylated during S-phase. Mo7e cells were incubated for 16 h in the presence or absence of SCF and then radiolabelled with $^{32}P[P_i]$ as described in the Experimental section. Cells were lysed, lysates were clarified and antiserum specific for Cdk2 was used for immunoprecipitation. Immune complexes were resolved with SDS/PAGE and transferred to Immobilon; autoradiography was performed to detect radiolabelled proteins. The lower portion shows the results of the cell cycle analysis. (**B**) Cdk2-associated Lyn is phosphorylated on serine residues. Phosphoamino acid analysis was performed as described in the Experimental section.

during SCF-induced cell cycle progression. Cells were radiolabelled with $^{32}P[P_i]$ after incubation overnight in the presence or absence of SCF. Similar to findings *in vitro*, Cdk2-associated Lyn was heavily phosphorylated in cells progressing through the cell cycle after stimulation with SCF (Figure 6A). Further, phosphoamino acid analysis demonstrated that Lyn was phosphorylated on serine residues during cell cycle progression (Figure 6B). The lower portion of Figure 6(A) shows the results of cell cycle analysis performed in parallel with the radiolabelling study. After deprivation of SCF, 90.9% of the cells were arrested in G_0/G_1, 6% were in S-phase and 3.1% in G_2/M. In contrast, when cells had been cultured overnight in SCF, 62.5% were in G_0/G_1, 29.4% in S-phase and 8.1% in G_2/M.

To assess the functional significance of Lyn activation during SCF-induced cell cycle progression we examined the effect of inhibiting Lyn activity with PP1. To determine the specificity of this drug, we first examined the effect of PP1 on both Lyn and c-Kit kinase activity. Cells were incubated overnight in medium or PP1 at a concentration of either 1 or 10 μM and kinase assays were performed with cell lysates. Figure 7(A) demonstrates that PP1 impaired Lyn activity and had no effect on c-Kit activity. In contrast, PP1 had no effect on the protein levels of either Lyn or c-Kit (Figure 7A, lower panels).

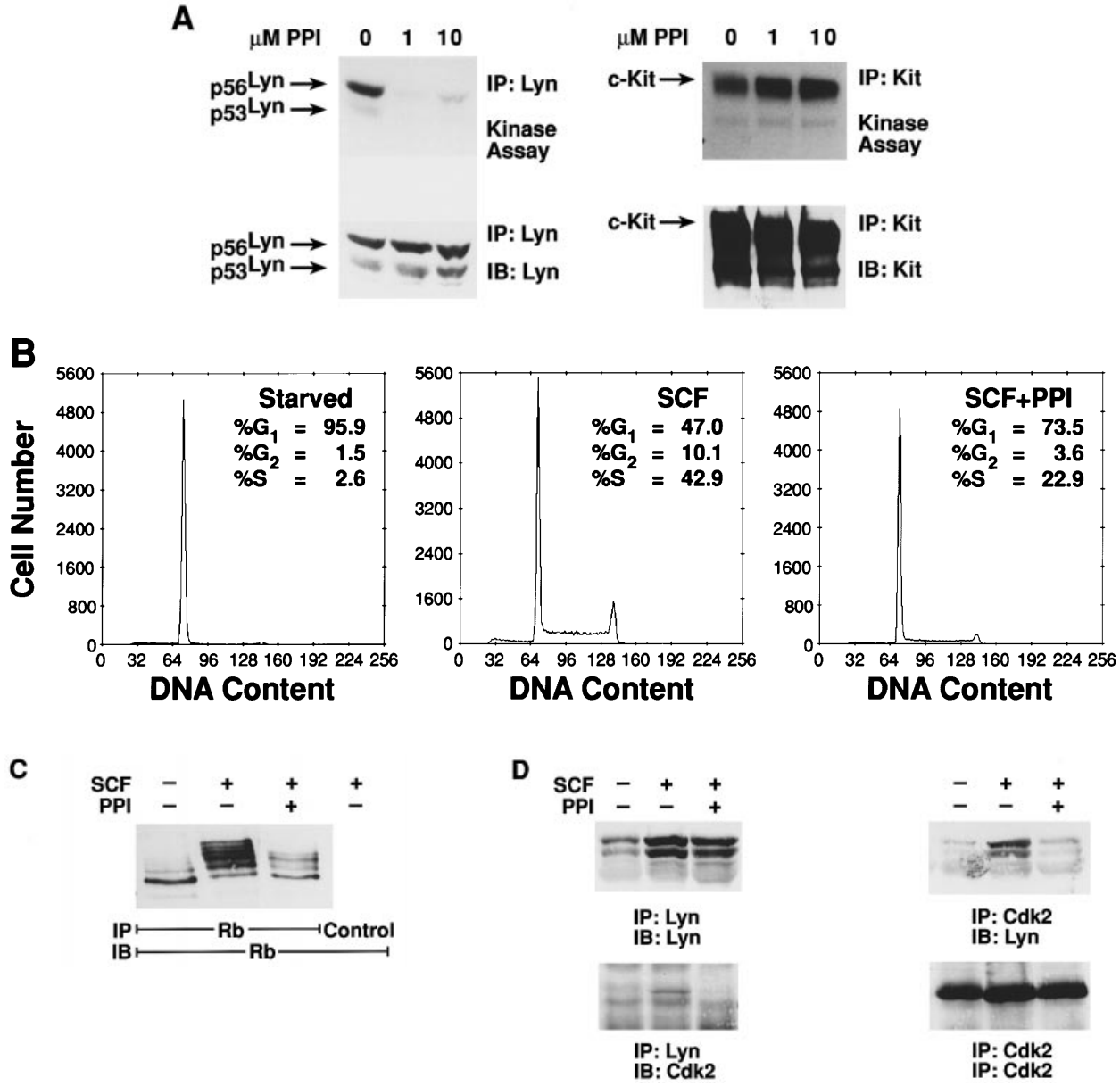

Figure 7 Inhibition of Lyn activity with PP1 reduces SCF-induced cell cycle progression

(**A**) PP1 inhibits the kinase activity of Lyn but not c-Kit. Mo7e cells were incubated overnight in the presence of the indicated concentrations of PP1. Cells were lysed and equivalent amounts of protein were immunoprecipitated with the indicated antibodies. Kinase assays on either c-Kit or Lyn immunoprecipitates were performed *in vitro*; samples were resolved with SDS/PAGE and transferred to Immobilon. Radiolabelled phosphoproteins were detected with autoradiography and are shown in the upper portion; the lower portion shows the results of immunoblotting with antibodies specific for either Lyn or c-Kit. (**B**) PP1 decreases the percentages of cells progressing through the cell cycle in response to SCF. Mo7e cells were incubated for 12 h with SCF. The cells were then incubated for 18 h in the absence of growth factor or with SCF in the presence or absence of PP1 (5 μM). Samples were then harvested for cell cycle analysis (results are shown in the top right of each panel) or lysates were prepared for analysis of Rb phosphorylation. (**C**) Inhibition of Lyn activity with PP1 decreases SCF-induced phosphorylation of Rb. Equivalent protein amounts of lysates from the experiment shown in (**B**) were immunoprecipitated (IP) with antibody specific for Rb or with an isotype-matched control, resolved with SDS-PAGE and transferred to Immobilon. Immunoblotting (IB) was performed with antiserum specific for Rb. (**D**) Treatment of cells with PP1 disrupts the association of Lyn and Cdk2. Lysates were prepared from cells grown in SCF in the presence or absence of PP1 (5 μM), as described in (**B**). Immunoprecipitations were performed with antiserum specific for either Lyn or Cdk2, as indicated. Immune complexes were resolved with SDS/PAGE and transferred to Immobilon. The upper portion of the Immobilon was immunoblotted with antiserum specific for Lyn and the lower portion was immunoblotted with antiserum specific for Cdk2.

To assess the effect of PP1 on SCF-induced cell cycle progression, Mo7e cells were cultured for 12 h with SCF in the presence or absence of PP1. Treatment with SCF resulted in 47% of the cells being in G_1, 42.9% in S-phase and 10.1% in G_2/M. The addition of PP1 and SCF resulted in 73.5% of the cells being in G_1, 22.9% in S-phase and 3.6% in G_2/M. The decrease in the percentages of cells reaching S-phase after treatment with PP1 suggests that Lyn activity has a role in the G_1/S transition during SCF-induced cell cycle progression.

Our previous studies have shown that Lyn is activated immediately after the treatment of cells with SCF. This corresponds to activation during either the G_0/G_1 transition or early G_1. The

present study demonstrates a second wave of Lyn activation hours after stimulation with SCF during middle to late G_1. To discern whether PP1 arrested cells in early or late G_1 we compared Rb phosphorylation in factor-deprived cells arrested in G_0/G_1, cells grown in SCF alone or cells grown in SCF and PP1. Rb is in a hypophosphorylated state during G_0/G_1 and its phosphorylation is increased as cells move through G_1 and the G_1/S transition. The hyperphosphorylated form of Rb migrates more slowly in an SDS/PAGE gel than the hypophosphorylated form. As expected, arrest of cells in G_0/G_1 through culture without SCF resulted in the rapidly migrating form of Rb (Figure 7C). Whereas growth for 12 h in SCF induced the slower-migrating form of Rb, SCF in the presence of PP1 resulted in an intermediate form of Rb that migrated between the hypophosphorylated and hyperphosphorylated forms found in starved or SCF-stimulated cells respectively (Figure 7C). These findings demonstrate that inhibition of Lyn activity increases the fraction of cells arrested in the middle of G_1.

One signalling component that is activated during G_1 and is critical for the G_1/S transition is Cdk2. Because we have found that Lyn and Cdk2 are associated in cells before and during the G_1/S transition (Figure 5), we examined the effect of PP1 on this interaction. The upper right panel of Figure 7(D) demonstrates that Lyn was co-immunoprecipitated with Cdk2 in cells arrested in G_0/G_1 through factor-deprivation. An increased amount of Lyn was also co-immunoprecipitated with Cdk2 during cell cycle progression. Inhibition of Lyn activity with PP1 decreased the amount of Cdk2-associated Lyn. The reciprocal experiment is shown in the bottom panel of Figure 7(D). Whereas Cdk2 was co-immunoprecipitated with Lyn during SCF-induced cell cycle progression, this association was disrupted after treatment of cells with PP1(Figure 7D, lower panel). These results demonstrate that the inhibition of Lyn activity by PP1 disrupts the Lyn/Cdk2 complex. Interestingly, this disruption is correlated with cell cycle arrest and suggests that the interaction of Lyn and Cdk2 might have a role in SCF-induced cell cycle progression.

DISCUSSION

We are interested in the signal transduction mechanisms of SCF, the ligand for the RTK c-Kit. Previously we found that the Src family member Lyn is highly expressed in SCF-responsive cell lines as well as normal haemopoietic progenitor cells. Treatment of haemopoietic cells with SCF induced increases in the tyrosine phosphorylation of Lyn and Lyn kinase activity within minutes of addition [19]. In the present study we examined Lyn kinase activity during the later events of SCF-induced cell cycle progression by using Mo7e cells.

Time-course studies comparing the kinetics of SCF-induced Lyn activation with cell cycle progression found that Lyn activity increased towards the middle of G_1 with activity peaking late in G_1 and maintained through the G_1/S transition (Figure 1). The activation of Lyn was also accompanied by two events critical for the G_1/S transition: increases in Cdk2 activity and the phosphorylation of Rb (Figure 2).

The drugs mimosine, aphidicolin and nocodazole have been used extensively to block cells at specific stages of the cell cycle [27–29]. We have shown that Lyn kinase activity is increased in cells arrested in early S-phase with aphidicolin in comparison with those arrested in either G_1 or M phase with mimosine and nocodazole respectively (Figure 3). When Mo7e cells were released from a mimosine block, increases in Lyn activity preceded the onset of S-phase by approximately 1 h (Figure 4). Taken together, these results demonstrate that the onset of S-phase is preceded by increases in Lyn activity.

Several lines of evidence have suggested that Src family members have a role in cell cycle progression induced by ligands interacting with RTKs in fibroblasts. Microinjection of fibroblasts with antiserum that impairs Src-family kinase activity inhibited DNA synthesis in response to PDGF, CSF-1 and EGF. Similarly, microinjection of kinase inactive mutants of Src, Fyn and Yes also impaired PDGF-, CSF-1- and EGF-induced S-phase in fibroblasts [17,18]. Interestingly, the SH3 domain of Src family members is required for fibroblast proliferation in response to RTK ligands [31,32]. We have shown previously that treatment of cells with either PP1 or anti-sense oligonucleotides specific for Lyn inhibits SCF-induced proliferation [19]. The present study demonstrates that the inhibition of Lyn activity with PP1 decreases the percentages of cells that enter S-phase in response to SCF (Figures 7A and 7B). Phosphorylation of Rb in cells grown in both SCF and PP1 was greater than that observed in cells arrested in G_0/G_1 and less than cells grown in SCF alone (Figure 7C). This suggests that the inhibition of Lyn activity increases the number of cells in the middle of G_1. It is important to note that PP1 does inhibit Src family members other than Lyn. Therefore, although Lyn is the most highly expressed Src family member in Mo7e cells, PP1 might have effects on SCF-induced cell cycle progression that are independent of Lyn inhibition [19].

The most extensive studies of the interaction of Src family members with cell cycle components have been with c-Src. c-Src associates with Cdc2 during mitosis and is phosphorylated on serine and threonine residues early in M-phase [33,34]. This is followed by the dephosphorylation of Tyr-527 and increases in c-Src catalytic activity [35]. Although both of these events have a role in optimal mitotic activation of c-Src, Cdc2-mediated phosphorylation of c-Src is not required for activation during mitosis. Lck, Lyn and Fyn have also been reported to associate with Cdc2 during mitosis of factor-independent T-cell lines [36]. Although we noted increases in Lyn autophosphorylation activity after arresting cells in early mitosis with nocodazole, maximal Lyn activity was observed late in G_1 and through the G_1/S transition (Figures 1, 3 and 4).

Several groups have reported that Lyn associates with both Cdk2 and Cdc2 in response to ionizing radiation or chemotherapeutic agents [30,37–39]. Interestingly, in this context, the Lyn-mediated phosphorylation of Tyr-15 of Cdk2 and Cdc2 decreased the activity of both of these kinases [30,38,39]. These studies suggest that Lyn has a role in arresting cells at the G_1/S and G_2/M checkpoints in response to specific genotoxins. Our work demonstrates the association of Lyn and Cdk2 during SCF-induced cell cycle progression (Figures 5–7). These findings suggest a regulatory loop between these two protein kinases during late G_1 and the G_1/S transition. In support of this possibility, Cdk2-associated Lyn is heavily phosphorylated on serine and threonine residues both *in vitro* and *in situ* during cell cycle progression (Figures 5E and 6). In addition, inhibition of Lyn activity with PP1 disrupts the association of Cdk2 and Lyn; this is correlated with an increased number of cells arrested in the middle of G_1 and fewer cells reaching S-phase (Figure 7D). Although Cdk2-mediated phosphorylation of Lyn might have a role in the activation of Lyn during SCF-induced cell cycle progression, as with c-Src, other signalling components are probably involved.

These results, in conjunction with previous work, suggest that Lyn is a component of signal transduction pathways mediating normal growth responses and can be appropriated by stress pathways mediating cell cycle arrest at critical checkpoints. Genotoxic agents such as UV irradiation have previously been shown to activate signal transduction components involved in proliferation, including RTKs and Src family members [40,41].

Further, a number of haemopoietic growth factors have also been shown to activate stress-activated protein kinases such as p38 mitogen-activated protein kinase and c-Jun N-terminal kinases [stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK)] [42,43]. These findings suggest bi-directional cross-talk between signal transduction pathways leading to growth and stress responses.

In summary, our studies have demonstrated that Lyn is activated during SCF-induced cell cycle progression. Peak increases in Lyn activity were observed late in G_1 and through the G_1/S transition. Lyn was associated with Cdk2 and was phosphorylated on serine and threonine residues early in S-phase both *in vitro* and *in situ*. Inhibition of Lyn activity with PP1 increased the fraction of cells in mid-G_1 and decreased the number of cells that reached S-phase. These findings demonstrate a strong correlation between activation of Lyn, association with Cdk2 and SCF-induced cell cycle progression. Studies addressing the role of Lyn in cell cycle progression are currently in progress.

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