**ABSTRACT**

At least 70% of small cell lung cancers (SCLCs) express the Kit receptor tyrosine kinase and its ligand, stem cell factor (SCF). In an effort to define the signal transduction pathways activated by Kit in SCLC, we focused on Src family kinases and, in particular, Lck, a Src-related tyrosine kinase that is expressed in hematopoietic cells and certain tumors, including SCLC. SCF treatment of the H526 cell line induced a physical association between Kit and Lck that, in vitro, was dependent on phosphorylation of the juxtamembrane domain of Kit. Stimulation of Kit with recombinant SCF resulted in a rapid 3- to 6-fold increase in the specific activity of Lck, which was similar in magnitude to the activation of Lek resulting from the cross-linking of the T-cell receptor complex of Jurkat cells. Lck activity peaked by 5 min after SCF addition, and the elevated activity persisted for at least 30 min in the presence of SCF, with kinetics similar to the activation of mitogen-activated protein kinase. PP1, an inhibitor of Src family kinases with selectivity for Lck, completely inhibited SCF-mediated growth but had little effect on insulin-like growth factor-1-mediated growth. PP1 antagonized both SCF-mediated proliferation and inhibition of apoptosis. PP1 had no effect on Kit kinase activity but was shown to block total Lck activity by at least 90% by immune complex kinase assay. Low levels of Src, Hck, and Yes were also expressed in the H526 cell line; only Yes showed a consistent increase in specific activity, which was also inhibited by PP1 following SCF treatment. These data demonstrate that, in the H526 SCLC cell line, Lck and, possibly, Yes are downstream of Kit in a signal transduction pathway; the inhibition by PP1 of SCF-mediated proliferation and inhibition of apoptosis suggests that Src family kinases are intermediates in the signaling pathways that regulate these processes.

**INTRODUCTION**

The c-kit gene encodes a tyrosine kinase growth factor receptor in the same subclass as the receptors for PDGF and CSF-1 (1). Kit's ligand is SCF (alternatively named mast cell growth factor or stem factor), a hemopoietic growth factor that, in conjunction with other hemopoietic growth factors, supports the proliferation and differentiation of multiple hemopoietic cell lineages from early precursors (2). In addition to hemopoietic cells, Kit is also normally expressed in melanocytes and germ cells, as well as a variety of solid tumors, including SCLC. In fact, over 70% of SCLC cell lines and tumor specimens coexpress Kit and its ligand, SCF (3-5), leading to the hypothesis that this coexpression constitutes an autocrine growth loop. Evidence for such an autocrine loop includes the observation that growth of selected SCLC cell lines is stimulated by exogenous SCF (6, 7). We have also recently demonstrated that reconstitution of SCF and Kit coexpression in a SCLC cell line leads to enhanced growth factor independence and that inhibition of Kit activation, using either a selective tyrosine kinase inhibitor or transfection of a dominant-negative c-kit gene, results in a loss of growth factor independence of several SCLC cell lines (7, 8), confirming that coexpression results in a functional autocrine loop. Because signal transduction initiated by Kit stimulation appears to play an important role in the growth factor independence of this cancer, we have been interested in defining downstream signaling events, particularly the role of Src family kinases in this process.

Although it has been known for many years that activation of Src family members occurs in a variety of human tumors, including SCLC (9), the role of Src family members in signaling from a variety of growth factor receptor tyrosine kinases has only recently been elucidated. In murine fibroblasts expressing the PDGFR or the CSF-1R, interaction with their respective ligands results in Src's physical association with the activated receptors and an increase in Src's specific activity (10, 11). The physical association between Src and the βPDGFR is mediated through the interaction of the SH2 domain and the sequences surrounding phosphorylated tyrosines 579 and 581 within the juxtamembrane domain of the βPDGFR (12). Microinjected anti-Src antibody or plasmids encoding a dominant inhibitory Src mutant block DNA synthesis mediated by PDGFR and CSF-1R stimulation, strongly suggesting that Src is an obligatory participant in the PDGFR and CSF-1 mitogenic pathway (13). Evidence also exists that Src activation enhances epidermal growth factor-mediated mitogenesis and that there is a physical association between Src and the EGFR following stimulation by epidermal growth factor (14, 15).

The Src-related nonreceptor protein tyrosine kinase Lck plays a critical role in the events leading to T lymphocyte activation. Through its interaction with the CD4 and CD8 coreceptor glycoproteins, Lck initiates signal transduction following engagement of the TCR complex (16, 17). Mice deficient in Lck are markedly depleted of T cells, and those T cells that do develop show a blunted response to TCR stimulation (18). Lck has also been shown to bind to the IL-2 receptor complex in T cells and participate in IL-2-mediated signaling (19). Although normal lck expression appears to be tightly restricted to lymphoid cells, some nonlymphoid tumor cells, including SCLC, express the gene (20). We felt that coexpression of the relatively hemopoietic-specific genes c-kit and lck in SCLC was intriguing and, perhaps, not purely coincidental. On the basis of the previously described interactions between the PDGFR and Src and their respective structural similarities to Kit and Lck, we undertook this study to determine whether Lck plays a role in Kit signal transduction in SCLC.

**MATERIALS AND METHODS**

**Cell Growth.** SCLC cell lines and Jurkat cells were grown in RPMI 1640 supplemented with 2 mM L-glutamine, with or without 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD); when cells were grown in the absence of serum, 0.1% BSA (Sigma Chemical Co., St. Louis, MO) was added...
to the medium. Where indicated, serum-free medium was supplemented with recombinant SCF (Intergen, Purchase, NY) or IGF-I (R&D, Minneapolis, MN) at the indicated concentrations. Cells were stimulated with SCF and IGF-I following preincubation in serum-free medium overnight. Cross-linking of the TCR on Jurkat cells was accomplished by preincubation with a monoclonal anti-CD3 antibody (Calbiochem, San Diego, CA) for 30 min at 4°C, followed by incubation with rabbit antimmunoglobulin IgG (Jackson Laboratories, West Grove, PA) for 5 min at 37°C. Cell growth was measured using the MTT (Sigma) colorimetric dye reduction method, an assay shown to correlate very well with viable SCLC cell number under the conditions used (21). Duplicate plates containing eight replicate wells per assay condition were seeded at a density of 1 × 10^4 cells in 0.1 ml of medium, and data were expressed as the change in absorbance at 540 nm over 72 h, relative to initial values obtained 3 h after plating. The selective tyrosine kinase inhibitor PP1 (Ref. 22; Calbiochem) was solubilized in DMSO; final concentration of DMSO in all cultures, including controls, was 0.1% in experiments using PP1. Trinitiated thymidine incorporation was measured after incubation in triplicate of 10^5 cells in 1 ml of serum-free medium containing 1 μCi/ml [3H]thymidine for 12 h. Cells were collected on glass fiber filters, extensively washed with cold 10% trichloroacetic acid, and counted in a liquid scintillation counter.

**IPs and Western Blotting.** K45 (NeoMarkers, Fremont, CA), YBS.B8 (PharMingen, San Diego, CA), and 3D9 (Boehringer-Mannheim, Indianapolis, IN) anti-Kit monoclonal antibodies directed against the extracellular domain were used for IP interchangeably, with approximately equal efficacy. For Lck IPs, rabbit polyclonal antisera directed against the NH2 terminus of Lck was purchased from PharMingen. For Hck and Yes IPs, rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); for Src, monoclonal clone 327 was obtained from Calbiochem. The IP procedure began with lysis of SCLC cells in a buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol, 0.2 mM NaVO4, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 10 μg/ml leupeptin using a Dounce homogenizer with a tight-fitting pestle; protein concentrations were determined by biocinchoninic acid assay (Pierce). The lysate, containing 1–5 mg of protein, was centrifuged for 10 min at 10,000 × g to obtain a soluble postnuclear supernatant. IP was initiated by addition of 10 μg of the specific antibody, followed by incubation for 2 h at 4°C and by an additional 2 h in the presence of protein A+G agarose. The IP was washed four times in lysis buffer and once in PBS and boiled in an equal volume of 2× SDS gel loading buffer. Western blotting was performed using standard procedures, with detection using the enhanced chemiluminescence system (Amersham, Arlington Heights, IL). Primary detection antibodies were as follows: rabbit polyclonal anti-Kit and monoclonal anti-Src (Calbiochem); monoclonal anti-N-terminal Lck, monoclonal anti-Hck, and affinity-purified rabbit polyclonal anti-Yes (Transduction Laboratories, Lexington, KY); and rabbit polyclonal anti-Erk 1 (Upstate Biotechnology, Inc., Saranac Lake, NY).

**Immune Complex Kinase Assays.** IPs were performed as above. The K45 monoclonal antibody was used for Kit kinase assays; the PharMingen rabbit polyclonal was used for Lck assays. After the protein A+G beads were washed with PBS, the pellet was aspirated to dryness, and 30 μl of kinase buffer (20 mM PIPES and 10 mM MnCl2) containing 10 μCi of (γ-32P)ATP was added. Twenty μg of acid denatured rabbit enolase (Sigma) were added to assays designed to measure phosphorylation of exogenous substrate. Reactions were carried out at room temperature for 2–5 min; longer incubations resulted in higher background. The reaction was terminated by addition of an equal volume of 2× SDS sample loading buffer and resolved on a 10% polyacrylamide gel. For calculation of Lck specific activity, two-thirds of the immunoprecipitate was analyzed by Western blotting, and one-third was used for kinase gel and the Western blot were analyzed by densitometry, and the kinase activity was precipitated. Lck specific activity in H526 increased following SCF stimulation to a similar degree to that seen after cross-linking the TCR of Jurkat cells.

**RESULTS**

**SCF Stimulation Results in a Rapid Increase in Lck-specific Activity.** To determine whether stimulation of Kit resulted in an alteration in the activity of Lck, H526 cells were stimulated with SCF for 5 min, and a Lck immune complex kinase assay was performed. For comparison, a Lck immune complex kinase assay was also performed after cross-linking the TCR complex of Jurkat cells by incubating with a monoclonal anti-CD3 antibody followed by rabbit antimmunoglobulin IgG. Fig. 1 illustrates that the degree of Lck activation induced by treating H526 cells with SCF was comparable to that of Lck from Jurkat cells.
induced by cross-linking of the TCR complex of Jurkat cells. The maximum increase in Lck specific activity in five independent experiments ranged between 3- and 6-fold, similar to the increase in Lck-specific activity reported previously after cross-linking the TCR (25). To determine whether SCF stimulation induced the physical association of Kit and Lck, H526 cells were stimulated with SCF for 5 min followed by Lck IP. The immunoprecipitate was then probed for the presence of Kit. As seen in Fig. 2, the amount of Kit that coprecipitated with Lck increased upon SCF stimulation. To confirm this finding, IP with Kit antibodies and detection with Lck antibodies was attempted, but cross-reaction of the secondary antibody with the heavy chain of the IP antibody, which comigrates with Lck (see Fig. 1), prevented us from drawing any conclusions using this approach, so we used an alternative one. It has been shown that Src associates with the βPDGFR via interactions between its SH2 domain and phosphotyrosines 579 and 581 in the juxtamembrane domain of the receptor (12). It has also been shown that the homologous region of Kit, including the conserved phosphotyrosines 568 and 570, specifically binds the Src family kinase Lyn, whereas other isolated cytoplasmic domains do not (23). We, therefore, determined whether Lck from H526 lysates would associate with either unphosphorylated or tyrosine-phosphorylated GST fusion proteins encoding the Kit juxtamembrane domain; the tyrosine phosphorylated fusion protein was produced in a bacterial strain containing an active tyrosine kinase. As demonstrated in Fig. 3, Lck specifically associates with the tyrosine-phosphorylated juxtamembrane domain of Kit. Thus, we have demonstrated that in vivo Lck associates with Kit in a ligand-dependent manner and in vitro the Kit juxtamembrane domain associates with Lck in a tyrosine phosphorylation-dependent manner. To determine the time frame over which Lck activation occurred, Lck immune complex assays were performed at successive intervals after SCF stimulation. Both autophosphorylation and phosphorylation of an exogenous substrate, rabbit muscle enolase, were measured in immune complex kinase assays. Fig. 4 demonstrates that Lck kinase activity began to rise within 1 min of SCF stimulation, peaked at 5 min after stimulation, and remained elevated for at least 30 min. To provide an internal standard for the progress of SCF-mediated signal transduction, activation of MAPK (within the same lysates used for

**Fig. 2.** Kit immunoprecipitated with Lck in a ligand-dependent manner. Top, Lck IP before and after stimulation of H526 with SCF immunoblotted with an anti-Kit antibody. Bottom, the same blot stained with an anti-Lck antibody. Cells were preincubated in serum-free medium overnight, split into two equal portions, one of which was stimulated with 100 ng/ml SCF for 5 min prior to IP. Control experiments using nonimmune rabbit IgG (data not shown) demonstrated a low level of nonspecific Kit adherence to the protein A + G beads, equivalent to that seen in NS cells (left). Therefore, although SCF enhances the association between Kit and Lek, we were not been able to determine whether or not a stable association exists prior to SCF treatment of the H526 cell line. These data are representative of four independent experiments.

**Fig. 3.** Lck associates with the tyrosine-phosphorylated juxtamembrane region of Kit. A. tyrosine-phosphorylated GST fusion protein encoding the Kit juxtamembrane region coprecipitated Lck from H526 lysates. H526 cells (2 × 10⁶) were lysed and clarified, and coprecipitations were performed for 3 h at 4°C. Coprecipitations were performed with either GST (GST Control) or the Kit juxtamembrane fusion protein (Juxta-GST), isolated from either BL21 (unphosphorylated) or TKX1 (tyrosine-phosphorylated) bacteria bound to glutathione-Sepharose. Proteins were resolved by SDS-PAGE, transferred to Immobilon, and immunoblotted (IB) with polyclonal antibody specific for GST. GST Fusion Protein arrow, phosphorylated and unphosphorylated Kit juxtamembrane fusion proteins. B. the blot in A was stripped and reprobed with polyclonal antibody specific for GST. GST Fusion Protein arrow, phosphorylated and unphosphorylated Kit juxtamembrane fusion proteins.
block its activation following SCF stimulation of H526 cells. The relative change in viable cell number over 72 h in the absence of added growth factors (NS) and in the presence of SCF and IGF-I was measured by MTT assay. IGF-I was chosen as a control because it is a ligand for a receptor tyrosine kinase, to which SCLC cells generally respond (26). PPl does show limited activity against the EGFR in vitro (IC50 was 50-fold greater than that for Lck; Ref. 22); however, SCLC cells, in general (27), and H526, in particular (data not shown), do not express the EGFR and, therefore, we could not use this receptor as a specificity control. Fig. 5 illustrates that 10 μM PPl completely blocked SCF-mediated growth, as well as autocrine growth of cells in the absence of growth factors, whereas IGF-I-mediated growth was only minimally affected at the identical concentration. The effect of PPl on IGF-I-mediated growth may be entirely accounted for by inhibition of basal autocrine growth because autocrine growth of the H526 cell line was also eliminated by direct inhibition of Kit kinase activity (7), suggesting that it is mediated by low-level endogenous SCF expression. Increasing the concentration of PPl to 25 μM resulted in significant cell death (P < 0.01) of cells grown in SCF, but no further significant change in the growth of the IGF-I treated cells, suggesting that the drug is not acting as a nonspecific toxin.

The accumulation of viable cells measured by MTT assay is determined by both the rate of cell proliferation and the rate of apoptosis. SCF enhances proliferation and inhibits apoptosis of SCLC in the absence of other growth factors (7, 8). To determine whether PPl antagonized both these effects, apoptosis and proliferation were assayed independently. Morphological evidence of apoptosis (see below) became apparent in cultures between 36 and 48 h after addition of PPl. To measure proliferation independently from apoptosis [3H]thymidine incorporation was determined during the first 18 h of culture in SCF and either DMSO vehicle or 25 μM PPl (Fig. 6). PPl eliminated (P < 0.05) SCF-stimulated [3H]thymidine incorporation.

**PP1 Selectively Blocks SCF-stimulated Growth.** To determine the functional significance of Lck activation, PP1 (22), a specific inhibitor of Src family kinases with selectivity for Lck, was used to determine Lck specific activity) was analyzed by gel shift criteria. Subsequent experiments have demonstrated that the relative level of MAPK activity measured by gel shift correlates well with relative activity measured using activation-specific antibodies or immune complex kinase assay. The kinetics of Lck activation were identical to those of MAPK activation, as demonstrated by the mobility shift of Erk 1 illustrated in Fig. 4C. Lck activity began to decrease between 30–60 min after SCF addition and was back to baseline activity at 24 h after addition (data not shown).

**PP1 Selectively Blocks SCF-stimulated Growth.** To determine the functional significance of Lck activation, PP1 (22), a specific inhibitor of Src family kinases with selectivity for Lck, was used to
INHIBITION OF SCLC GROWTH BY PPI

Fig. S. Morphological appearance of H526 cells exposed to 25 μM PPI. Cells were seeded at 10^5 cells/ml and grown for 48 h in serum-free medium containing 100 ng/ml SCF (A and B) or 20 ng/ml IGF-I (C and D) in the presence of DMSO vehicle (A and C) or 25 μM PPI (B and D) and photographed using phase contrast optics (×200). Note not only the markedly decreased cell number in the SCF- and PPI-treated culture (B) but also the cell shrinkage and membrane blebbing suggestive of apoptosis. Also note the enhanced cellular aggregation in the IGF-I- and PPI-treated culture (D).

Incorporation by PPI-treated cultures was less than that of NS control cultures, consistent with a role for endogenous SCF in promoting autocrine growth of this cell line. To determine whether PPI enhanced apoptosis at latter times, TUNEL assays were performed on cells grown in SCF for 48 h with either 25 μM PPI or vehicle alone. Nuclear labeling was confirmed by fluorescence microscopy and quantitated by flow cytometry. Fig. 7 illustrates a marked increase in fluorescence of cells incubated with PPI, indicating enhanced apoptosis. Thus, PPI antagonizes both the proliferative and antiapoptotic effects of SCF.

The morphological appearance of H526 cells treated with 25 μM PPI is illustrated in Fig. 8. Cultures stimulated with SCF and IGF-I in the absence of PPI had an identical appearance consisting of loose three dimensional branching aggregates, previously defined as “variant” SCLC morphology (28). Cultures treated with SCF and PPI showed not only a marked decrease in cell number but also cell shrinkage and membrane blebbing, indicative of apoptosis. Similar morphological changes were obtained when cells were incubated in the presence of SCF and a direct Kit kinase inhibitor (7). Cultures treated with IGF-I and PPI did not show an increase in apoptotic morphology but instead showed a condensation of the loose cellular aggregates into tight spheroids, reminiscent of “classic” SCLC morphology (28). This latter observation suggests that, in addition to playing a role in SCF-mediated proliferation and inhibition of apoptosis, Src family kinases may also regulate adherence of these cells.

PPI Inhibits Lck and Yes but not Kit Activation. To confirm the inhibitor’s specificity for Src family kinases, Lck immune complex kinase assays were performed following SCF stimulation in the presence and absence of PPI. Following SCF stimulation in the presence of PPI, Lck specific activity was reduced to 5–10% of the control (Fig. 9, A and B). It should be noted that this represents a minimum estimate for Lck inhibition in vivo because the inhibitor was only

Fig. 9. PP1 blocked Lck but not Kit activation. H526 cells were incubated in serum-free medium overnight and stimulated for 5 min with 100 ng/ml SCF after a 30-min preincubation with DMSO vehicle or 10 μM PP1. Lck was then immunoprecipitated and an immune complex kinase assay (A) and Western blot (B) were performed. Lck activity was reduced to 5–10% of the vehicle control by PP1. To determine whether Kit activity was affected by the drug, Kit was immunoprecipitated following SCF stimulation in the presence or absence of drug and an immune complex kinase assay (C) and Western blot (D) were performed. Kit specific activity was not affected. These data are representative of two independent experiments.
Inhibition of SCLC growth by PPI

Table 1: Kinase activity assays for Hck, Src, and Yes.

<table>
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<tr>
<th>Kinase</th>
<th>NS</th>
<th>SCF</th>
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<tr>
<td>Hck</td>
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<td>Src</td>
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<td>Yes</td>
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In the presence of DMSO vehicle or 10 μM PPI (Fig. 10), the effects of PPI for anti-CD3 cross-linking of the TCR was 0.5 μM, for the specific inhibitor PPI. Hanke et al. (22) demonstrated that the IC50 of activity is likely to be necessary for SCF-mediated growth using the stimulation of the H526 cell line, we demonstrated that Lek activity, was unaffected. More importantly, we demonstrated that, among these, only Yes showed an increase in activity following SCF treatment that could be inhibited by PPI. Although it is difficult to compare the relative amounts of different proteins detected with antibodies having different affinities, we estimate that these other Src family kinases are expressed at a maximum of 10–20% the level of Lck in the H526 cell line. Thus, although Yes activation may play a role in SCF-mediated growth, based on a strictly quantitative argument, it is likely that Lck plays the major role. It is also important to point out that, although we did not see Src or Hck activation following SCF stimulation of the H526 cell line, we cannot conclude that these kinases are not capable of being stimulated by Kit. The lack of any increase in specific activity could have been due to the low levels of protein expression in the H526 cell line. For instance, if these kinases were engaged in high affinity interactions with proteins other than Kit, there may not have been any excess to interact with Kit following SCF treatment. In fact, we have noted a parallel increase in both Lck and Src specific activity after SCF treatment of platelets.5 Other SCLC cell lines do express high levels of activated Src (9), and in some, Lck expression is very low or nonexistent (20). Considering the high prevalence of the SCF/Kit autocrine loop, it is possible, perhaps likely, that, in other SCLC cell lines, Kit mediates activation of Src. However, because H526 is the only cell line in our possession that responds to exogenous SCF, allowing us to precisely correlate Kit and Lck activation, we must limit our conclusions to this cell line.

To confirm the specificity of PPI in the H526 cell line, we demonstrated that Lck activity was markedly inhibited by PPI, whereas Kit activity was unaffected. More importantly, we demonstrated that IGF-I-mediated growth was only minimally affected by 10 μM PPI, which inhibited SCF-mediated growth completely. Furthermore, increasing the dose of PPI beyond 10 μM had no significant effect on IGF-I-mediated growth, making it unlikely that the drug was functioning as a nonspecific toxin. We hypothesize that the inhibition of growth by PPI in the presence of IGF-1 is, in large part, due to inhibition of the endogenous SCF/Kit autocrine loop for two reasons. First, the extent of growth inhibition by PPI in the presence of IGF-I is approximately equivalent to the growth inhibition induced by PPI in the absence of growth factors (Fig. 5). Second, the selective Kit inhibitor AG1296 had the same effect on growth in the presence and absence of IGF-I; that is, AG1296 also inhibited IGF-I-mediated growth to the same extent it inhibited growth in the absence of growth factors (7). These observations suggest that growth mediated by the SCF/Kit autocrine loop and IGF-I are additive and that inhibition of

Lly. present prior to cell lysis and not during the IP, washing, or kinase assay, during which time the effect of the inhibitor could have been attenuated. To make certain that the inhibitor did not directly affect Kit activity, parallel Kit immune complex kinase assays was carried out following SCF stimulation in the presence and absence of PPI. Kit activity, as measured by autophosphorylation, was not affected by the presence of the inhibitor (Fig. 9, C and D). Thus, the inhibition of SCF-mediated growth by PPI is not a result of any direct effect on Kit.

In addition to Lck, low levels of Src itself, as well as Hck and Yes, are also expressed in the H526 cell line. Immune complex kinase assays on these family members were performed before and after SCF stimulation in the presence of DMSO vehicle or 10 μM PPI (Fig. 10). Only Yes showed a consistent increase in specific activity after SCF treatment. This increase in specific activity was also inhibited by PPI, although not to the same extent as that seen for Lck. However, it is again important to point out that the in vitro assay of relative specific activity may underestimate the degree of inhibition in vivo because of the prolonged absence of drug during the IP and washing.

Discussion

Several important conclusions can be drawn from the above data. This is the first demonstration of a rapid increase in Lck specific activity following stimulation of a receptor tyrosine kinase. The degree of SCF-mediated activation is comparable to that produced in T-cells after engagement of the TCR, strongly suggesting it is biologically relevant. This increase in Lck specific activity is temporally related to a ligand-dependent physical association between Lck and Kit. This physical association appears to be mediated through tyrosine phosphorylation of the juxtamembrane domain of Kit containing tyrosines 568 and 570, which are homologous to phosphotyrosines 579 and 581 of the βPDGFR, which constitute the Src binding site (12). Recently, it has been demonstrated that the Src family kinase Lyn is activated by Kit in hemopoietic cells and that the SH2 domain of Lyn binds to the juxtamembrane tyrosine residues of Kit in a ligand-dependent manner (23). Taken together, these data suggest a strong conservation of the interaction between the juxtamembrane domain of this class of receptor tyrosine kinases and the Src family, further suggesting an important physiological role for this interaction.

In addition to demonstrating that Lck was activated following SCF stimulation of the H526 SCLC cell line, we demonstrated that Lck activity is likely to be necessary for SCF-mediated growth using the specific inhibitor PPI. Hanke et al. (22) demonstrated that the IC50 of PPI for anti-CD3 cross-linking of the TCR was 0.5 μM; for the physiologically mediated T-cell proliferative responses induced following antigen stimulation and the mixed lymphocyte reaction, IC50s were on the order of 4–5 μM. Although we did not determine an IC50, because we observed slight inhibition of SCF-mediated proliferation at 1 μM and complete inhibition at 10 μM (Fig. 5), the effects of PPI on SCF-mediated proliferation appear comparable to those on physiological Lck-dependent proliferation of T cells. Although it was demonstrated that in vitro PPI had some selectivity for Lck and Fyn, other Src family kinases were also inhibited at higher concentrations (22). Fyn expression was not detected by Western blot in the H526 cell line (data not shown), but low-level expression of Src, Hck, and Yes was detected. Immune complex kinase assays demonstrated that, among these, only Yes showed an increase in activity following SCF treatment that could be inhibited by PPI. Although it is difficult to compare the relative amounts of different proteins detected with antibodies having different affinities, we estimate that these other Src family kinases are expressed at a maximum of 10–20% the level of Lck in the H526 cell line. Thus, although Yes activation may play a role in SCF-mediated growth, based on a strictly quantitative argument, it is likely that Lck plays the major role. It is also important to point out that, although we did not see Src or Hck activation following SCF stimulation of the H526 cell line, we cannot conclude that these kinases are not capable of being stimulated by Kit. The lack of any increase in specific activity could have been due to the low levels of protein expression in the H526 cell line. For instance, if these kinases were engaged in high affinity interactions with proteins other than Kit, there may not have been any excess to interact with Kit following SCF treatment. In fact, we have noted a parallel increase in both Lck and Src specific activity after SCF treatment of platelets.5 Other SCLC cell lines do express high levels of activated Src (9), and in some, Lck expression is very low or nonexistent (20). Considering the high prevalence of the SCF/Kit autocrine loop, it is possible, perhaps likely, that, in other SCLC cell lines, Kit mediates activation of Src. However, because H526 is the only cell line in our possession that responds to exogenous SCF, allowing us to precisely correlate Kit and Lck activation, we must limit our conclusions to this cell line.

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5 G. W. Krystal and E. N. Dessypris, unpublished observations.

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Kit signal transduction in the presence of IGF-I decreases total growth by the contribution of Kit activation. Although the present data do not prove this hypothesis, the same phenomenon observed with two inhibitors of Kit signal transduction having different mechanisms of action makes it less likely that the effects on growth in the presence of IGF-I are due to nonspecific toxic effects. However, we cannot completely rule out the possibility that PPI inhibits SCF and IGF-I-mediated growth by inhibiting enzymes in addition to the Src family kinases.

The precisely role of Lck in the mitogenic and antiapoptotic signal transduction pathways in SCLC remains to be determined. It has been demonstrated in numerous studies using hemopoietic cells and transfected fibroblasts that Kit activates the Ras-MAPK cascade (2), and we have confirmed this in SCLC cells. The virtually identical time course of MAPK and Lek activation suggests that Lck could be involved in MAPK activation. However, it has recently been demonstrated that one of the critical roles for Src in PDGFR and CSF-1R signaling is the activation of Myc expression. Inhibition of PDGFR or CSF-1R-mediated DNA synthesis by a dominant inhibitory allele of Src or by a COOH-terminal antibody could be prevented by ectopic expression of Myc (29). It was also demonstrated that activation of AP-1 transcriptional activity, accomplished through the Ras-MAPK cascade, was also required for PDGF- and CSF-1R-mediated DNA synthesis. Thus, for mitogenic stimulation of fibroblasts by PDGFR or CSF-1, activation of apparently parallel Ras-MAPK and Src-Myc signaling pathways is required. The present study demonstrates that, analogous to its closely related receptors, Kit can signal through the Src family member Lck. Eisenman and Cooper (30) have speculated that Src family kinases may activate Myc expression by phosphorylating proteins involved in the regulation of transcriptional elongation, which is a major mechanism by which Myc expression is regulated by growth factor stimulation (31). Myc family amplification and/or overexpression is a universal feature of SCLC biology (27), and we have previously demonstrated that loss of the block to transcriptional elongation is responsible for enhanced Myc family expression in SCLC cell lines lacking gene amplification (32). Thus, activation of Lck and other Src family kinases could provide a link between autocrine stimulation of Kit and altered Myc expression.

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