Src Family Kinase Activity Is Required for Signal Transducer and Activator of Transcription 3 and Focal Adhesion Kinase Phosphorylation and Vascular Endothelial Growth Factor Signaling in Vivo and for Anchorage-dependent and -independent Growth of Human Tumor Cells

A. Douglas Laird,1 Guangmin Li, Katherine G. Moss, Robert A. Blake, Martin A. Broome, Julie M. Cherrington, and Dirk B. Mendel


Abstract
The Src family kinases (SFKs) Src and Yes are believed to play critical roles in tumor growth, angiogenesis, invasion, and dissemination. Using a panel of highly selective and structurally diverse Src inhibitors, we found that phosphorylation of signal transducer and activator of transcription 3 [STAT3 (Y705)] and focal adhesion kinase [FAK (Y861)] was SFK dependent in cultured human colon, breast, lung, and ovarian tumor cells. These findings were reproduced in vivo in target modulation studies using tumors derived from fibroblasts overexpressing activated Src. Additionally, treatment of mice with multiple Src inhibitors resulted in inhibition of phosphorylation of FAK (Y861) and of a putative Src autophosphorylation epitope (Y419) in HT-29 human colon tumor xenografts. Next we pharmacologically examined the requirement for SFKs in asynchronous proliferation of human tumor cells. At concentrations sufficient to selectively inhibit Src, structurally diverse Src inhibitors inhibited growth of cultured human colon, breast, and lung cells on plastic under low serum conditions. In addition, these compounds inhibited anchorage-independent growth of HT-29 human colon tumor cells in soft agar. The role of SFK activity in vascular endothelial growth factor signaling was also evaluated. Inhibition of SFK signaling using structurally distinct Src inhibitors resulted in complete inhibition of vascular endothelial growth factor-dependent vascular permeability in vivo. These data demonstrate that STAT3 (Y705) and FAK (Y861) phosphoepitopes are SFK-dependent in tumor cells and reveal a requirement for SFK function in tumor cell proliferation and vascular permeability.

Introduction
The SFKs play a central role in the transduction of extracellular signals acting through activated RTKs, G-protein-coupled hormone receptors, and integrins to downstream cellular effectors (1). Three SFKs (Src, Yes, and Fyn) are widely expressed, whereas others (Lck, Lyn, Fgr, Hck, and Blk) have more restricted patterns of expression and are found primarily in hematopoietic cells (1–3). A series of elegant knockout studies has identified a limited number of functions uniquely dependent on the activity of single family members, most notably bone resorption (Src), T-cell development and signaling (Lck), and multiple B-cell signal pathways [Lyn (2–4)]. However, when coexpressed in less specialized cell types, a certain level of functional redundancy between family members is likely.

Src and Yes have been implicated in several human cancers (5). Although activating genetic mutations in SFKs are rare (they have been detected for Src in a small subset of advanced colon cancers and in a single endometrial cancer), high levels of Src activation secondary to the activation of oncogenic RTKs, such as EGF receptor and Met, are common (5–7). In colon cancer, Src activity increases with disease progression, and Src activity has recently been demonstrated to be an independent negative prognostic indicator for disease progression and patient survival (8, 9). Moreover, in patients with disseminated colon cancer, elevated Yes activity in metastases predicted poor survival (10). Overexpression/activation of Src has also been reported in other tumor types, including breast and pancreatic cancer (5).

Src activation has been reported to be associated with numerous facets of the transformed phenotype, including proliferation, survival, protease production, migration, and invasion (1, 5). STAT3 and FAK are implicated in cancer development, growth, and metastasis and have been reported to be substrates of SFKs (11, 12). In addition, SFKs have been implicated in VEGF-dependent endothelial cell...
Signaling, transducing signals leading to endothelial cell survival and VEGF-dependent vascular permeability (13).

A requirement for SFK function in G1-S progression after treatment with PDGF and EGF has been demonstrated in fibroblasts using microinjection of mutant Src proteins and treatment with a selective small molecule Src kinase inhibitor, SU6656 (14–16). However, the evidence supporting a role for Src in maintaining the transformed phenotype in cancer cells, in phosphorylation of STAT3 and FAK, and in VEGF-dependent vascular permeability is less than decisive for several reasons. First, some studies depended on overexpression of activated Src mutants, which may exhibit qualitative as well as quantitative differences in signaling relative to native SFKs. Second, in other studies, putative inhibition of Src function was achieved using only a single inhibitor or single structural class of small molecule kinase inhibitor (17), leaving open the possibility that compound-dependent rather than target-dependent effects were being observed. In general, observed cellular responses were not tightly correlated with modulation of SFK activity, and in many cases the cellular readouts used were inadequate [e.g., asynchronous proliferation in the presence of high levels of serum (17) or total phosphotyrosine levels of signaling proteins with complex phosphorylation patterns (18)]. Third, an otherwise outstanding study using genetic and pharmacological means to implicate Src in VEGF-dependent signaling (19) was flawed by reliance on an older tyrosine kinase inhibitor that is insufficiently selective to distinguish SFKs from many other kinases (Ref. 1; Table 1; data not shown).

One approach to address these concerns and to decisively elucidate the importance of SFKs in cancer would be to use several highly selective and structurally diverse small molecule inhibitors in parallel, looking for common biological responses to treatment. Using this strategy, we addressed the following three important questions: (a) Are STAT3 and FAK phosphorylation truly SFK dependent in cells representing multiple tumor types? (b) What are the consequences of pharmacological inhibition of SFK activity on anchorage-dependent and -independent proliferation of cultured tumor cells? and (c) Is there a requirement for SFK function in Fik-1/KDR signaling? We found that SFK activity is required for STAT3 and FAK phosphorylation in culture and in vivo, for tumor cell anchorage-dependent and -independent growth, and for VEGF-induced vascular permeability.

**Materials and Methods**

**Inhibitors.** The inhibitors used in these studies were adenine mimetics, which bind in the vicinity of the ATP-binding pocket of their target kinases. CGP77675 is a selective inhibitor of SFKs, whose biochemical activities and structure have been reported previously (18). SU5416 is a selective inhibitor of the VEGF receptor Fik-1/KDR (20). SU6668 is a selective inhibitor of Fik-1/KDR, PDGF receptor β, and fibroblast growth factor receptor 1 (21). SU11333 and SU11336 are derivatives of SU6656 (Fig. 1), with a similar selectivity profile (16), but they exhibit enhanced potency relative to SU6656 in biochemical and cellular assays. SU12470 represents a third class of inhibitor, structurally distinct from both CGP77675 and SU11333/SU11336. In biochemical assays SU11333, SU11336, and SU12470 are highly selective for SFKs over a large panel of other tyrosine and serine/threonine kinases. Selected biochemical IC₅₀ values for the SFK inhibitors used in this study are summarized in Table 1. Biochemical IC₅₀ values for the previously published Src inhibitors PP1 and PP2 (22) are included for comparison.

**Cell Culture.** Cell culture reagents were obtained from Life Technologies, Inc. (Gaithersburg, MD). All human tumor cell lines were obtained from the American Type Culture Collection (Manassas, VA), with the exception of NCI-HT-29 human colorectal adenocarcinoma cells and MDA-MB-468 human breast adenocarcinoma cells (National Cancer Institute, Bethesda, MD). Colo205 human colon carcinoma cells were grown in RPMI 1640 supplemented with 10% FBS, 1 mM sodium pyruvate, and 10 mM HEPES. MDA-MB-468 cells were grown in DMEM supplemented with 10% FBS. A549 human lung carcinoma cells were grown in F12K nutrient mixture supplemented with 10% FBS. SKOV3TP5 human ovarian carcinoma cells and NCI-HT-29 cells were grown in McCoy’s 5A medium (modified) supplemented with 10% FBS. NIH 3T3 cells stably overexpressing a mutated, fully activated human Src (YS30F; Ref. 16) were maintained in DMEM supplemented with 10% FCS. Cells were propagated
ard cell culture techniques. Loading was confirmed by immunoblotting of parallel blots for STAT3 and phosphoepitopes on STAT3 (Y705) and Caveolin-1 (Y14). Comparable loading was confirmed by immunoblotting of parallel blots for STAT3 and Caveolin-1.

Cells were lysed in an ice-cold buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl₂, 1.0 mM EDTA, 10% glycerol, 1% Triton X-100, 10 mM Na₃VO₄, 1 mM NaF, 1 μM leupeptin, 0.3 μM aprotinin, 1 μM pepstatin A, 10 μM bestatin, and 1.4 mM E-64. Lysates were cleared by centrifugation at 4°C for 15 min at 14,000 rpm in a microcentrifuge, and protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). For immunoprecipitation, 1 mg of whole cell lysate was incubated with 3 μg of antibody and protein A-agarose-conjugated beads (Boehringer Mannheim) with continuous rocking at 4°C for 2 h. Beads were pelleted and washed three times with cold HNTG buffer [20 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, and 0.1% Triton X-100] before being resuspended in an equal volume of 2× Laemmli sample buffer [100 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 200 mM DTT, and 0.2% bromphenol blue] and heated at 95°C for 5 min. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated primary antibodies in Tris-buffered saline containing 0.1% Triton X-100 and 5% nonfat dry milk, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham) and visualization using enhanced chemiluminescence reagents (Amersham).

**Antibodies.** α-Phospho-STAT3 (Y705), α-total STAT3, α-phospho-SFK (Y419), α-phospho-p44/p42 Erk (T202/Y204), and α-total p44/p42 Erk antibodies were purchased from New England Biolabs/Cell Signaling Technology (Beverly, MA). α-phospho-FAK (Y861) and α-total Src antibodies were purchased from Biosource International (Camarillo, CA). α-total FAK, α-phospho-caveolin, and α-total caveolin antibodies were purchased from Transduction Laboratories (Lexington, KY).

**Immunoblotting and Immunoprecipitation Analyses.** Cells were lysed in an ice-cold buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl₂, 1.0 mM EDTA, 10% glycerol, 1% Triton X-100, 10 mM Na₃VO₄, 1 mM NaF, 1 μM leupeptin, 0.3 μM aprotinin, 1 μM pepstatin A, 10 μM bestatin, and 1.4 mM E-64. Lysates were cleared by centrifugation at 4°C for 15 min at 14,000 rpm in a microcentrifuge, and protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). For immunoprecipitation, 1 mg of whole cell lysate was incubated with 3 μg of antibody and protein A-agarose-conjugated beads (Boehringer Mannheim) with continuous rocking at 4°C for 2 h. Beads were pelleted and washed three times with cold HNTG buffer [20 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, and 0.1% Triton X-100] before being resuspended in an equal volume of 2× Laemmli sample buffer [100 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 200 mM DTT, and 0.2% bromphenol blue] and heated at 95°C for 5 min. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated primary antibodies in Tris-buffered saline containing 0.1% Triton X-100 and 5% nonfat dry milk, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham) and visualization using enhanced chemiluminescence reagents (Amersham).

**Growth Assays.** Cells grown on plastic (anchorage-dependent growth assay) were plated at a density of 7,500 cells/well (HT-29) or 12,000 cells/well (MDA-MB-468 cells) in 96-well plates on day 0. Compounds were added on day 1, and the cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 2 days. Total cell protein was determined by staining cells with sulforhodamine B (Sigma, St. Louis, MO), and IC₅₀ and LD₅₀ values were determined for quadruplicate wells as described previously (23). For the soft agar assay (anchorage-independent growth assay), each well of a 24-well culture dish was coated with 0.5 ml of bottom agar mixture [McCoy’s 5A medium (modified), 10% FBS or 0.5% FBS, 0.6% agar]. After the bottom layer had solidified, 0.5 ml of a top agar mixture [McCoy’s 5A medium (modified), 10% FBS or 0.5% FBS, 0.3% agar] containing HT-29 cells (1,200 cells/well) along with the indicated inhibitors was added to each well. An additional 0.5-ml layer of medium [McCoy’s 5A medium (modified), 10% FBS or 0.5% FBS] was added over the top of the solidified agar layer. Plates were incubated for 2 weeks before being stained overnight at 37°C in an atmosphere of 5% CO₂ in a solution of 1 μg/ml iodonitrotetrazolium salt (Sigma) in 50% ethanol. Colonies were counted at X4 magnification, and the average number of colonies/triplicate wells was calculated.

**In Vivo Target Modulation Studies.** NIH 3T3 cells stably overexpressing mutated, fully activated human Src (Y530F) were grown to 80% confluence and treated with 0.05, 0.5, or 5 μM SU11333 or 5 μM SU6668 (as a negative control) for 24 h in complete growth medium. Cell lysates were prepared, and 30 μg of whole cell lysate was resolved by SDS-PAGE and immunoblotted as described in the Fig. 1 legend. An evaluation similar to that described in A was subsequently performed for FAK (Y861).
or HT-29 cells were implanted s.c. into athymic mice [5 \times 10^5 Src (Y530F) cells/implant and 5 \times 10^6 HT-29 cells/implant]. Tumors were established to approximately 500 mm^3 in volume. Mice were given a single i.p. injection of SU11333 dissolved in 50 \mu l of DMSO or 50 \mu l of DMSO alone. At the indicated time points, tumors were resected and subdivided; half of each tumor was fixed for subsequent histological and immunohistochemical evaluation (see below), and half was snap-frozen, pulverized, homogenized in lysis buffer, and analyzed by Western blotting as described above.

**Immunohistochemistry.** Freshly resected tumor pieces to be evaluated by immunohistochemistry were fixed in 10% buffered formalin for 24 h and then transferred to 70% ethanol. These specimens were subsequently embedded in paraffin and sectioned. Phospho-FAK was detected using a 1:640 dilution of a polyclonal phospho-specific FAK (Y861) antibody (Biosource International) and visualized using a biotinylated antirabbit antibody (Vector Laboratories, Burlingame, CA). All immunostained sections were counterstained using hematoxylin.

**Miles Assay for Vascular Permeability.** The Miles assay for vascular permeability (13, 24) was adapted as follows. Mice were treated i.p. with the indicated inhibitors dissolved in 50 \mu l of DMSO or with 50 \mu l of DMSO alone. Three hours later, 100 \mu l of a 2.2% solution of FITC-dextran (M_r 150,000; Sigma) were administered i.v. via the tail vein. One h later, mice received intradermal injection (in duplicate sites on their backs) with 400 ng of VEGF (human recombinant VEGF165; R&D Systems, Minneapolis, MN) dissolved in 20 \mu l of PBS and (in adjacent duplicate sites) with PBS alone. After an additional 30 min, the extent of VEGF-dependent dye leakage from the vasculature into skin was assessed visually and photographed using fluorescence microscopy.

**Results**

**SU11333 Inhibits Putative SFK-dependent Phosphoepitopes in Fibroblasts Engineered to Overexpress an Activated Src Allele (Y530F).** In published studies, STAT3 (Y705), Caveolin-1 (Y14), and FAK (Y861) have been proposed as potential SFK substrates (11, 25, 26). Consistent with this, we found that phosphorylation at these phosphoepitopes is elevated in NIH 3T3 cells stably overexpressing an activated Src allele (Y530F) relative to cells stably overexpressing wild-type Src, cells that were vector transfected, or untransfected NIH 3T3 cells (Fig. 2; data not shown).
We set out to determine whether pharmacological inhibition of Src kinase activity, initially in the NIH 3T3 cells engineered to overexpress activated Src and subsequently in human tumor cells, would result in decreased phosphorylation at these phosphoepitopes. Activated Src was overexpressed in NIH 3T3 fibroblasts and multiple human tumor cell lines.

**Fig. 6.** SU11333 treatment inhibits phosphorylation of STAT3 (Y705) and FAK (Y861) phosphoepitopes in tumors derived from fibroblasts engineered to overexpress an activated Src allele. 

A, NIH 3T3 cells stably overexpressing a mutated, fully activated human Src (Y530F) were implanted s.c. into athymic mice, and tumors were established to approximately 500 mm³ in volume. Mice were treated i.p. with vehicle (DMSO) alone or SU11333 at 50 or 150 mg/kg. Tumors were resected 1 h after treatment, frozen, pulverized, and homogenized in lysis buffer as described in “Materials and Methods.” Fifty µg of whole cell protein lysate were analyzed by immunoblotting for FAK (Y861) and STAT3 (Y705) as described in “Materials and Methods.” Protein levels of FAK and STAT3 were also determined by immunoblotting of duplicate blots using antibodies for total FAK and STAT3. Each lane represents data from a separate mouse. Predose (untreated) and vehicle-treated controls are included.

B, tumor specimens were prepared, and phospho-FAK (Y861) was visualized immunohistochemically as described in “Materials and Methods.” Slides were evaluated and photographed at ×400 magnification. Images from representative animals are shown, including inset panels at higher magnification (×800) showing more cellular detail.

**Fig. 7.** SU11333 and CGP77675 inhibit phosphorylation of FAK (Y861) and Src (Y419) phosphoepitopes in HT-29 human colon tumor xenografts. HT-29 cells were implanted s.c. into athymic mice, and tumors were established to approximately 500 mm³ in volume. Mice were treated i.p. with vehicle (DMSO) alone, SU11333 at 100 mg/kg, or CGP77675 at 40 and 100 mg/kg. Tumors were resected 4 h after treatment, frozen, pulverized, and homogenized in lysis buffer. Fifty µg of whole cell lysates were analyzed by immunoblotting for FAK (Y861) and the putative Src autophosphorylation epitope Y419 as described in “Materials and Methods.” Protein levels of FAK and Src were also determined by immunoblotting of duplicate blots using antibodies for total FAK and Src. Each lane represents data from a separate mouse. Predose (untreated) and vehicle-treated (Veh) controls are included.

Erk2 (Fig. 3A), demonstrating that inhibition of STAT3, FAK, and Caveolin-1 phosphorylation was selective.

**STAT3 and FAK Phosphorylation Is SFK dependent in Cultured Human Tumor Cells.** Based on the above data, we set out to evaluate the effect of Src inhibition on FAK, STAT3, and Caveolin-1 phosphorylation in multiple human tumor cell types. However, because the Caveolin-1 (Y14) phosphoepitope was present at much lower levels in human tumor cells than in fibroblasts engineered to overexpress Src and because this phosphoepitope [unlike FAK (Y861) and STAT3 (Y705)] was not widely detectable in archival human tumors (data not shown), it was not investigated further. We evaluated the effects of multiple structurally distinct Src inhibitors on STAT3 and FAK phosphorylation in five diverse human tumor cell lines (HT-29 colon adenocarcinoma, Colo205 colon carcinoma, MDA-MB-468 breast adenocarcinoma, A549 lung carcinoma, and SKOV3TP5 ovarian carcinoma). The experiments were performed under both low (0.5% FBS) and high (10% FBS) serum conditions, and low serum conditions were thought to better mimic autocrine growth conditions in vivo. Treatment with SU11333, SU11336, and CGP77675 resulted in potent and dose-dependent submicromolar inhibition of tyrosine phosphorylation.
Src Kinase Phosphorylates STAT3 and FAK in Vivo

Table 2  Structurally diverse selective Src inhibitors inhibit proliferation of human tumor cells grown under low serum conditions

<table>
<thead>
<tr>
<th>Compounds</th>
<th>HT-29</th>
<th>MDA-MB-468</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low serum</td>
<td>High serum</td>
</tr>
<tr>
<td></td>
<td>IC_{50} (μM)</td>
<td>LD_{50} (μM)</td>
</tr>
<tr>
<td>SU11333</td>
<td>0.8</td>
<td>3.9</td>
</tr>
<tr>
<td>SU11336</td>
<td>1.2</td>
<td>2.7</td>
</tr>
<tr>
<td>SU12470</td>
<td>0.8</td>
<td>5.5</td>
</tr>
<tr>
<td>CGP77775</td>
<td>0.8</td>
<td>5.0</td>
</tr>
</tbody>
</table>

SU11333 Inhibits STAT3 and FAK Phosphorylation in Tumors Grown in Vivo. The studies described above, which demonstrated that STAT3 (Y705) and FAK (Y861) are SFK dependent, were performed in cultured cells. However, the signals driving phosphorylation of STAT3 and FAK in tumor cells in vivo could potentially be quite different, reflecting differences in cell adhesion, morphology, and signaling. To address this possibility, we evaluated the ability of SU11333 to inhibit phosphorylation of these targets in vivo. Tumors were established in athymic mice from the NIH 3T3 cells engineered to overexpress activated Src. As shown in Fig. 6A, a single dose of SU11333 was able to inhibit STAT3 and FAK phosphorylation in a dose-dependent manner relative to pretreatment and vehicle-treated controls, indicating that these phosphoepitopes are SFK dependent in these engineered tumors in vivo. Comparable levels of inhibition were evident 1 (Fig. 6A) and 5 h (data not shown) after treatment. Similar results were seen when Fak (Y861) phosphorylation changes were assessed by immunohistochemistry (Fig. 6B).

The effects of Src inhibition were also evaluated in mice bearing HT-29 human colon cancer xenografts (these tumors have naturally high levels of Src activity but are not engineered to overexpress an activated Src allele). We found that phosphorylation at FAK (Y861) was partially inhibited by both SU11333 and CGP77775 (Fig. 7). Moreover, as in cultured HT-29 cells (Fig. 4A), the putative Src autophosphorylation epitope Y419 was also inhibited with potency similar to that seen for FAK (Y861). These tumors express very low levels of STAT3, so the effects of Src inhibition on STAT3 signaling could not be explored in this model.

Src Inhibitors Suppress Anchorage-dependent Growth of Human Tumor Cells. To explore the potential requirement for SFKs in tumor cell proliferation, we evaluated the effect of four different SFK inhibitors in 2-day proliferation assays using HT-29, MDA-MB-468, and A549 cells. As shown in Table 2, we found that multiple Src compounds potently inhibited the growth of HT-29 and MDA-MB-468 cells under low serum (autocrine) conditions. These effects were seen at dosages consistent with those required to inhibit Src-dependent signaling (see the results above). In contrast, growth under high serum conditions was not inhibited at target-selective concentrations. These data suggest

phosphorylation of FAK (Y861) under both low and high serum conditions in HT-29 cells. STAT3 was expressed at very low levels in this cell line, so potential changes in its phosphorylation status could not be accurately assessed. Src Y419 is located in the Src catalytic domain and is reported to be the major Src autophosphorylation site. We found that this phosphoepitope was inhibited by the compounds to an extent comparable with that seen with FAK (Y861) under low serum conditions (Fig. 4A) but was less susceptible in cells grown under high serum conditions (Fig. 4B).

SU11333 dose-dependently inhibited STAT3 (Y705) and FAK (Y861) phosphorylation in MDA-MB-468 (Fig. 5A) and A549 cells (Fig. 5B) in both low and high serum conditions with submicromolar potency. Similar results were obtained after treatment of Colo205 cells and SKOV3TPS cells with SU11333 (data not shown). Hence, phosphorylation at STAT3 (Y705) and FAK (Y861) appears to be highly SFK dependent in multiple human tumor cell lines.
that Src kinase activity is required for autocrine growth of HT-29 and MDA-MB-468 cells. A similar trend was also observed for A549 cells (data not shown).

**Src Inhibitors Suppress Anchorage-independent Growth of HT-29 Human Colon Tumor Cells.** Anchorage-independent growth is a hallmark of the transformed state (27). Therefore, to explore the contribution of SFKs to anchorage-independent growth, we evaluated the effect of three different SFK inhibitors on HT-29 human colon tumor cell colony formation in soft agar. Treatment with SU11333 resulted in dose-dependent suppression of the growth of HT-29 cells in soft agar under both low and high serum conditions (Fig. 8; Table 3). Again, the submicromolar potency of SU11333 in this assay is consistent with the concentrations of compound required to inhibit SFK signaling in HT-29 cells (Fig. 2). Similarly, SU11336 and CGP77675 were also potent inhibitors of colony formation (Table 3). In general, all three inhibitors appeared somewhat more potent in inhibiting anchorage-independent growth than in inhibiting anchorage-dependent growth (compare Table 3 with Table 2).

**SU11333 Inhibits VEGF-dependent Vascular Permeability in Mice.** To address the potential requirement for ability in Mice.

**Table 2.**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Compounds</th>
<th>Concentration (μM)</th>
<th>Total no. of colonies</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Serum</td>
<td>SU11336</td>
<td>0.2</td>
<td>141</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>141</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0</td>
<td>100</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>CGP77675</td>
<td>0.2</td>
<td>187</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>133</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0</td>
<td>47</td>
<td>82</td>
</tr>
<tr>
<td>Low Serum</td>
<td>SU11333</td>
<td>0.2</td>
<td>73</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>58</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>SU11336</td>
<td>0.2</td>
<td>54</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>49</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>CGP77675</td>
<td>0.2</td>
<td>250</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>164</td>
<td>37</td>
</tr>
</tbody>
</table>

**Table 3.**

| HT-29 cells (1200 cells/well) were seeded in soft agar along with the indicated inhibitors as described in “Materials and Methods.” Plates were incubated for 2 weeks and stained overnight in a solution of 1 μg/ml iodonitrotetrazolium salt. Colonies were counted at ×4 magnification, and for each treatment group the total number of colonies in triplicate wells was calculated (variation was minimal between wells within groups).

**Discussion**

Phosphorylation at tyrosine 705 results in dimerization and activation of STAT3, a transcription factor that is implicated in the expression of several genes important in cancer, including myc and vegf (15, 28). The ability of diverse SFK inhibitors to potently inhibit phosphorylation at STAT3 (Y705) in diverse cancer cells in culture demonstrates that SFKs are probably the major physiological effectors of phosphorylation at this phosphoepitope. These data are consistent with a previous report that STAT3 activation is Src dependent in unstimulated breast cancer cells (29). In contrast with solid tumors, STAT3 is likely to be predominantly activated by Janus kinase family members in leukemia (30).

The fak locus is frequently amplified in human cancers, and FAK expression is elevated in sarcomas and in colon, prostate, and breast tumors (12). FAK is activated after integrin engagement and mediates integrin-induced Src activation by relieving negative regulatory conformational constraints in Src. In turn, FAK is itself phosphorylated and activated by Src (12). FAK contains numerous phosphoepitopes, and the kinases responsible for phosphorylation of each phosphoepitope are not completely characterized. The ability of multiple independent SFK inhibitors to suppress phosphorylation of FAK (Y861) in diverse tumor cells in culture and in vivo indicates that this phosphoepitope is a site of regulation of FAK by SFKs. The observation that phosphorylation at STAT3 (Y705) and FAK (Y861) was readily pharmacologically reversible strongly, albeit indirectly, suggests that the enhanced phosphorylation seen at these phosphoepitopes in Src Y530F-transformed fibroblasts (Fig. 3) occurs via a direct mechanism i.e., direct phosphorylation by Src, rather than being secondary to cell transformation per se. These data are consistent with the observation that, in biochemical phosphorylation assays using isolated c-Src and FAK proteins, Y861 is the predominant site of phosphorylation of FAK by Src (31).

A requirement for Src in G1-S progression in fibroblasts after treatment with PDGF or EGF has been demonstrated (14). However, the role of Src kinase activity in proliferation of tumor cells has not been rigorously addressed. Previous studies using synthetic kinase inhibitors have demonstrated inhibition of cell growth, but the interpretation of these data is unclear because cells were grown in the presence of high levels of serum (17). We found that, when cultured under low serum conditions, the proliferation of colon, breast, and lung tumor cells was inhibited by diverse SFK inhibitors at compound concentrations similar to those required to inhibit SFK-dependent phosphorylation of STAT3 and FAK (Table 2; data not shown). In contrast, in cells cultured under high serum conditions, inhibition was not seen at compound concentrations considered to be highly selective. This divergence does not stem from differences in levels of soluble proteins (which might bind compounds) in the culture medium because all four compounds evaluated can inhibit Src-
Src Kinase Phosphorylates STAT3 and FAK in Vivo

Three different SFK inhibitors potently inhibited HT-29 colony formation in soft agar under both high and low serum conditions (Table 3). Consistent with the results for anchorage-dependent growth, greater potency was evident for colonies grown under low serum conditions, although strong inhibition was seen at selective concentrations of compound under high serum conditions as well. These effects on anchorage-independent growth are consistent with a previous report demonstrating that antisense RNA-mediated inhibition of Src expression in HT-29 cells inhibits their subsequent growth as s.c. tumor xenografts in nude mice (32). It has been hypothesized that abnormally activated SFKs can substitute for appropriate cell-matrix contacts, thereby permitting aberrant growth of cancer cells under anchorage-independent conditions (33). Src has been implicated in VEGF-dependent angiogenesis at the level of VEGF production (34, 35). Interestingly, it has recently been demonstrated that v-Src-dependent induction of VEGF expression is mediated via STAT3 (28). It has also become apparent that SFKs may serve as important downstream effectors of signaling via activated Flk-1/KDR (13). Consistent with this hypothesis, administration of the semi-selective SFK inhibitor PP1 partially inhibited VEGF-induced vascular permeability in mice after stroke injury (19). However, interpretation of these data is complicated by the fact that PP1, unlike SU11333 and CGP77675, potently inhibits Flk-1/KDR in addition to SFKs (Table 1). Here we demonstrated that administration of either SU11333 or CGP77675 completely inhibited VEGF-dependent vascular permeability in mouse skin (Fig. 9). These data strongly support the hypothesis that SFKs are indispensable mediators of the VEGF-dependent vascular permeability signal cascade and are consistent with a published report demonstrating that mice deficient in Src or Yes exhibit reduced VEGF-dependent vascular permeability (13). Potential SFK effectors in this pathway include the gap junction protein connexin 43 (36).

We recognize that the small molecule inhibitors that we have used in these studies exhibit limited selectivity for SFKs over other kinases evaluated (Table 1). Therefore, we cannot exclude the possibility that the pharmacological effects we have documented may partially reflect effects on kinases other than SFKs. However, the fact that common biological effects were seen with several structurally diverse inhibitors administered at selective concentrations strongly supports the argument that the effects we have seen in vitro and in vivo are predominantly SFK dependent.

In conclusion, in this work, the use of multiple highly selective SFK inhibitors in culture and in vivo has decisively demonstrated the central involvement of SFK signaling in phosphorylation of signaling molecules important in cancer (STAT3 and FAK), in tumor cell proliferation, and in VEGF-dependent signaling.

Acknowledgments

We thank Sara Courtneidge, Nancy Pryer, and Chris Liang for helpful discussions and Barbara Remley for expert assistance in preparation of this manuscript.
References


Src Family Kinase Activity Is Required for Signal Transducer and Activator of Transcription 3 and Focal Adhesion Kinase Phosphorylation and Vascular Endothelial Growth Factor Signaling \textit{in Vivo} and for Anchorage-dependent and -independent Growth of Human Tumor Cells

A. Douglas Laird, Guangmin Li, Katherine G. Moss, et al.

\textit{Mol Cancer Ther} 2003;2:461-469.

Updated version
Access the most recent version of this article at:
http://mct.aacrjournals.org/content/2/5/461

Cited articles
This article cites 33 articles, 12 of which you can access for free at:
http://mct.aacrjournals.org/content/2/5/461.full.html#ref-list-1

Citing articles
This article has been cited by 22 HighWire-hosted articles. Access the articles at:
/content/2/5/461.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.