Src Activation Is Not Necessary for Transforming Growth Factor (TGF-\(\beta\))-mediated Epithelial to Mesenchymal Transitions (EMT) in Mammary Epithelial Cells

PP1 DIRECTLY INHIBITS TGF-\(\beta\) RECEPTORS I AND II

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Epithelial to mesenchymal transitions (EMTs) are key events during embryonic development and cancer progression. It has been proposed that Src plays a major role in some EMT models, as shown by the overexpression of viral Src (v-Src) in epithelial cells. It is clear that Src family kinases can regulate the integrity of both adherens junctions and focal adhesions; however, their significance in EMT, especially in the physiological context, remains to be elucidated. Here we showed that Src is activated in transforming growth factor-\(\beta\) (TGF-\(\beta\))-mediated EMT in mammary epithelial cells and that the Src family kinase inhibitor, PP1, prevents EMT. However, neither a more specific Src family kinase inhibitor, SU6656, nor a dominant-negative Src inhibited TGF-\(\beta\)-1-mediated EMT, leading us to speculate that Src activation is not an essential component of TGF-\(\beta\)-1-mediated EMT. Unexpectedly, PP1 prevented Smad2/3 activation by TGF-\(\beta\), whereas SU6656 did not. Most interestingly, an \textit{in vitro} kinase assay showed that PP1 strongly inhibited the TGF-\(\beta\) receptor type I, and to a lesser extent, the TGF-\(\beta\) receptor type II. Taken together, our data indicated that PP1 interferes with TGF-\(\beta\)-1-mediated EMT not by inhibiting Src family kinases but by inhibiting the Smad pathway via a direct inhibition of TGF-\(\beta\) receptor kinase activity.

Epithelial to mesenchymal transitions (EMTs) \(^{3}\) are phenomena whereby epithelial cells convert to motile fibroblastic cells. EMTs play crucial roles both in embryonic development and in cancer progression \((1–4)\). A number of \textit{in vitro} model systems have been developed to study EMT, and many cellular changes that accompany EMT have been described, including actin reorganization, dissolution of cell-cell junctions, induction of focal adhesions, and increased cell motility \((5,6)\). The model system we have used previously in our laboratory and continue to use for these studies is the mouse mammary epithelial cell line, NMuMG, which undergoes EMT in response to transforming growth factor-\(\beta\) (TGF-\(\beta\)) \((7)\).

TGF-\(\beta\) has diverse functions with regard to cell proliferation, differentiation, and migration \((8–10)\). In particular, TGF-\(\beta\) signaling plays a dual role in cancer progression. It functions as a growth suppressor in normal epithelial cells, and thus, TGF-\(\beta\) receptors are considered to be tumor suppressors. However, in some tumor cells, TGF-\(\beta\) has a tumor-promoting effect, which contributes to tumor invasion and metastasis. This tumor-promoting effect of TGF-\(\beta\) signaling is thought to be related to its ability to induce EMT \((11–14)\).

There is evidence that TGF-\(\beta\) induces EMT via the Smad pathway in injury-induced EMT in lens epithelium and in NMuMG cells \((15–17)\). However, this latter finding is controversial since Bhowmick et al. \((18)\) suggested that TGF-\(\beta\)-mediated EMT is independent of the Smad pathway. Indeed, several non-Smad pathways, including those involving Rho, Ras, Type 2 protein serine/threonine phosphatase, and TGF-\(\beta\)-activated kinase 1/extracellular signal-regulated kinase kinase 1 (TAK1/MEKK1), have been reported to be downstream of TGF-\(\beta\) receptors \((19)\). TGF-\(\beta\)-mediated EMT is one of the best-studied \textit{in vitro} models of EMT and thus has been used in many studies focusing on defining the mechanisms that mediate the phenotypic changes associated with EMT. Thus, far, several downstream pathways, including RhoA \((18)\), phosphatidylinositol-3-kinase \((20)\), mitogen-activated protein kinase (MAPK) \((21,22)\), Rac1 \((21)\), Ras \((23)\), integrin-linked kinase (ILK) \((24,25)\), and Notch1/Notch \((26)\), have been implicated in TGF-\(\beta\)-mediated EMT.

One characteristic event in TGF-\(\beta\)-mediated EMT is the formation of focal adhesions, where various tyrosine kinases and their substrates reside. One such kinase is the non-receptor tyrosine kinase, c-Src, a mediator of signal transduction that regulates cell proliferation, differentiation, adhesion, and migration \((27–31)\). It has been shown that c-Src is involved in the turnover of focal adhesions and also in disruption of adherens junctions \((32,33)\). Likewise, oncosgenic v-Src disrupts cadherin-dependent cell-cell adhesions in rat 3Y1 fibroblasts \((34,35)\). Furthermore, v-Src and an active mutant of c-Src induce EMT in Madin-Darby canine kidney cells and KMC12C colon cancer cells, respectively \((36,37)\). Moreover, Src is required for epidermal growth factor-induced EMT in NBT-II rat bladder carcinoma cells \((38)\). Since c-Src has been reported to be activated by TGF-\(\beta\) in various types of cells \((17,39–42)\), it has been hypothesized that c-Src activity is likely one driving force.
Role of Src in EMT

behind TGF-β-mediated EMT. Given the discrepancies in the literature, the role Src plays in TGF-β-mediated EMT has yet to be elucidated, and even whether its activation is necessary for EMT remains unclear. Here we used the NMuMG mouse mammary epithelial cell model system to rule out a central role for Src activity in TGF-β1-mediated EMT.

MATERIALS AND METHODS

Reagents, Antibodies, and Cultured Cells—All reagents were from Sigma or Fisher unless otherwise indicated. Mouse monoclonal antibodies (mAbs) against N-cadherin (13A9) and β-catenin (15B8) have been described (43, 44). Anti-Src mouse mAb (327) was from Oncogene Research Products, San Diego, CA. Anti-phospho Src family (Tyr-416) rabbit polyclonal antibody (pAb) and anti-phospho-Smad2 (Ser-465/Ser-467) rabbit pAb were from Cell Signaling Technology, Beverly, MA. Anti-phosphotyrosine mouse mAb (PY20), anti-paxillin mouse mAb, anti-p120 catenin mouse mAb, anti-focal adhesion kinase (FAK) mouse mAb, anti-FAK phospho-specific (Tyr-397) mouse mAb, and anti-Smad2/3 mouse mAb were from Pharmingen. Anti-FAK phospho-specific (Tyr-577) rabbit pAb was from BIOSOURCE, Camarillo, CA. Anti-mouse E-cadherin rat mAb (ECCD-2) and anti-ZO-1 rabbit pAb were from Zymed Laboratories Inc., San Francisco, CA. Anti-fibronectin rabbit pAb was from Sigma. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mouse mAb was from Abcam, Cambridge, MA. Mouse NMuMG/E9 cells were established as described previously (7) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 4.5 g/liter glucose, and 10% fetal bovine serum (Invitrogen), 20 ng/ml insulin, and 500 ng/ml hydrocortisone. TGF-β1-mediated EMT. Given the discrepancies in the literature, the role Src plays in TGF-β-mediated EMT has yet to be elucidated, and even whether its activation is necessary for EMT remains unclear. Here we used the NMuMG mouse mammary epithelial cell model system to rule out a central role for Src activity in TGF-β1-mediated EMT.

Detergent Extraction, SDS-PAGE, Immunoblots, and Immunoprecipitations—Monolayers of cultured cells were washed with ice-cold PBS and extracted on ice with TNE buffer (10 mM Tris-HCl, pH 8.0, 0.5% Nonidet P-40, 1 mM EDTA) containing 2 mM orthovanadate, 20 μM calyculin A, and 2 mM phenylmethylsulfonyl fluoride. Extracts were centrifuged at 20,000 × g for 15 min at 4 °C, and the supernatant was collected. Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad). In some experiments, SDS sample buffer (2% SDS, 10% glycerol, 62.5 mM Tris-HCl, pH 6.8) was used for extraction followed by sonication, and the protein concentration was determined using the DC protein assay kit (Bio-Rad). Cell extracts were resolved by SDS-PAGE and immunoblotted as described (43). TNE cell extracts were used for immunoprecipitation as described (7). Immunoblots were quantified by densitometry using Adobe® PhotoShop® histogram.

Immunofluorescence Microscopy—For most experiments, cells were fixed with HistoChoice tissue fixative (Amresco, Solon, OH) and processed as described previously (48). For anti-Smad2/3 and when double staining included TRITC-conjugated phalloidin, the cells were fixed in 3.7% formaldehyde for 15 min, permeabilized with 0.2% Triton X-100 in PBS for 15 min, and blocked in 10% goat serum in PBS. For immunostaining with anti-phospho Src family antibody (Tyr-416), cells were fixed with ice-cold 10% trichloroacetic acid for 15 min, permeabilized with 0.2% Triton X-100 in PBS for 15 min, and blocked in 10% goat serum in PBS (49). Cells were examined on a Zeiss Axiosvert 200M microscope (Göttingen, Germany) equipped with an ORCA-ER digital camera (Hamamatsu, Houston, TX). Images were collected and processed using OpenLab software from Improvision Inc. (Boston, MA).

In Vitro Protein Kinase Assay—In vitro kinase assays were done essentially as described (50, 51). cDNAs encoding mouse TGF-β receptor type I and type II were obtained from ATCC (GenBank™ accession numbers BC063260 and BC052629, respectively). The carboxyl-terminal fragments (nucleotides 449–1519 of TGF-β type I receptor and nucleotides 880–2007 of the TGF-β type II receptor) were amplified by PCR, subcloned into the TOPO TA cloning system, and moved into pGST-Parallel-1 vector (52) to produce glutathione S-transferase (GST) fusions of the carboxyl-terminal residues of TGF-β receptor type I and type II for expression in Escherichia coli. PCR products were sequenced and shown to encode the wild type amino acid sequence. Logarithmically growing E. coli (JM109; Promega, Madison, WI) transformed with pGST-parallel recombinants were incubated with 0.3 mM isopropyl β-D-thiogalactopyranoside for 15 h at 23 °C. Cells were then pelleted, resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride), and sonicated on ice. After centrifugation, the supernatant was mixed with glutathione-agarose and gently shaken for 30 min at 4 °C. After washing with wash buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol), 2 mM phenylmethylsulfonyl fluoride), and sonicated on ice. After centrifugation, the supernatant was mixed with glutathione-agarose and gently shaken for 30 min at 4 °C. After washing with wash buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol), the GST fusion proteins were eluted with wash buffer containing 10 mM glutathione. The kinase reactions for TGF-β receptor types I and II (M MP Biomedicals, Irvine CA), 2 pmol of TGF-β type I or type II receptor protein, and serial concentrations of PP1 at 30 °C for 30 min. After incubation, the reaction mixture was mixed with SDS sample buffer and resolved by SDS-PAGE. The 32P signals corresponding to autophosphorylated TGF-β receptors were analyzed by autoradiography, and the inhibitory effects of PP1 were measured as a decrease in autophosphorylation of the receptors. Autoradiographic films were quantified by densitometry using Adobe® PhotoShop® histogram.

RESULTS

The Src Inhibitor PP1 Prevents TGF-β1-mediated EMT in NMuMG/E9 Cells—In this study, we used NMuMG/E9 cells, which are a clone of the NMuMG mouse mammary epithelial cell line. The E9 clone was established in our laboratory and has a highly epithelial morphology (7). The phosphorylation level of a known autophosphorylation site in the activation loop of mouse c-Src (Tyr-418, using the initiating methionine as amino acid
FIGURE 1. Src is activated in response to TGF-β1 and its activity is inhibited by PP1. A, NMuMG/E9 cells pretreated for 30 min with Me2SO (DMSO) as a control or 10 μM PP1 were incubated with or without TGF-β1 (5 ng/ml) for 1 day. TNE extracts were analyzed for phospho Src (Tyr-418) (p Src (418Y)) or c-Src, and GAPDH by immunoblots. B, phospho Src and total Src were quantified and normalized to GAPDH from three independent experiments and graphed showing the standard deviation. The phosphorylation level of c-Src at Tyr-418 was significantly elevated in response to TGF-β1 (Tβ1), and PP1 pretreatment suppressed the phosphorylation level to nearly that of untreated cells. C, NMuMG/E9 were stained for phospho Src (Tyr-418) (a and b) and paxillin (c and d) in the absence (a and c) or presence (b and d) of TGF-β1 (5 ng/ml for 1 day). Activated Src accumulated in the focal adhesions that were induced by TGF-β1. Photographs were taken using a ×40 dry objective: bar = 10 μm. D, NMuMG/E9 cells were pretreated with Me2SO or PP1 and incubated with or without TGF-β1 (1 ng/ml) for 2 days. Total RNA was analyzed by RT-PCR for c-Src, N-cadherin, Snail, SIP1, and GAPDH (as a control).

1) is correlated with its enzymatic activity (53). Analysis using anti-phospho-specific antibody (p418Y of c-Src) demonstrated that phosphorylation of c-Src at Tyr-418 was elevated by TGF-β1 treatment in NMuMG/E9 cells, and this phosphorylation was inhibited by 10 μM Src family kinase (SFK) inhibitor PP1 (Fig. 1, A and B). The total c-Src level was slightly elevated upon TGF-β1 treatment. The increase is likely regulated at the post-transcriptional level since no increase in c-Src mRNA was detected by RT-PCR in TGF-β1-treated NMuMG/E9 cells (Fig. 1D). Immunostaining using anti-phospho-specific antibody (p418Y of c-Src) showed that activated c-Src was translocated to newly formed focal adhesions in TGF-β1-treated NMuMG/E9 cells, where it co-localized with paxillin, a marker of focal adhesions (Fig. 1C). These data indicated that c-Src was activated by TGF-β1 in NMuMG/E9 cells.

To investigate the significance of Src activation in TGF-β1-mediated EMT in NMuMG/E9 cells, we asked whether the SFK inhibitor PP1 would prevent not only Src activation but also EMT itself in NMuMG/E9 cells. In a previous report (54), 50 nM PP1 failed to prevent EMT in TGF-β1-treated NMuMG cells. We confirmed this finding by showing that in our system, 1 μM PP1 did not show any effect on the cells (data not shown). However, 10 μM PP1 did significantly inhibit EMT in our system. We have previously shown that TGF-β1 treatment of NMuMG/E9 cells induced expression of N-cadherin, Snail, and SIP1 mRNA (7). Here we show that PP1 pretreatment inhibited the induction of each of these genes (Fig. 1D). In addition, TGF-β1 treatment induced formation of stress fibers and focal adhesions and dissolution of cell-cell junctions in NMuMG/E9 cells, and these effects were also abrogated by 10 μM PP1 (Fig. 2). Moreover, at the protein level, PP1 inhibited the induction of the mesenchymal markers N-cadherin and fibronectin and the reduction of the epithelial marker ZO-1 (see Figs. 4 and 6).

TGF-β1-induced Tyrosine Phosphorylation of Known c-Src Substrates Is Inhibited by PP1—TGF-β1 treatment of NMuMG/E9 cells induced focal adhesions, and several of the components of focal adhesions are substrates for c-Src. To confirm that c-Src functioned in the cellular context of TGF-β1-mediated EMT, we investigated the tyrosine phosphorylation level of known c-Src substrates, including FAK, paxillin, and p120 catenin, in the absence or presence of PP1 pretreatment. Tyr-397 of FAK is a major autophosphorylation site that provides a docking site for the SH2 domain of Src family kinases (55, 56). Tyr-577 of FAK, which is located in the kinase domain, is phosphorylated by Src (57). In addition to the elevation of FAK protein level by TGF-β1, immunoblots using phospho-specific antibodies revealed that TGF-β1 treatment significantly raised the phosphorylation level on Tyr-577 and slightly raised the phosphorylation level on Tyr-397 (Fig. 3, A and B). PP1 treatment strongly inhibited phosphorylation of Tyr-577, which is a consensus Src phosphorylation site. However, phosphorylation at Tyr-397, which is an autophosphorylation site rather than a Src site, was not significantly changed (Fig. 3, A and B).

When the Src substrate, paxillin, is phosphorylated, it migrates more slowly on SDS-PAGE, thus, we can get an indication of the phosphorylation status of paxillin from immunoblots of total cell lysates. Phosphorylation of paxillin was up-regulated in the presence of TGF-β1 (Fig. 3C, arrow), and this was diminished by PP1 (Fig. 3C, left panel). To confirm that paxillin was phosphorylated on tyrosine, we immunoprecipitated cell extracts with anti-phosphotyrosine (PY-20) and blotted back with anti-paxillin (Fig. 3C, right panel). Indeed, PP1 inhibited phosphorylation of paxillin.

Immunoprecipitation with antibodies against p120 catenin, another Src substrate, followed by immunoblotting with anti-phosphotyrosine (PY-20) showed that p120 catenin was also tyrosine phosphorylated by TGF-β1 treatment of NMuMG/E9 cells and that PP1 also inhibited its
Thus, the results shown in Fig. 3 clearly demonstrate that TGF-β1 treatment induced c-Src activation and tyrosine phosphorylation of known c-Src substrates and that PP1 treatment interfered with phosphorylation of each of these substrates. These data point out: 1) that c-Src is activated when NMuMG/E9 cells undergo EMT in response to TGF-β1; 2) that a number of known Src substrates that are thought to be involved in the biological changes cells undergo during EMT are phosphorylated in response to TGF-β1-induced EMT.
in NMuMG/E9 cells; and 3) that the Src family kinase inhibitor, PP1, inhibits TGF-β1-induced EMT and inhibits phosphorylation of the relevant Src kinase substrates.

More Specific Src Inhibitors Do Not Interfere with TGF-β1-mediated EMT—The above data suggested that Src activation was necessary for TGF-β1-mediated EMT in NMuMG/E9 cells. However, some recent reports have shown that PP1 is not entirely specific for Src family kinases and is able to inhibit other kinases such as c-Abl and platelet-derived growth factor receptor (PDGFR) (58–60). These reports prompted us to use other inhibitors to examine whether Src activity was truly necessary for TGF-β1-mediated EMT. Thus, we investigated another SFK inhibitor, SU6656, which is known to be more specific for Src family kinases (61, 62). When NMuMG/E9 cells were treated with PP1, cell-cell contacts became tighter and, even in the presence of TGF-β1, tight cell-cell contacts were preserved. Moreover, although some peripheral cells in small islands became slightly elongated, similar to control TGF-β1-treated cells, most cells maintained their morphology in the presence of TGF-β1 (Fig. 4A, compare panel d with panel b). In contrast, SU6656-treated NMuMG/E9 cells showed elongated morphological changes together with a dispersed phenotype that included loosed cell-cell junctions when treated with TGF-β1 and were almost identical to control cells (Fig. 4A, compare panel f with panel b). As shown in Fig. 4B, both PP1 and SU6656 inhibited Src activation (phosphorylation at Tyr-418). However, PP1 interfered with induction of N-cadherin and fibronectin (Fig. 4B, mesenchymal markers) and down-regulation of ZO-1 (an epithelial marker; see Fig. 6), which were all induced by TGF-β1 treatment in control cells, whereas SU6656 did not prevent any of these classical hallmarks of EMT (Fig. 4B). To confirm that SU6656 did in fact inhibit the activity of Src, we examined the phosphorylation state of the downstream Src target, FAK. Indeed, both PP1 and SU6656 inhibited TGF-β1-mediated phosphorylation of FAK (Fig. 4C, 577Y). To further rule out a role for Src in TGF-β1-mediated EMT in NMuMG/E9 cells, we expressed a dominant-negative form of Src and then examined morphological and biochemical features of EMT. Cells expressing the dominant-negative form of Src were indistinguishable from mock-transduced cells (Fig. 5A). In addition, dominant-negative Src did not inhibit the TGF-β1-induced increase in N-cadherin expression (Fig. 5, B and C). Importantly, dominant-negative Src effectively prevented activation of Src as shown by decreased phosphorylation on Tyr-418 of Src and decreased phosphorylation on Tyr-577 of the Src substrate, FAK (Fig. 5, B and C). Together, these data indicate that Src activation is not necessary for the process of TGF-β1-mediated EMT in NMuMG/E9 cells.
The Potential PP1 Target, c-Abl, Is Not Essential for TGF-β1-mediated EMT—Several non-Src kinases have been reported to be inhibited by PP1 including c-Abl, which is proposed to play an important role in actin cytoskeleton regulation (63). Thus, we investigated whether the activity of c-Abl kinase was necessary for TGF-β1-induced EMT in NMuMG/E9 cells. In contrast to PP1, 10 μM specific c-Abl inhibitor, STI-571 (also called imatinib mesylate or Gleevec), did not interfere with the breakdown of cell-cell junctions or stress fiber formation as shown by immunostaining for ZO-1 and F-actin (Fig. 6A). In addition, STI-571 did not inhibit fibronectin induction or ZO-1 reduction (Fig. 6B), suggesting that c-Abl activity is not necessary for TGF-β1-mediated EMT in NMuMG/E9 cells, and thus, although its activity is likely inhibited by PP1, c-Abl is not the relevant target of PP1 in this system.

PP1 Inhibits TGF-β1-induced Smad Activation via Direct Inhibition of Type I and Type II TGF-β Receptors—In our system, PP1 interfered with many aspects of EMT, including morphological changes, actin reorganization, the breakdown of cell-cell junctions, transcriptional regulation of N-cadherin, Snail and SIP1, and Src activation, suggesting that PP1 likely acts early in the process of TGF-β1-mediated EMT rather than late. Surprisingly, PP1 significantly interfered with rapid Smad activation in response to TGF-β1. That is, both Smad2/3 nuclear translocation (Fig. 7A and supplemental Fig. 1) and Smad2 phosphorylation (Ser-465/Ser-467) (Fig. 7B) were inhibited by PP1. Because SU6656 did not inhibit Smad activation by TGF-β1 (Fig. 7B), this effect cannot be ascribed to the inhibition of Src family kinases. Thus, the inhibitory effect of PP1 on TGF-β1-mediated EMT seemed to include inhibition of the Smad pathway.

The most likely mechanism for inhibition of the Smad pathway would be direct inhibition of the kinase activity of the TGF-β receptors. A critical residue for PP1 to fit into the ATP binding pocket of mouse c-Src is threonine 340 (corresponding to v-Src threonine 338). If the side chain of the amino acid in this position is small, such as with threonine, serine, cysteine, valine, alanine, or glycine, PP1 can fit into the pocket and inhibit the kinase activity. If the side chain is large, such as with methionine or phenylalanine, PP1 is not an effective inhibitor (64, 65). Thus, as shown in Fig. 8A, PP1 inhibits the activity of Src family members as well as other kinases that have a small residue in this “gatekeeper” position (66). PP1 is not specific for tyrosine kinases as the serine/threonine kinase SAPK2b/p38 is also inhibited by PP1. Kinases with a large residue in this position, such as ZAP-70, Jak2, Ca²⁺/calmodulin-dependent kinase IIα (CamKIIα), and cyclin-dependent kinase 2 (Cdk2), are not inhibited by PP1 (65, 67). Both TGF-β receptor types I and II have appropriate amino acids in the gatekeeper position, making them potential targets for PP1. Thus, we examined the possibility that PP1
directly inhibited the kinase activity of TGF-β receptor type I and/or TGF-β receptor type II.

We cloned the carboxyl-terminal fragments (corresponding to amino acids 148–503 of the mouse TGF-β type I receptor and amino acids 218–592 of the mouse TGF-β type II receptor) into pGST-Parallel-1 vector to produce the active kinase domain in E. coli as described previously (50, 51). Fusion proteins were purified on a glutathione-agarose column, and in vitro kinase assays were done using 2 pmol of TGF-β type I or type II receptor protein and serial concentrations of PP1 at 30 °C for 30 min. After incubation, the reaction was mixed with SDS sample buffer and resolved by SDS-PAGE. The 32P signals of autophosphorylated TGF-β receptors were analyzed by autoradiography (Fig. 8B), and the inhibitory effects of PP1 were determined by a decrease in signal (Fig. 8C). PP1 effectively inhibited the kinase activity of the type I TGF-β receptor (IC50 ~0.4 μM); however, inhibition of the TGF-β type II receptor was weaker (IC50 ~3 μM).

In total, the data presented here showed that although Src was activated when NMuMG/E9 cells were treated with TGF-β, its activity was not essential for EMT to occur. It is likely that PP1 prevented EMT by directly inhibiting the kinase activities of the TGF-β receptors.

DISCUSSION

TGF-β1 treatment increased the overall level of tyrosine phosphorylation in NMuMG/E9 cells, and this additional phosphotyrosine noticeably accumulated in focal adhesions. Indeed, tyrosine phosphorylation on the autophosphorylation site of Src (Tyr-418 of mouse c-Src) was elevated in TGF-β1-treated NMuMG/E9 cells (Fig. 1), and tyrosine phosphorylation of known Src substrates (FAK, paxillin, and p120 catenin) was also increased (Fig. 3). These data clearly indicated that TGF-β1 treatment of NMuMG/E9 cells induced Src activation. Nonetheless, Src activation itself was not essential for TGF-β1-mediated EMT because SU6656, which effectively inhibited Src activity, failed to directly inhibit the kinase activity of TGF-β1-mediated EMT. A, NMuMG/E9 cells, pretreated with Me2SO (DMSO) (a–d), 10 μM PP1 (e–h), or 10 μM STI-571/Gleevec (I–l), were incubated in the absence (a, b, e, f, i, and j) or presence (c, d, g, h, k, and l) of TGF-β1 (2 ng/ml) for 1 day and stained for ZO-1 (a, c, e, g, i, and k) and F-actin (b, d, f, h, j, and l). Photographs were taken using a ×40 dry objective; bar = 10 μm. B, TNE extracts from similarly treated cells were immunoblotted for fibronectin, ZO-1, and GAPDH.
prevent EMT. This was not restricted to mouse mammary cells as we obtained similar results with the human mammary epithelial cell line, MCF10A. That is, in MCF10A cells, which undergo TGF-β-mediated EMT (and show elevated levels of active Src), PP1 prevented EMT, whereas SU6656 did not (supplemental Fig. 2).

Introduction of v-Src or constitutively active c-Src is sufficient to induce EMT in some types of epithelial cells (36, 37). However, in other epithelial cells, v-Src or constitutively active c-Src induces transformation, which is characterized by changes to a more rounded cell shape and a reduction in stress fibers but is quite different from classical EMT (68). Src-induced transformation has been well characterized in fibroblasts, and these investigations have implicated several pathways. FAK phosphorylation by active Src might induce disassembly of Src/FAK complexes, degradation of FAK, and disruption of focal adhesions (32). Moreover, regulation of p190 RhoGAP by active Src might induce actin cytoskeleton disruption (69, 70). These different cellular responses to Src activation seem to be dependent on the cell type, but the mechanisms whereby Src acts differently in different cell types have not been elucidated. Interestingly, forced expression of constitutively active c-Src (Y527F) in NMuMG/E9 cells induced a transformation-like phenotype rather than EMT, providing further evidence that Src is not responsible for the phenotypic changes involved in TGF-β-mediated EMT (data not shown).

Since neither the specific Src inhibitor, SU6656, nor the dominant-negative Src prevented TGF-β1-mediated EMT in NMuMG/E9 cells, whereas PP1 did, an obvious question was how PP1 interfered with TGF-β1-mediated EMT and which of its targets were relevant to EMT. As recent reports have suggested that PP1 is not specific for Src family kinases and is able to inhibit several other kinases including c-Abl, we first speculated that PP1 interfered with EMT by inhibiting c-Abl. Indeed, a recent report showed that c-Abl is responsible for TGF-β-induced morphological changes, cell proliferation, and extracellular matrix-induced gene expression in some fibroblasts (71). It is true that TGF-β did activate c-Abl in NMuMG/E9 cells (data not shown). However, the specific c-Abl inhibitor, STI-571, did not prevent TGF-β1-mediated EMT, indicating that the activity of Abl is not critical to EMT, and thus, it is not the relevant target for PP1 in this system (Fig. 6). A very interesting and unexpected observation was that PP1 interfered with TGF-β1-induced Smad activation, whereas the specific Src inhibitor SU6656 did not, clearly suggesting that the inhibitory effect of PP1 against Smad activation was not through Src inhibition (Fig. 7).

PP1 inhibits kinases that have a gatekeeper residue, which allows PP1...
to fit into the ATP binding site (64, 72). As shown in Fig. 8, type I and type II TGF-β receptors have a serine or a threonine residue, respectively, in the gatekeeper position that could enable PP1 to bind to these kinases, prompting us to examine the possibility that PP1 might directly inhibit the kinase activity of the TGF-β receptors. In vitro kinase assays showed that indeed, PP1 does very effectively inhibit the kinase activity of the type I TGF-β1 receptor and also inhibits the kinase activity of the type II receptor to a lesser degree. Thus, the inhibitory effect of PP1 against TGF-β-mediated EM Tie was likely due to direct inhibition of the receptors. The concentration of PP1 that effectively inhibits the TGF-β receptors in cells is ~10-fold higher than the concentration that is effective in vitro. This parallels the inhibition of Src by PP1 as the reported IC₅₀ for inhibition of autophosphorylation of c-Src in cells is about 3 μM (73), whereas the IC₅₀ for in vitro kinase assays is 0.17 μM (67). The discrepancy in effectiveness, both for Src and for the TGF-β receptors, between in vitro and in vivo experiments is likely due to the limited ability of PP1 to cross the plasma membrane.

PP1 has been widely used as a Src family kinase inhibitor to implicate Src in many cellular events. Our data pointed out that results obtained using PP1 should be treated very carefully, especially when these experiments are examining TGF-β signaling. The similar Src family kinase activities such as cell-cell adhesion and cellular motility, careful attention must be paid to its specificity.
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