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Cell Cycle Signaling by Endothelin-1 Requires Src Nonreceptor Protein Tyrosine Kinase

Rangnath Mishra, Yuan Wang, and Michael S. Simonson

Division of Nephrology and Hypertension, Department of Medicine, School of Medicine, Case Western Reserve University and University Hospitals of Cleveland, Cleveland, Ohio

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ABSTRACT

Cross-talk between G protein-coupled receptors and protein tyrosine kinases is well established, but the phenotypic consequences of these signaling interactions are not completely understood. To investigate the role of Src family kinases in mitogenic signaling by G protein-coupled receptors, we used genetic and pharmacological inhibition of Src to study cell growth in response to endothelin-1. We found that dominant-negative Src and COOH-terminal Src kinase blocked mesangial cell growth in response to endothelin-1, whereas growth induced by v-Ras was unaffected. Endothelin-1-induced cell growth was blocked by the pharmacological Src antagonist 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) but not by the inactive analog 4-amino-7-phenylpyrazol[3,4-*d*]pyrimidine. RNA interference knockdown of Src with on-target but not with off-target small interfering RNAs also inhibited growth in cells treated with endothelin-1. Dominant-negative Src prevented growth in cells activated by platelet-derived growth factor alone or in combination with endothelin-1, which suggests that Src integrates mitogenic signals from diverse classes of cell surface receptors. To further explore the role of Src in mitogenic signaling by G protein-coupled receptors, we sought to determine whether endothelin-1 induced cyclin D1 by a Src-based mechanism. We found that endothelin-1 increased cyclin D1 protein, which was blocked by preincubation with the Src antagonist PP2 and with the protein kinase C antagonist bisindolylmaleimide I. These results provide evidence for a Src- and protein kinase C-based pathway of mitogenic signaling by endothelin-1 receptors that involves cyclin D1.

Although mitogenic signaling is usually associated with receptor tyrosine kinases, many potent mitogens (e.g., endothelin, angiotensin II, and thrombin) stimulate cell proliferation by activating their cognate G protein-coupled receptors in a cell type-specific manner (Adams and Brown, 2001; Marinissen and Gutkind, 2001). Growth control by G protein-coupled receptors has been implicated in normal development and in pathological processes such as cardiac and vascular remodeling, inflammation, and cancer (Adams and Brown, 2001; Marinissen and Gutkind, 2001). Mitogenic signaling by G protein-coupled receptors requires a complex network of highly interacting cell signaling effectors such as mitogen-activated protein kinases and Rho proteins. Ligands that bind to G protein-coupled receptors also activate a broad spectrum of protein tyrosine kinases (Luttrell et al., 1999), but the role of tyrosine kinases in mitogenic signaling by these ligands is not well understood.

Endothelin-1 is a vasoconstrictor peptide that also regulates gene expression and growth of vascular and nonvascular cells (Miyauchi and Masaki, 1999; Kedzierski and Yanagisawa, 2001). Accumulating evidence suggests that endothelin-1 contributes to growth and compensatory remodeling of vascular and myocardial cells in vivo (Miyauchi and Masaki, 1999; Remuzzi et al., 2002; Amiri et al., 2004). One of the important effectors of cell signaling by endothelin-1 seems to be cross-talk between the G protein-coupled ET_A/ET_B receptors and the Src nonreceptor tyrosine kinase. Endothelin-1 rapidly activates the tyrosine kinase activity of Src (Force and Bonventre, 1992; Simonson and Herman, 1993; Simonson et al., 1996a), and Src seems to participate in endothelin-1-directed signaling events such as induction of the *c-fos* transcription factor (Simonson et al., 1996b). The

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ABBREVIATIONS: ET, endothelin; Csk, COOH-terminal Src kinase; siRNA, small interfering RNA; PP2, 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; PP3, 4-amino-7-phenylpyrazol[3,4-*d*]pyrimidine; BIS, bisindolylmaleimide I; PDGF, platelet-derived growth factor; HMC, human mesangial cell; DMEM, Dulbecco's modified Eagle's medium; BrdU, bromodeoxyuridine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; RNAi, RNA interference; ANOVA, analysis of variance; PKC, protein kinase C; CDK, cyclin-dependent kinase; MAPK, mitogen-activated protein kinase.

fact that Src is an important effector in cell signaling by endothelin-1 is underscored by the finding that cell transformation by v-Src selectively amplifies activation of phospholipase C and Ca^{2+} signaling by endothelin-1 in fibroblasts (Mattingly et al., 1992). Despite the apparent importance of endothelin-1 receptor-Src cross-talk in cell signaling, the ability of Src to participate in mitogenic signaling by endothelin-1 remains unclear.

In this study, we investigated whether Src or other Src family kinases participate in signaling cascades linking G protein-coupled endothelin-1 receptors to DNA synthesis and cell proliferation in mesangial cells, an important vascular target cell for endothelin-1 in vivo (Sorokin and Kohan, 2003). We report here that endothelin-1-stimulated cell growth is blocked by a dominant-negative Src mutant, by COOH-terminal Src kinase (Csk), by a pharmacological Src antagonist, and by siRNA knockdown of Src. Moreover, Src activity was required for induction of the cyclin D1 protein in cells treated with endothelin-1. The findings emphasize the importance of cross-talk between endothelin-1 receptors and Src in propagating signals that regulate cell cycle progression.

Materials and Methods

Reagents. Antibodies used for Western blotting were obtained as follows: phospho-Src (PTyr416)- and cyclin D1-specific antibodies were from Cell Signaling Technology Inc. (Beverly, MA); total Src antibody was from BioSource International (Camarillo, CA); and anti-β-Actin antibody from Sigma-Aldrich (St. Louis, MO). PP2, a cell-permeable pharmacological inhibitor of Src, and its inactive analog PP3, were also from Sigma-Aldrich. Bisindolylmaleimide I (BIS) was from Calbiochem (San Diego, CA). Platelet-derived growth factor (PDGF)-BB fraction was obtained from R&D Systems (Minneapolis, MN).

Human Mesangial Cell Culture. Human mesangial cells (HMCs), purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 17% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 5 ng/ml selenite, and 5 μg/ml each of insulin and transferrin in a CO_2 incubator at 37°C in the presence of 5% CO_2. Characterization was performed by phase contrast microscopy and by immunostaining for intermediate filaments and surface antigens as described previously (Schultz et al., 1988). In brief, cells were positive for desmin, vimentin, and myosin, but they did not stain for factor VIII, keratin, or common leukocyte antigen.

Measurements of DNA Synthesis in Transiently Transfected Cells. To study the role of Src in endothelin-1-stimulated DNA synthesis, mesangial cells in six-well plates were transfected, and the cells successfully transfected were identified by staining for β-galactosidase as described previously (Simonson et al., 1995; Wang et al., 2003). Cells were transfected with 0.1 μg of pRSVβGal and with 2 μg of the following plasmids as described previously (Simonson et al., 1996b): pSrcK−, SrcK+, pCMVCsk, pMv-Src, and v-Ha-Ras. Incorporation of 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich) into newly synthesized DNA in transiently transfected cells (i.e., β-galactosidase-positive cells) was also assessed exactly as described previously (Simonson et al., 1995; Wang et al., 2003). In brief, after transfection cells were labeled for 24 h with 20 μM BrdU, monolayers were then washed twice with ice-cold Dulbecco's phosphate-buffered saline and fixed for 5 min on ice with 2.0% formaldehyde/0.2% glutaraldehyde in Dulbecco's phosphate-buffered saline. Cells expressing β-galactosidase were detected by histochemical analysis (Simonson et al., 1995; Wang et al., 2003). Under these conditions, 3 to 5% of mesangial cells were transfected (i.e., β-galactosidase-positive). BrdU incorporation into DNA was then identified by immunocytochemistry using 6 μg of IgG/ml of a specific monoclonal antibody (Roche Diagnostics, Indianapolis, IN) as described previously (Simonson et al., 1995; Wang et al., 2003). Cells were visualized under bright-field microscopy (Nikon Diaphot) with a neutral filter. Red cells (i.e., β-galactosidase-positive) with dark nuclei (BrdU-positive) and light nuclei (BrdU-negative) were counted, and percentage of inhibition of DNA synthesis was calculated as $(N_v - N_e)/N_v$, where N_v is percentage of BrdU-positive nuclei in cells transfected with vector only, and N_e is percentage of BrdU-positive nuclei in cells transfected with expression plasmid. Data are the mean percentage of positive cells calculated from three independent experiments in which >300 transfected cells per experiment were analyzed. Statistical significance for the percentage data were analyzed by the χ^2 test using InStat for Macintosh (GraphPad Software Inc., San Diego, CA).

Measurement of BrdU Incorporation in Cells Treated with PP2 or PP3. To study the effect of a pharmacological Src inhibitor on endothelin-1-induced DNA synthesis, cells were plated in six-well plates. Cells, serum starved for 24 h in DMEM/0.5% FBS for 24, were pretreated with 10 μM PP2 or PP3 for 1 h before adding either 100 nM endothelin-1 or 20% FBS. Cells were fixed and stained for BrdU incorporation as described above.

Western Blotting. Western blotting for PTyrSrc(416) and cyclin D1 in HMCs was performed as reported previously by us with minor modifications (Mishra et al., 2003). In brief, cells were washed with ice-cold PBS and scraped in CHAPS extraction buffer (50 mM PIPES/HCI, pH 6.5, 2 mM EDTA, 0.1% CHAPS, 20 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 5 mM dithiothreitol, 2 mM sodium-pyrophosphate, 1 mM Na_3VO_4, and 1 mM NaF). An aliquot was saved for protein determination using the DC assay (Bio-Rad, Hercules, CA). An equal volume of 2× SDS sample buffer was immediately added to the extraction buffer, and Western blotting was carried out using antibodies for human pTyr416Src or cyclin D1. The membranes were reprobed for either total Src or β-actin to confirm equal protein loading. As described previously (Mishra et al., 2004), the Western blots were analyzed by densitometry in NIH Image by normalizing values for the relevant protein to the highest value within each experiments (maximum level 1).

siRNA-Mediated Src Gene Knockdown by RNAi. A pool of four complementary siRNA oligonucleotide duplexes targeted specifically to human Src (GenBank NM_005417) and designed to leave 2-base pair overhangs were obtained from Dharmacon Inc. (Lafayette, CO). The algorithms for determining the siRNA sequences have been described in detail previously (Reynolds et al., 2004). The siRNAs were introduced into mesangial cells with LipofectAMINE 2000 (Invitrogen), according to the manufacturer's instructions. To determine the most effective dose of siRNA in mesangial cells, transfection was carried out with increasing concentrations of siRNA from 0 to 50 nM. In brief, mesangial cells were plated at 50 to 70% density in 35-mm Petri dishes a day before transfection. Cells were incubated with siRNA duplexes at final concentrations ranging from 2.5 to 50 nM in 2 ml of low serum-containing Opti-MEM I (5% FBS) for 18 h. After the incubation with siRNAs in low-serum medium, the level of serum was adjusted to that in complete medium, and cells were grown for 48 h. Total Src was assessed by Western blotting to determine the amount of Src siRNA that knocked down the Src protein level most effectively. In parallel, nontarget siRNA in equivalent concentrations was used to control for off-target changes in the level of Src in mesangial cells. Src siRNA at a final concentration of 20 nM was found to be most effective in knocking down the level of Src without any significant ill effect on cell survival or on the level of β-actin protein. Therefore, siRNA at a concentration of 20 nM was used for further studies of ET-1- induced mesangial cell proliferation. After transfecting the cells with siRNA, cells were allowed to recover and grow for 48 h in complete medium and then serum starved for 24 h in DMEM/0.5% FBS before proceeding with cell counts by

hemocytometer. Statistical significance was calculated by ANOVA and Bonferroni multiple correction.

Results

We used cultured human mesangial cells to investigate the potential role of Src in mitogenic signaling by endothelin-1. Mesangial cells derive from the glomerular microvasculature where endothelin-1 participates in the compensatory growth of mesangial cells and remodeling of the glomerular capillaries after renal injury (Sorokin and Kohan, 2003). We previously showed that ET_A G protein-coupled receptors stimulate Src activity and tyrosine phosphorylation in these cells (Simonson and Herman, 1993; Simonson and Rooney, 1994; Simonson et al., 1996a). In addition, Src is required for induction of *c-fos* by endothelin-1 in mesangial cells (Simonson et al., 1996b). Using an activation state-specific antibody against PTyr416 Src, we found that endothelin-1 induced a rapid and robust activation of Src in human mesangial cells (Fig. 1, A and B). Maximal Src activation occurred at 10 min and declined slowly thereafter (Fig. 1B). These results suggest that the cell signaling pathways linking endothelin-1 receptors to putative long-term events such as cell proliferation are intact in mesangial cells and might involve Src protein tyrosine kinases.

To determine whether Src contributes to mitogenic signaling by endothelin-1, we first used a dominant-negative mutant strategy. A kinase-inactivating mutation in Src (Lys295 to Met, SrcK−) forms a dominant-negative Src protein, which effectively blocks signaling by some but not all growth factor receptors in NIH 3T3 fibroblasts. Using a transient transfection assay to identify the effects of transfected genes on DNA synthesis, endothelin-1 increased the percentage of cells with BrdU+ nuclei when added to cells with vector alone (Fig. 2, A and B, no addition, 23.4%; ET-1, 57.4%). In contrast, in cells transfected with the Src dominant-negative mutant SrcK−, ET-1 did not increase the percentage of BrdU+ cells (Fig. 2B, Con, 18.6%; ET-1, 20.5%). Two additional control experiments confirmed that SrcK− did not block DNA synthesis in a nonspecific manner. First, as a control for the specificity of SrcK−, we note that the number of BrdU+ cells was not altered significantly in cells stimulated with FBS or in cells transfected with v-Ras (Fig. 2B), which we have previously shown lies downstream in the ET-1-Src signaling pathway (Herman and Simonson, 1995; Simonson et al., 1996b). Second, the effects of dominant-negative mutants should be reversible, and we showed that overexpression of v-Src reversed inhibition of endothelin-1-stimulated DNA synthesis by Src K− (Fig. 2B). Together, these findings suggest that the dominant-negative actions of Src K− are specific and that Src contributes to mitogenic signaling by endothelin-1 in mesangial cells.

Additional evidence that Src contributes to mitogenic signaling by endothelin-1 was obtained by transfecting cells with a vector Csk, which phosphorylates the COOH-terminal tyrosine in Src and in other Src-family kinases and inactivates the kinases by maintaining the repressed conformation (Bjorge et al., 2000). Gene targeting and other studies confirm that Csk negatively regulates Src activity in vivo (Bjorge et al., 2000). In cells transfected with vector alone, endothelin-1 increased the number of BrdU+ nuclei (Fig. 3, A and B, Con, 31.8%; ET-1, 58.1%). Transfection with Csk attenuated the endothelin-1-stimulated increase in BrdU+ cells (Fig.

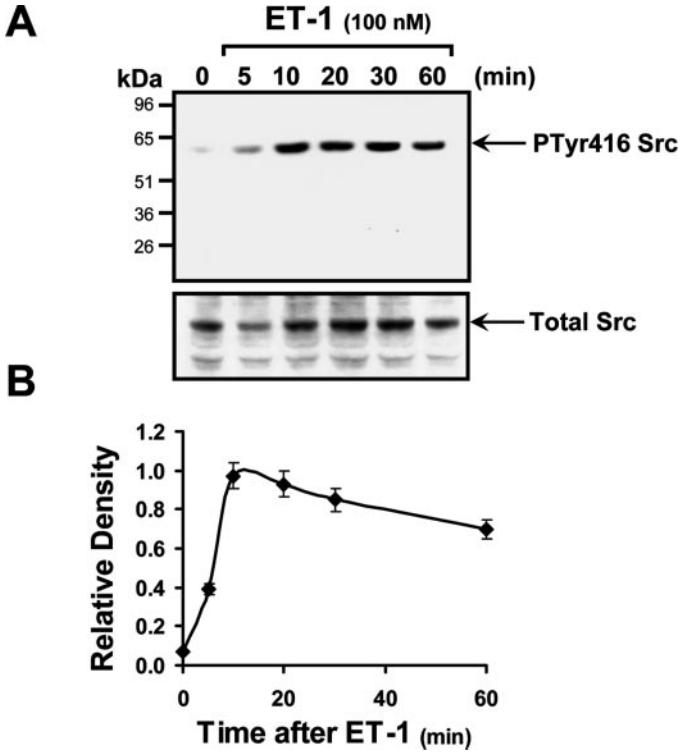

Fig. 1. ET-1 activates Src in human mesangial cells. A, ET-1 was added to quiescent mesangial cells, and the activation state-specific phosphorylation of Src (i.e., PTyr416) was measured by Western blotting at the times indicated. The blot was reprobed with an antibody that recognizes total Src to ensure that an equivalent amount of Src was present in each lane. B, densitometric analysis of Western blots from $n = 4$ independent experiments illustrates the temporal activation of Src by ET-1.

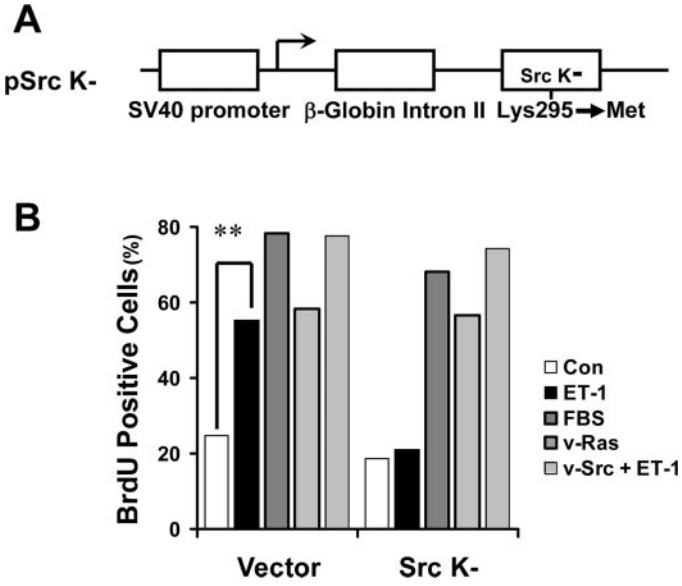

Fig. 2. Expression of a dominant negative SrcK− mutant blocks ET-1-stimulated DNA synthesis. A, schematic diagram of the expression vector used to transfect mesangial cells with the dominant negative mutant of Src (Lys295 mutated to Met). B, incorporation of BrdU, an indicator of DNA synthesis, was measured in cells transfected with vector alone or with SrcK− and treated with media alone (Con), 100 nM ET-1, or 10% FBS. As controls for possible nonspecific effects, cells were cotransfected with vectors expressing constitutively active v-Ras or v-Src as described under *Materials and Methods*. Statistical significance versus control was calculated by the χ^2 test for percentage data. **, $p < 0.01$.

3B, Con, 30.1%; ET-1, 36.5%). Csk partially inhibited the mitogenic response to FBS but had no effect on DNA synthesis in cells transfected with v-Ras (Fig. 3B). Together, these results with Csk support the hypothesis that Src or other Src family kinases contribute to mitogenic signaling by endothelin-1.

To obtain independent evidence that Src contributes to mitogenic signaling by endothelin-1, we used a selective pharmacological antagonist of Src, PP2, and its inactive analog PP3 (Hanke et al., 1996). In the presence of PP3, ET-1 stimulated a 1.7-fold increase in BrdU+ nuclei (Fig. 4, Con, 31.9%; endothelin-1, 55.1%). However, consistent with a role for Src, PP2 completely prevented the increase in BrdU+ cells when endothelin-1 was added (Fig. 4, Con, 30.4%; endothelin-1, 33.1%). PP2 had no effect on the increase in BrdU+ nuclei in cells treated with FBS, which suggested that PP2 did not nonspecifically inhibit DNA synthesis (Fig. 4).

One potential difficulty in interpreting the studies described above is that SrcK−, Csk, and PP2 might significantly block the activity of other Src family kinases because of the structural homology between members of the Src family. We therefore turned to RNA interference to knockdown the Src gene and determine the effect of on-target and off-target siRNAs for human Src on cell growth stimulated by ET-1. Forty-eight hours after transfection with a pool of four siRNA complexes specifically directed against human Src, total Src protein was significantly reduced by 5 nM siRNA complexes, and concentrations from 10 to 50 nM effectively knocked down Src protein below detectable levels by Western blotting (Fig. 5A). As controls for specificity of Src knockdown, we note that the level of β-actin protein was unaffected by the on-target Src siRNAs, with suggests that the siRNA complexes as these low concentrations did not induce an interferon response that generally blocks protein synthesis. Addition of nontargeting siRNA complexes at 20 and 50 nM did not reduce total Src or β-actin levels (Fig. 5A). The spe-

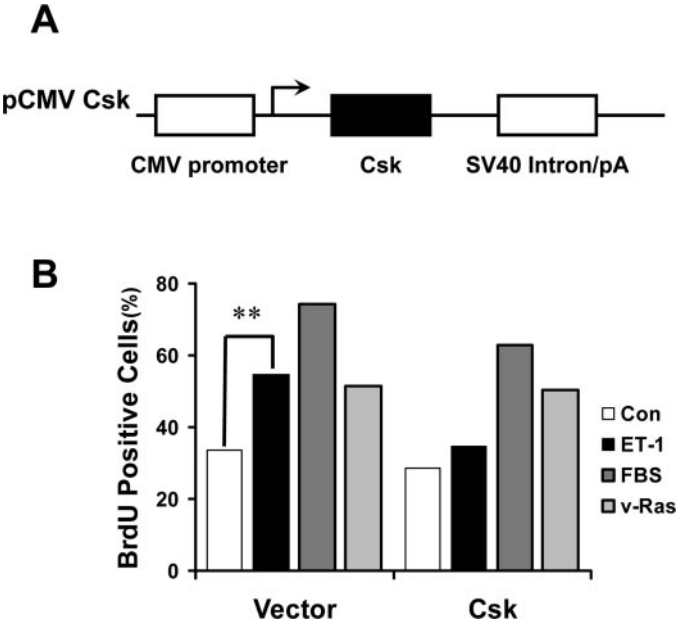

Fig. 3. The Src-inactivating kinase Csk blocks ET-1-stimulated DNA synthesis. A, schematic diagram of the plasmid used for constitutive expression of Csk in mesangial cells. B, BrdU incorporation was measured in cells transfected with vector alone or with Csk and treated with 100 nM ET-1 or 20% FBS. Cells were also cotransfected with a vector expressing constitutively active v-Ras. Data are the mean percentage of BrdU+ cells from $n = 4$ independent experiments with statistical significance calculated by the χ^2 test versus control. **, $p < 0.01$.

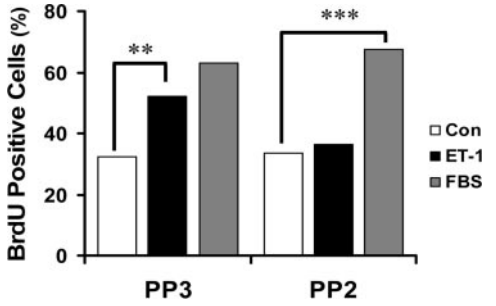

Fig. 4. Pharmacological inhibition of Src activity prevents ET-1- but not FBS-induced DNA synthesis. BrdU incorporation was assessed in ET-1- or FBS-stimulated mesangial cells pretreated with the Src inhibitor PP2 (10 μM for 1 h) or with an equivalent concentration of its inactive analog PP3. Data are the mean of $n = 4$ independent experiments with statistical significance calculated by the χ^2 test versus no addition. **, $p < 0.01$; ***, $p < 0.001$.

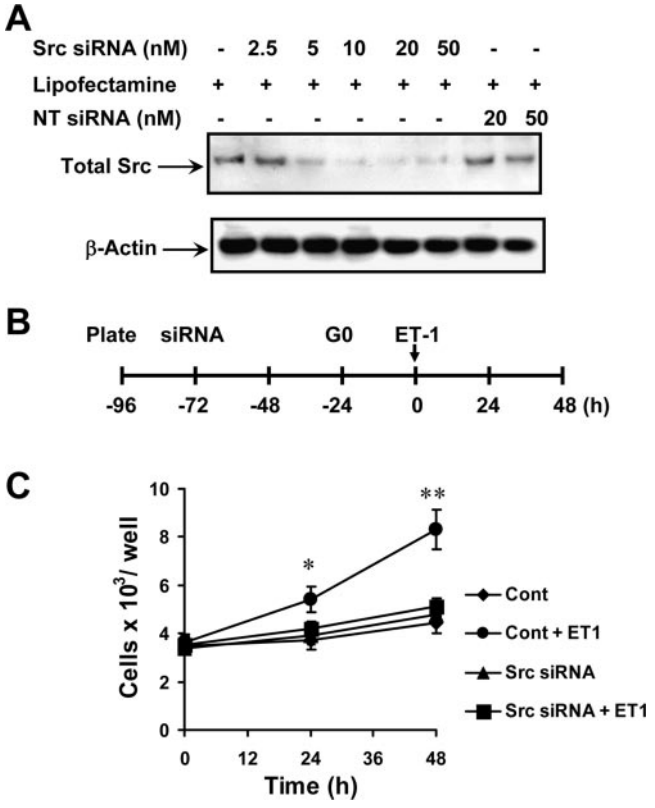

Fig. 5. Knockdown of Src by Src siRNA blocks ET-1-induced proliferation of mesangial cells. A, as detailed under *Materials and Methods*, mesangial cells were transfected with increasing amounts of on-target Src siRNA or with nontarget (NT) siRNA for 48 h before assessment of total Src protein by Western blotting. Results are representative of three independent experiments with similar results. B, schematic illustration of the experimental design for testing the effects of Src siRNA on ET-1-induced proliferation. G_0 indicates initiation of a 24-h period in serum-free medium to render the cells quiescent before addition of ET-1 at time 0 and cell counts at 24 and 48 h thereafter shown below in C. C, cell counts of human mesangial cells treated with media alone (Cont), media plus 100 nM ET-1, and media containing 20 nM Src siRNA with or without ET-1. Data are mean ± S.D. from three independent experiments, and statistical differences between treatment groups at 24 and 48 h were determined by ANOVA with Bonferroni multiple comparison post tests. *, $p < 0.05$; **, $p < 0.01$.

cific and robust reduction of Src protein by siRNAs at 48 h provides a reliable basis for analyzing the role of Src in mitogenic signaling by ET-1.

Endothelin-1 was added 72 h after mock transfection or transfection with the Src siRNAs, and cell counts were measured thereafter at 24 and 48 h (Fig. 5B). When added to mock-transfected cells, endothelin-1 stimulated a time-dependent increase of cell number at 24 and 48 h (Fig. 5C). In contrast, addition of endothelin-1 to cells transfected with Src siRNAs failed to increase cell number (Fig. 5C). These results are consistent with our previous genetic and pharmacological approaches to block Src and strongly support the hypothesis the Src plays a critical role in cell proliferation in mesangial cells treated with endothelin-1.

In pathological remodeling of the glomerulus, mesangial cells are exposed to multiple mitogens in addition to endothelin-1, including PDGF (Haas et al., 1999). We therefore sought to determine whether the role of Src in mitogenic signaling was restricted to G protein-coupled receptors or whether Src also participates in mitogenic signaling by the PDGF receptor tyrosine kinase. PDGF stimulated a rapid but more transient activation of Src than did endothelin-1 (compare Fig. 6A with Fig. 1). In cells transfected with vector alone, saturating concentrations of PDGF increased the percentage of BrdU-positive cells from 20.7 to 62.4% (Fig. 6B). In contrast, transfection with dominant-negative Src prevented any increase in DNA synthesis in cells treated with PDGF (Fig. 6B). The percentage of BrdU+ cells was unaffected by transfection with wild-type SrcK+. To determine whether Src-independent pathways could be recruited when both mitogens were present, we measured DNA synthesis in cells treated with saturating concentrations of both endothelin-1 and PDGF. The percentage of BrdU+ cells was nearly identical when either PDGF or PDGF plus endothelin-1 was added (Fig. 6C). It is noteworthy that DNA synthesis in response to addition of both mitogens was effectively blocked by transfection with SrcK− (Fig. 6C). These results are consistent with the idea that both PDGF and endothelin-1 use

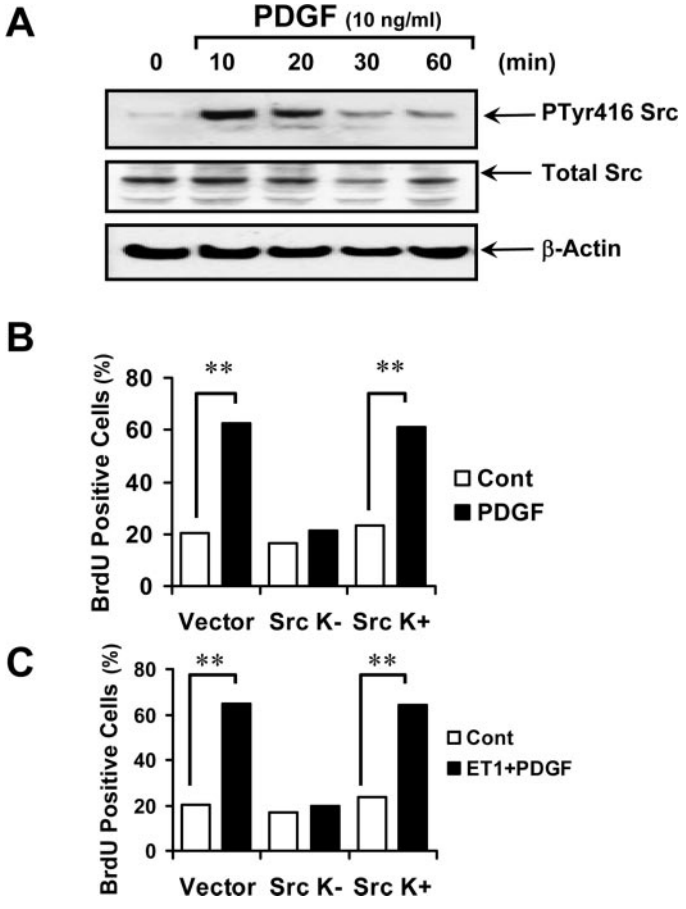

Fig. 6. PDGF-induced mesangial cell DNA synthesis is blocked by dominant-negative SrcK−. A, PDGF was added to quiescent mesangial cells and Src activity was assessed by Western blotting at the times indicated. The blot was reprobed for total Src and for β-actin. Results are representative of three independent experiments. B, PDGF (10 ng/ml) was added to mesangial cells transfected with vector alone, SrcK−, or wild-type SrcK+, and the number of BrdU+-transfected cells was calculated. C, 100 nM ET-1 plus PDGF (10 ng/ml) were added simultaneously to cells transfected with vector, SrcK−, or SrcK+. The number of BrdU+-transfected cells was than analyzed as described above. Statistical significance versus vector alone was calculated by the χ^2 test versus no addition with three independent experiments in both B and C. **, $p < 0.01$.

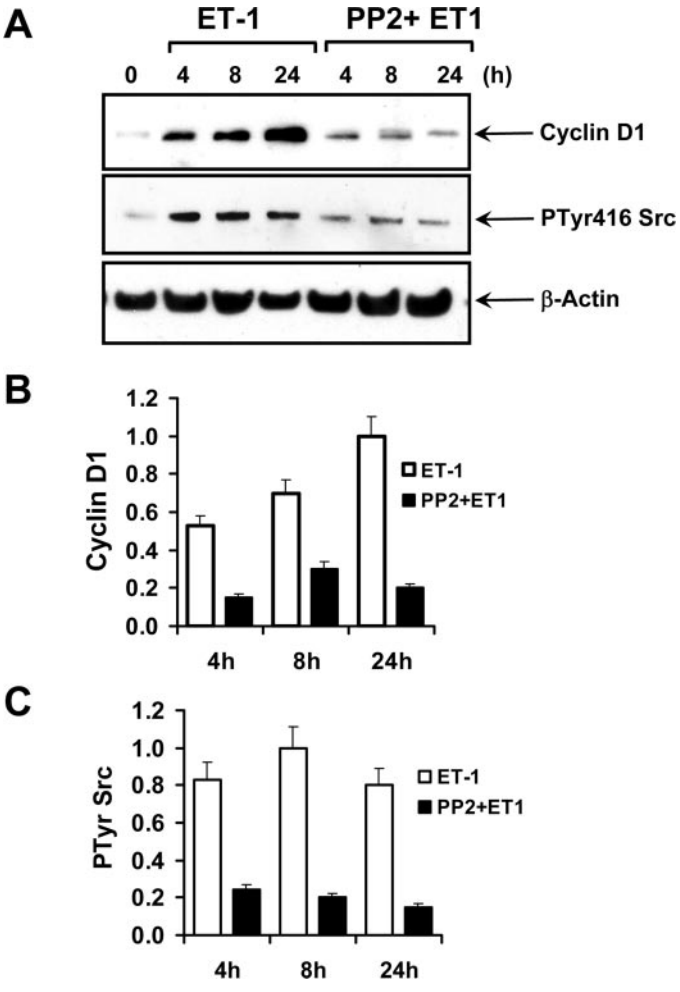

Fig. 7. ET-1 increases cyclinD1 protein by a Src-based signaling pathway. A, Quiescent mesangial cells were treated with 100 nM ET-1 alone or in cells pretreated for 1 h with a 10 μM concentration of the pharmacological Src inhibitor PP2. The level of cyclinD1 protein and of active Src was measured by Western blotting in equivalent amounts of mesangial cell protein as indicated by constant levels of β-actin. Levels of cyclinD1 protein (B) or of PTyr416Src (C) were analyzed by densitometry from three independent experiments and confirm that PP2 reduced Src activation and the level of cyclinD1 at each time point. All levels for cyclinD1 (B) or PTyrSrc (C) were significantly lower (at least $p < 0.01$) in PP2-treated cells compared with ET-1 alone as calculated by t tests at 4, 8, and 24 h.

Src in mitogenic signaling in mesangial cells and that Src is required even when both mitogens were present simultaneously.

To better understand the mechanisms by which a Src-based endothelin-1 signal results in cell cycle progression, we sought to determine whether Src is required for stimulation of cyclinD1 synthesis by endothelin-1. Transcriptional upregulation of D-type cyclins occurs in G_1, when they associate with and activate CDK4 or CDK6 to promote cell cycle progression. Endothelin-1 stimulated a 3.7-fold increase in cyclinD1 protein after 4 h and a 8.3-fold increase after 24 h (Fig. 7, A and C). Pretreatment with the pharmacological Src inhibitor PP2 attenuated the increase in cyclinD1 protein at 4, 8, and 24 h in cells treated with endothelin-1 (Fig. 7, A and B). We also confirmed that PP2 attenuated the endothelin-1-stimulated increase in PTyr416 Src (Fig. 7, A and C). The inactive Src antagonist PP3 had no effect on endothelin-1-stimulated cyclinD1 protein (data not shown). These results suggest that Src activation is required for endothelin-1 stimulation of cyclinD1, which in turn contributes to cell cycle progression in mesangial cells.

We next sought to determine whether protein kinase C (PKC), an important effector of ET-1 signaling, also contributed to cyclinD1 induction by ET-1 in mesangial cells. The elevation of cyclinD1 protein by ET-1 in mesangial cells was markedly attenuated by BIS, a selective inhibitor of conventional type PKCs (Fig. 8, A and B). In addition, BIS blocked mesangial cell proliferation in response to ET-1 (Fig. 8C). BIS alone had no effect on mesangial cell growth. Together, these results suggest that PKC is also required for induction of cyclinD1 by ET-1. To determine the relationship between PKC and Src in the ET-1 signaling pathway, we sought to determine whether inhibition of PKC would block activation of Src by ET-1. In fact, preincubation with BIS completely prevented Src activation by ET-1 (Fig. 9, A and B). To demonstrate that BIS prevented activation of Src by PKCs, we also showed that BIS prevented Src activation by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (Fig. 9C). These results suggest that PKC is upstream of Src in the ET-1 signaling pathway.

Discussion

The G protein-coupled endothelin-1 receptors evoke signaling cascades leading to cell cycle progression and cell growth (Sorokin and Kohan, 2003). Previous studies have shown that endothelin-1 receptors activate nonreceptor protein tyrosine kinases such as Src, Pyk2, and focal adhesion kinase (Sorokin and Kohan, 2003), but the role of specific nonreceptor protein tyrosine kinases in mitogenic signaling by endothelin-1 has not been extensively studied. The goal of the present study was to determine whether Src or Src family kinases contribute to mitogenic signaling by endothelin-1 in human mesangial cells. Our results, based on independent genetic and pharmacological approaches to antagonize Src activity, strongly suggest that Src participates in a pathway that propagates signals from endothelin-1 receptors to elements of the cell cycle regulatory machinery that promote proliferation. Src or closely related Src family kinases seem to participate in a pathway specifically linking endothelin-1 to induction of the cyclinD1 gene, an important regulatory step increasing CDK activity and cell cycle progression.

Src Kinase and Mitogenic Signaling by Endothelin-1. We have previously shown that endothelin-1 increases Src activity in mesangial and other cell types (Simonson and Herman, 1993; Simonson et al., 1996a). An additional rationale for this study was that Src participates in ET-1-directed signaling events that might be linked to mitogenesis such as induction of the *c-fos* transcription factor (Simonson et al., 1996b); in addition, Schieffer et al. (1997) have previously shown that electroporation of Src-neutralizing antibodies in vascular smooth muscle cells blocks proliferation induced by ET-1 (Schieffer et al., 1997). Our approach was to test the role of Src by using both genetic (dominant-negative mutant, Csk, and Src siRNA) and pharmacological (PP2) methods that antagonize Src-dependent signaling by independent mechanisms. Transfection with dominant-negative Src or with Csk effectively prevented DNA synthesis in response to endothelin-1. Several observations suggest that dominant-

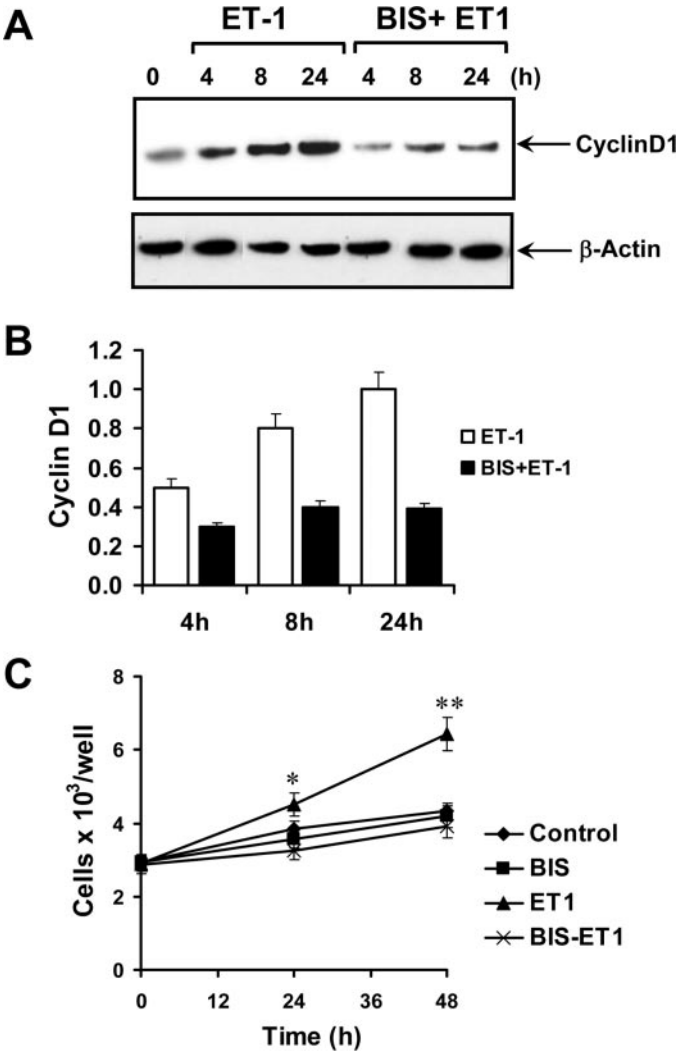

Fig. 8. Protein kinase C contributes to induction of cyclinD1 by endothelin-1. A, endothelin-1 was added alone or in cells preincubated with 5 μM selective PKC inhibitor BIS. Levels of cyclinD1 protein were measured by Western blotting and the densitometric analysis of cyclin D1 from three independent experiments is presented in B. C, quiescent mesangial cells were treated with endothelin-1 alone, BIS alone, BIS plus endothelin-1, or medium alone (control). Cell counts are presented as mean ± S.D. from three independent experiments, and statistical differences between treatment groups at 24 and 48 h were determined by ANOVA with Bonferroni multiple comparison post tests. *, $p < 0.05$; **, $p < 0.01$.

negative Src or Csk did not block endothelin-1-stimulated mitogenesis by nonspecifically inhibiting cell cycle progression. First, transfection with the empty vector alone did not alter endothelin-1-stimulated DNA synthesis, and transfection with wild-type SrcK+ similarly did not alter mitogenic signaling by endothelin-1. Second, the inhibitory effects of dominant-negative Src were reversed by overexpression of v-Src. Third, dominant-negative SrcK− did not alter DNA synthesis in cells treated with FBS, which presumably contains growth factors that signal by Src-independent mechanisms. Fourth, Ras acts downstream of Src in endothelin-1 signaling in mesangial cells (Herman and Simonson, 1995; Simonson et al., 1996b), and transfection with SrcK− or with Csk did not block DNA synthesis in response to a constitutively activate v-Ras construct. Together, these results support the hypothesis that Src helps propagate endothelin-1 postreceptor signals that drive mitogenesis in mesangial cells.

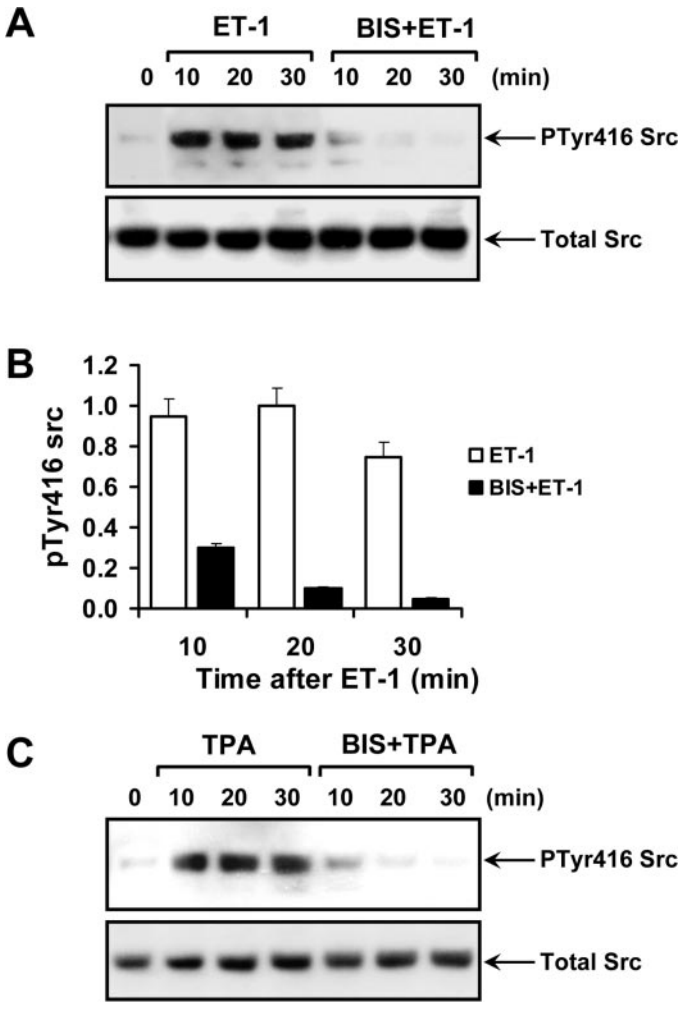

Fig. 9. Protein kinase C activity is required for Src activation by endothelin-1. A, to determine whether PKC was upstream of Src in signaling by endothelin-1, quiescent mesangial cells were treated with 100 nM endothelin-1 or endothelin-1 and 5 μM BIS. Src activation was measured by Western blotting using the activation state-specific antibody. Equivalent amounts of total Src protein were present in each lane. B, Levels of PTyr416Src were analyzed by densitometry from three independent experiments and confirm that BIS reduced Src activation at each time point. C, To confirm that BIS could inhibit activation of Src by PKC, 100 nM 12-*O*-tetradecanoylphorbol-13-acetate was added to quiescent cells or to cells preincubated with 5 μM BIS. PTyr416 Src levels were measured by Western blotting, and similar results were observed in three experiments.

Another observation that supports a role for Src in mitogenic signaling by endothelin-1 is that PP2 but not PP3 effectively prevents DNA synthesis in cells exposed to endothelin-1. PP2 is a selective inhibitor of Src family kinases that binds to Thr338 in c-Src and prevents derepression of the inactive conformation. PP2 prevented endothelin-1-stimulated DNA synthesis at concentrations that have minimal effects on other types of protein tyrosine kinases such as c-Abl and the epidermal growth factor receptor (Hanke et al., 1996). Furthermore, the finding that PP3, which is almost structurally identical to PP2 but lacks significant Src inhibition, failed to block endothelin-1-stimulated DNA synthesis suggests that the inhibition by PP2 was related to its Src inhibitory properties.

One potential problem in interpreting these results is that SrcK−, Csk, and PP2 can inhibit other closely related members of the Src family such as Fyn and Lyk. Mesangial cells express abundant amounts of p62 Yes, but endothelin-1 does not activate Yes protein tyrosine kinase activity in these cells (Simonson et al., 1996b). However, given the potential interactions of Src−, Csk, and PP2 with other Src family kinases, we cannot formally rule out their possible involvement in mitogenic signaling by endothelin-1. To help address this issue, we turned to RNAi, which has the potential to specifically knock down single members of gene families while having little or no effect on closely related genes (McManus and Sharp, 2002). Using a pool of siRNA complexes designed to specifically knockdown human Src, we reduced the level of Src protein to nearly undetectable levels by 48 after transfection. Endothelin-1 increased mesangial cell growth when added to mock-transfected cells. When cells transfected with the Src siRNA were made quiescent and then treated with endothelin-1, the proliferative response was inhibited, consistent with an essential role for Src in mitogenic signaling. We note that 20 nM Src siRNAs did not reduce β-actin levels, as would be expected if the siRNAs induced a nonspecific, interferon type of response that could globally block mRNA translation. Further evidence for a specific, on-target effect of the Src siRNAs was that identical concentrations of an off-target siRNA, which according to bioinformatic criteria has at least four mismatches to any known human gene (including Src family genes) and which at 20 nM did not significantly alter the expression of human genes analyzed globally by microarray (Dharmacon Inc., personal communication), did not reduce total Src or β-actin levels. This off-target siRNA also interacts with the RNA-induced silencing complex and so serves as an important negative control for any nonspecific but RNA-induced silencing complex-dependent gene silencing effects. Thus, the results with Src siRNA provide strong support for the hypothesis that Src participates in mitogenic signaling by endothelin-1.

Induction of CyclinD1 by an Endothelin-1/Src-Based Signaling Cascade. To further characterize how the endothelin-1/Src-based signaling cascade induces proliferation in mesangial cells, we studied the role of Src in elevating cyclinD1 protein in cells treated with endothelin-1. The D-type cyclins are critical integrators of mitogenic signaling, and their expression is one of the important endpoints of the Ras-MAPK pathway (Obaya and Sedivy, 2002). Fluctuations in the protein level of the D-type cyclins (cyclinD1, D2, and

D3) represent the primary mechanism for regulation of CDK4 and CDK6. When growth factors are added to quiescent cells, D-type cyclins are the first to be expressed, and by forming active heterodimeric complexes with CDK4 and CDK6, they help drive cells through G_1 into S phase (Obaya and Sedivy, 2002). The importance of cyclinD1 regulation by ET-1 derives in part from the fact that D-type cyclins are rate-limiting for the formation of active Cdk4 and Cdk6 complexes (Obaya and Sedivy, 2002). In addition, induction of cyclinD1 was previously shown to be necessary for endothelin-1-induced proliferation in mesangial cells (Terada et al., 1998), and induction of cyclin D1 by endothelin-1 in astrocytes is blocked by PP2 (Koyama et al., 2004). Thus, regulation of D-type cyclin expression by endothelin-1 represents a potential step for integration of mitogenic signals propagated by endothelin-1 receptors.

Using pharmacological inhibition of Src, we showed that endothelin-1-stimulated Src activity is essential for increasing the level of cyclinD1 in mesangial cells. Endothelin-1 stimulated a robust increase in cyclinD1 protein, which was essentially abolished by preincubation with PP2 but not with the inactive PP3. Under the same conditions, we confirmed that PP2 greatly reduced PTyr 416 phosphorylation of Src. Together, these results provide strong support for the endothelin-1/Src-based signaling pathway in elevating cyclinD1 in mesangial cells and promoting cell cycle progression through G_1 into S phase. A previous study in NIH 3T3 cells reported that the PKC inhibitor calphostin C failed to block cyclinD1 induction by endothelin-1 (Suzuki et al., 1999). In contrast, our results suggest that PKC plays an important role upstream of Src in the pathway by which ET-1 induces cyclinD1. The reason for the apparent difference between the previous report (Suzuki et al., 1999), and our data in mesangial cells are not immediately apparent but might result from the use of different PKC inhibitors or a cell type-specific difference in PKC-cyclin D1 signaling in mesangial cells versus NIH 3T3 cells. Although we did not specifically test the putative role of the Ras-MAPK pathway in cyclinD1 induction, previous work has convincingly linked this pathway to induction of D-type cyclins in a variety of cell types (Obaya and Sedivy, 2002), and we have previously shown that Src functions upstream of Ras in mesangial cells (Herman and Simonson, 1995; Simonson et al., 1996b). In fact, extracellular signal-regulated kinase activity is required for induction of cyclinD1 and for DNA synthesis in many cell types. It seems likely, therefore, that the endothelin-1/Src-based signal induces cyclinD1 by a Ras-MAPK pathway, although additional experiments are required to prove this hypothesis.

In summary, our results provide support for the importance of an endothelin-1/Src-based signaling cascade in DNA synthesis and cell cycle progression in mesangial cells. By extension, it seems likely that endothelin-1/Src-based signaling contributes to growth and compensatory remodeling of vascular and myocardial cells in vivo, which has been implicated in fibroproliferative response of these cells to injury (Miyauchi and Masaki, 1999; Remuzzi et al., 2002; Amiri et al., 2004). Finally, these results point to a widening role for nonreceptor protein tyrosine kinases in propagating mitogenic signals from G protein-coupled receptors.

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References

Adams JW and Brown JH (2001) G-proteins in growth and apoptosis: lessons from the heart. *Oncogene* **20:**1626–1634.

Amiri F, Virdis A, Neves MF, Iglarz M, Seidah NG, Touyz RM, Reudelhuber TL, and Schiffrin EL (2004) Endothelium-restricted overexpression of human endothelin-1 causes vascular remodeling and endothelial dysfunction. *Circulation* **110:**2233–2240.

Bjorge JD, Jakymiw A, and Fujita DJ (2000) Selected glimpses into the activation and function of Src kinase. *Oncogene* **19:**5620–5635.

Force T, Kyriakis JM, Auruch J, Bonventre JU (1991) Endothelin, vasopressin, and angiotensin II enhance tyrosine phosphorylation by protein kinase C-dependent pathways in glomerular mesangial cells. *J Biol Chem* **266:**6650–6656.

Haas CS, Schocklmann HO, Lang S, Kralewski M, and Sterzel RB (1999) Regulatory mechanism in glomerular mesangial cell proliferation. *J Nephrol* **12:**405–415.

Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollock BA, and Connelly PA (1996) Discovery of a novel, potent and Src family-selective tyrosine kinase inhibitor. *J Biol Chem* **271:**695–701.

Herman WH and Simonson MS (1995) Nuclear signaling by endothelin-1: a Ras pathway for activation of the c-fos serum response element. *J Biol Chem* **270:**11654–11661.

Kedzierski RM and Yanagisawa M (2001) Endothelin system: the double-edged sword in health and disease. *Annu Rev Pharmacol Toxicol* **41:**851–876.

Koyama Y, Yoshioka Y, Shinde M, Matsuda T, and Baba A (2004) Focal adhesion kinase mediates endothelin-induced cyclin D3 expression in rat cultured astrocytes. *J Neurochem* **90:**904–912.

Luttrell LM, Daaka Y, and Lefkowitz RJ (1999) Regulation of tyrosine kinase cascades by G-protein-coupled receptors. *Curr Opin Cell Biol* **11:**177–183.

Marinissen MJ and Gutkind JS (2001) G-protein-coupled receptors and signaling networks: emerging paradigms. *Trends Pharmacol Sci* **22:**368–376.

Mattingly RR, Wasilenko WJ, Woodring PJ, and Garrison JC (1992) Selective amplification of endothelin-stimulated inositol 1,4,5-trisphosphate and calcium signaling by v-Src transformation of Rat-1 fibroblasts. *J Biol Chem* **267:**7470–7477.

McManus MT and Sharp PA (2002) Gene silencing in mammals by small interfering RNAs. *Nat Rev Genet* **3:**737–747.

Mishra R, Emancipator SN, Miller C, Kern T, and Simonson MS (2004) Adipose differentiation related protein and regulators of lipid homeostasis identified by gene expression profiling in murine *db/db* diabetic kidney. *Am J Physiol* **286:**F913–F921.

Mishra R, Leahy P, and Simonson M (2003) Gene expression profile of endothelin-1-induced growth in glomerular mesangial cells. *Am J Physiol* **285:**C1109–C1115.

Miyauchi T and Masaki T (1999) Pathophysiology of endothelin in the cardiovascular system. *Annu Rev Physiol* **61:**391–415.

Obaya AJ and Sedivy JM (2002) Regulation of cyclin-Cdk activity in mammalian cells. *Cell Mol Life Sci* **59:**126–142.

Remuzzi G, Perico N, and Benigi A (2002) New therapeutics that antagonize endothelin: promises and frustrations. *Nat Rev Drug Dis* **1:**986–1001.

Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, and Khvorova A (2004) Rational siRNA design for RNA interference. *Nature (Lond) Biotech* **22:**326–330.

Schieffer B, Drexler H, Ling B, and Marrero MB (1997) G protein-coupled receptors control vascular smooth muscle cell proliferation via pp60c-Src and p21ras. *Am J Physiol* **272:**C2019–C2030.

Schultz PJ, DiCorleto PE, Silver BJ, and Abboud HE (1988) Mesangial cells express PDGF mRNAs and proliferate in response to PDGF. *Am J Physiol* **255:**F674–F684.

Simonson MS and Herman WH (1993) Protein kinase C and protein tyrosine kinase activity contribute to mitogenic signaling by endothelin-1: cross-talk between G protein-coupled receptors and pp60c-Src. *J Biol Chem* **268:**9347–9357.

Simonson MS, LePage DF, and Walsh K (1995) Rapid characterization of growth-arrest genes in transient transfection assays. *Biotechniques* **18:**434–442.

Simonson MS and Rooney A (1994) Characterization of endothelin receptors in mesangial cells: evidence for two functionally distinct endothelin binding sites. *Mol Pharmacol* **46:**41–50.

Simonson MS, Wang Y, and Herman WH (1996a) Ca^{2+} channels mediate protein tyrosine kinase activation by endothelin-1. *Am J Physiol* **270:**F790–F797.

Simonson MS, Wang Y, and Herman WH (1996b) Nuclear signaling by endothelin-1 requires Src protein tyrosine kinases. *J Biol Chem* **271:**77–82.

Sorokin A and Kohan DE. Physiology and pathophysiology of endothelin-1 in renal mesangium. *Am J Physiol* **285:**F579–F589.

Suzuki E, Nagata D, Kakoki M, Hayakawa H, Goto A, Omata M, and Hirata Y (1999) Molecular mechanisms of endothelin-1-induced cell cycle progression. *Circ Res* **84:**611–619.

Terada Y, Inoshita S, Nakashima O, Yamada T, Tamamori M, Ito H, Sasaki S, and Marumo F (1998) Cyclin D1, p16 and retinoblastoma gene regulate mitogenic signaling of endothelin in rat mesangial cells. *Kidney Int* **53:**76–83.

Wang Y, Mishra R, and Simonson MS (2003) Ca^{2+}/calmodulin-dependent protein kinase II stimulates *c-fos* transcription and DNA synthesis by a Src-based mechanism in glomerular mesangial cells. *J Am Soc Nephrol* **14:**28–36.

Address correspondence to: Dr. Michael S. Simonson, Department of Medicine, Division of Nephrology, Biomedical Research Bldg., Rm. 427, Case Western Reserve University, 2109 Adelbert Rd., Cleveland, OH 44106. E-mail: mss5@po.cwru.edu