

Cell Cycle Signaling by Endothelin-1 Requires Src Non-Receptor Protein Tyrosine Kinase*

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Non-standard Abbreviations: HMC, human mesangial cells; Csk, COOH-terminal Src kinase;

BrdU, bromodeoxyuridine; PP2, 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine ; siRNA, small interfering RNA; RISC, RNA-induced silencing complex; PDGF, platelet-derived growth factor; PKC, protein kinase C BIS, bisindolylmaleimide I.

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 pharmacologic 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 PP2 but not by the inactive analog PP3. RNAi knockdown of Src with on-target but not with off-target siRNAs 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 asked 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.

INTRODUCTION

Although mitogenic signaling is usually associated with receptor tyrosine kinases, many potent mitogens (endothelin, angiotensin II, thrombin, etc) 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 non-vascular cells (Kedzierski and Yanagisawa, 2001; Miyauchi and Masaki, 1999). Accumulating evidence suggests that endothelin-1 contributes to growth and compensatory remodeling of vascular and myocardial cells *in vivo* (Amiri et al., 2004; Miyauchi and Masaki, 1999; Remuzzi et al., 2002). One of the important effectors of cell signaling by endothelin-1 appears to be cross-talk between the G protein-coupled ET_A/ET_B receptors and the Src non-receptor 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 appears to participate in endothelin-1-directed signaling events such as induction of the *c-fos* transcription factor (Simonson et al., 1996b). The 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 pharmacologic 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 from Cell Signaling Technology (Beverly, MA); total Src antibody from Biosource International (Camarillo, CA); and anti- β -Actin antibody from Sigma Chemical Co. (St. Louis, MO). PP2 (4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine), a cell-permeable pharmacological inhibitor of Src, and its inactive analog PP3, were also from Sigma. Bisindolylmaleimide I (BIS) was from Calbiochem (San Diego, CA). PDGF-BB was obtained from R & D Systems, (Minneapolis, MN).

Human Mesangial Cell Culture: Human mesangial cells (HMC), purchased from Cambrex Bioscience Inc. (Walkersville, MD), were maintained in Dulbecco's modified essential medium (DMEM, Gibco-BRL, 11885) supplemented with 17% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μ g/ml streptomycin, 5 ng/ml selenite, and 5 μ g/ml each of insulin and transferrin in a CO₂ incubator at 37°C in the presence of 5% CO₂. Characterization was performed by phase contrast microscopy and by immunostaining for intermediate filaments and surface antigens as described previously (Schultz et al., 1988). Briefly, cells were positive for desmin, vimentin, and myosin, but 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 6-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 μ g pRSV β Gal and with 2 μ g of the following plasmids as described (Simonson et al., 1996b): pSrcK-, SrcK+, pCMVCsk, pMv-Src, v-Ha-Ras. Incorporation of 5-bromo-2-deoxyuridine (BrdU, Sigma) into newly synthesized DNA in transiently transfected cells (i.e., β -galactosidase

positive cells) was also assessed exactly as previously described (Simonson et al., 1995; Wang et al., 2003). Briefly, after transfection cells were labeled for 24 h with 20 μ M BrdU, monolayers were then washed twice with ice-cold DPBS and fixed for 5 min on ice with 2.0% formaldehyde/0.2% glutaraldehyde in DPBS. Cells expressing β -galactosidase were detected by histochemical analysis (Simonson et al., 1995; Wang et al., 2003). Under these conditions 3-5 % of mesangial cells were transfected (i.e., β -galactosidase positive). BrdU incorporation into DNA was then identified by immunocytochemistry using 6 μ g IgG/ml of a specific monoclonal antibody (Roche Applied Sciences, 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 % inhibition of DNA synthesis was calculated as : $(N_V - N_E)/N_V$, where N_V =% BrdU positive nuclei in cells transfected with vector only, and N_E =% 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 was analyzed by the Chi-squared test using InStat for Macintosh (GraphPad, 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 6 well plates. Cells, serum starved for 24 h in DMEM-0.5% FBS for 24, were pretreated with 10 μ M of PP2 or PP3 for 1h before adding either 100 nM of 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 HMC was performed as previously reported by us with minor modifications (Mishra et al., 2003). Briefly, cells were

washed with ice cold PBS and scraped in CHAPS extraction buffer (50 mM Pipes/HCl, pH 6.5, 2 mM EDTA, 0.1% Chaps, 20 µg/ml leupeptin, 10 µg/ml pepstatin A, 10 µg/ml aprotinin, 5mM DTT, 2mM Na pyrophosphate, 1mM Na₃VO₄, and 1mM NaF). An aliquot was saved for protein determination using the DC assay (Biorad, Hercules, CA). An equal volume of 2X 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 previously described (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 bp overhangs were obtained from Dharmacon Inc. Lafayette, CO (Cat. # M-003175). The algorithms for determining the siRNA sequences have been described in detail (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 0-50 nM. Briefly, mesangial cells were plated at 50-70% density in 35 mm petri dishes a day prior to 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-MEMI (5%FBS) for 18h. 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, non-target siRNA (Cat. # D-001210) 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 48h in complete medium 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 following 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. 1A,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 employed 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. 2A,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 non-specific 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 over-expression of v-Src reversed inhibition of endothelin-1-stimulated DNA synthesis by Src K- (Fig. 2B). Collectively, 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 encoding COOH-terminal Src kinase (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. 3A,B, Con, 31.8%; ET-1 58.1%). Transfection with Csk attenuated the endothelin-1-stimulated increase in BrdU+ cells (Fig. 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). Taken 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 non-specifically 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 due to 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 of the siRNA complexes, and concentrations from 10 – 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 at these low concentrations did not induce an interferon response that generally blocks protein synthesis. Addition of non-targeting siRNA complexes at 20 and 50 nM did not reduce total Src or β -actin levels (Fig. 5A). The specific 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

pharmacologic 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 platelet-derived growth factor (PDGF) (Haas et al., 1999). We therefore asked 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 were added (Fig. 6C). Importantly, 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 utilize 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 asked whether Src is required for stimulation of cyclinD1 synthesis by endothelin-1. Transcriptional up-regulation of D-type cyclins occurs in G1, 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. 7A,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. 7A,B). We also confirmed that PP2 attenuated the endothelin-1-stimulated increase in PTyr416 Src (Fig. 7A,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 asked 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. 8A,B). In addition, BIS blocked mesangial cell proliferation in response to ET-1 (Fig. 8C). BIS alone had no effect on mesangial cell growth. Taken 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 asked 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. 9A, B). To demonstrate that BIS prevented activation of Src by PKCs, we also showed that BIS prevented Src activation by the phorbol ester TPA (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 non-receptor protein tyrosine kinases such as Src, Pyk2, and focal adhesion kinase (Sorokin and Kohan, 2003), but the role of specific non-receptor protein tyrosine kinases in mitogenic signaling by endothelin-1 has not been extensively studied. The goal of the present study was to determine if 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 appear 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 and coworkers 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, Src siRNA) and pharmacologic (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-negative Src or Csk did not block endothelin-1-stimulated mitogenesis by non-specifically 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 over-expression 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. Collectively, these results support the hypothesis that Src helps propagate endothelin-1 post-receptor signals that drive mitogenesis in mesangial cells.

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 de-repression 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 suggest 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 knockdown 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 of the Src siRNAs did not reduce β -actin levels, as would be expected in the siRNA induced a non-specific, 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 4 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 (personal communication, Dharmacon), did not reduce total Src or β -actin levels. This off-target siRNA also interacts with the RNA-induced silencing complex (RISC) and so serves as an important negative control for any non-specific but RISC-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 G1 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. Taken 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 G1 into S phase. A previous study in NIH3T3 cells reported that the PKC inhibitor calphostin C failed to block cyclin D1 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 is 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 NIH3T3 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, ERK 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 (Amiri et al., 2004; Miyauchi and Masaki, 1999; Remuzzi et al., 2002). Finally, these results point to a widening role for non-receptor protein tyrosine kinases in propagating mitogenic signals from G protein-coupled receptors.

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FOOTNOTES

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FIGURE LEGENDS

Figure 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.

Figure 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 non-specific effects, cells were co-transfected with vectors expressing constitutively active v-Ras or v-Src as described in Materials and Methods. Statistical significance versus control was calculated by the Chi-square test for percentage data. ** $p<0.01$.

Figure 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 co-transfected 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 Chi-square test versus control ** $p<0.01$.

Figure 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 μ M for 1 hr) or with an equivalent concentration of its inactive analog PP-3. Data are the mean of n=4 independent experiments with statistical significance calculated by the Chi-square test versus no addition ** p <0.01, *** p <0.001.

Figure 5. Knockdown of Src by Src siRNA blocks ET-1-induced proliferation of mesangial cells. (A) As detailed in Materials and Methods, mesangial cells were transfected with increasing amounts of on-target Src siRNA or with non-target (NT) siRNA for 48 h before assessment of total Src protein by Western blotting. Results are representative of 3 independent experiments with similar results. (B) Schematic illustration of the experimental design for testing the effects of Src siRNA on ET-1-induced proliferation. G₀ 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 \pm SD from 3 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.

Figure 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 3 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) ET-1 (100 nM) plus PDGF (10 ng/ml) were added

simultaneously to cells transfected with vector, SrcK-, or SrcK+. The number of BrdU+ transfected cells was then analyzed as above. Statistical significance versus vector alone was calculated by the Chi-square test versus no addition with 3 independent experiments in both (B) and (C) ** $p<0.01$.

Figure 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 10 μ M 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 3 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 to ET-1 alone as calculated by *t*-tests at 4, 8, and 24 h.

Figure 8. Protein kinase C contributes to induction of cyclinD1 by endothelin-1. (A) Endothelin-1 was added alone or in cells preincubated with 5 μ M of the selective PKC inhibitor BIS. Levels of cyclinD1 protein were measured by Western blotting and the densitometric analysis of cyclin D1 from 3 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 \pm SD from 3 independent experiments, and statistical differences between treatment groups at 24 and 48 h were determined by ANOVA with Bonferroni multiple comparison post tests. * $p<0.05$; ** $p<0.01$.

Figure 9. Protein kinase C activity is required for Src activation by endothelin-1. (A) To determine if 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 μ M 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 3 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 TPA was added to quiescent cells or to cells preincubated with 5 μ M BIS. PTyr416 Src levels were measured by Western blotting, and similar results were observed in 3 experiments.

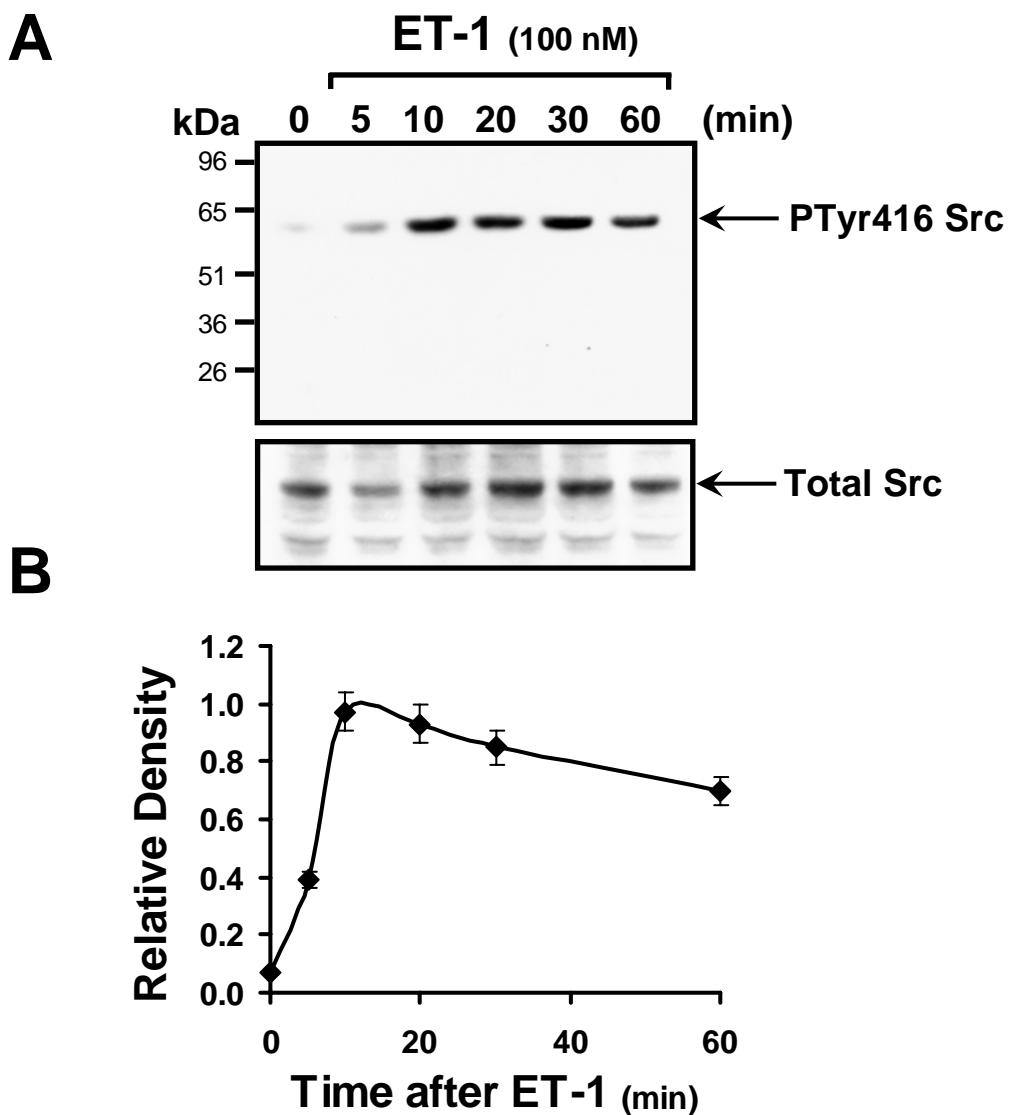


Figure 1

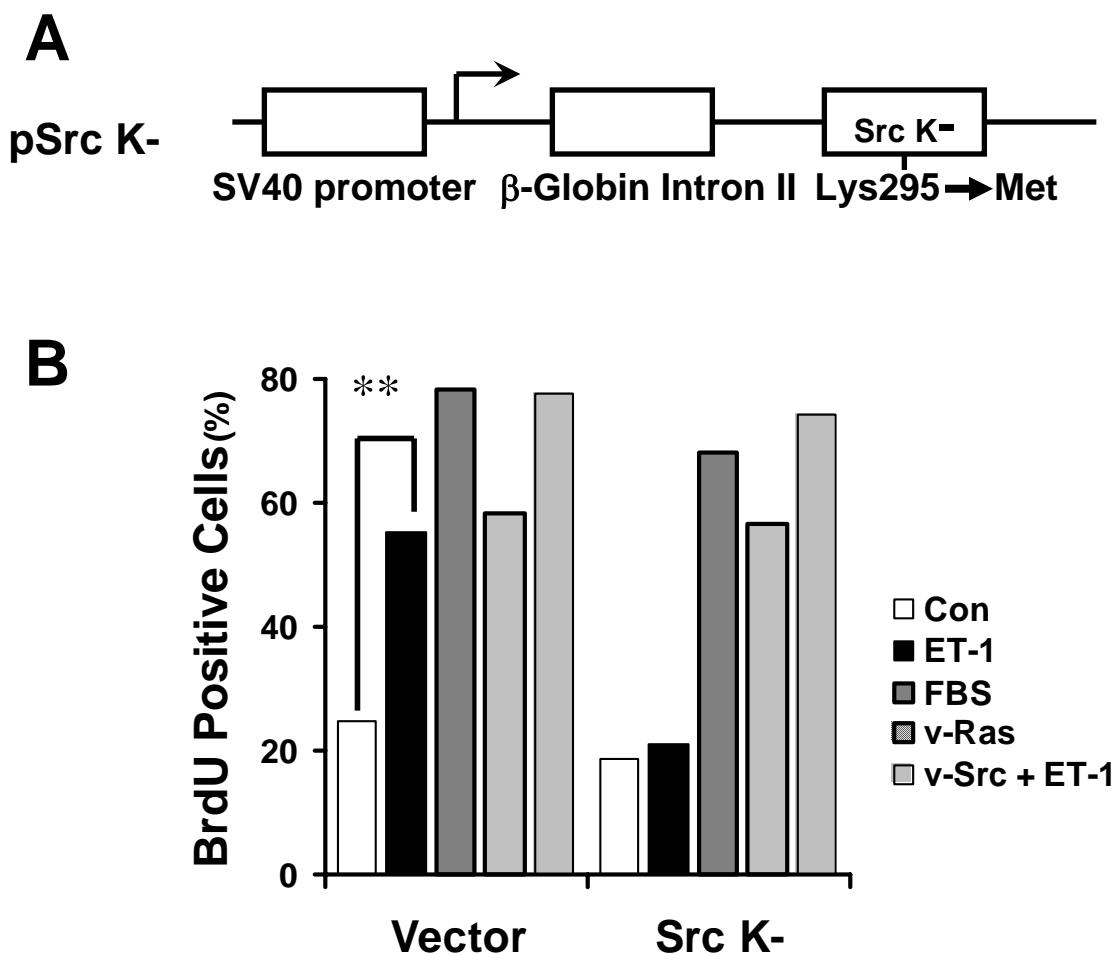


Figure 2

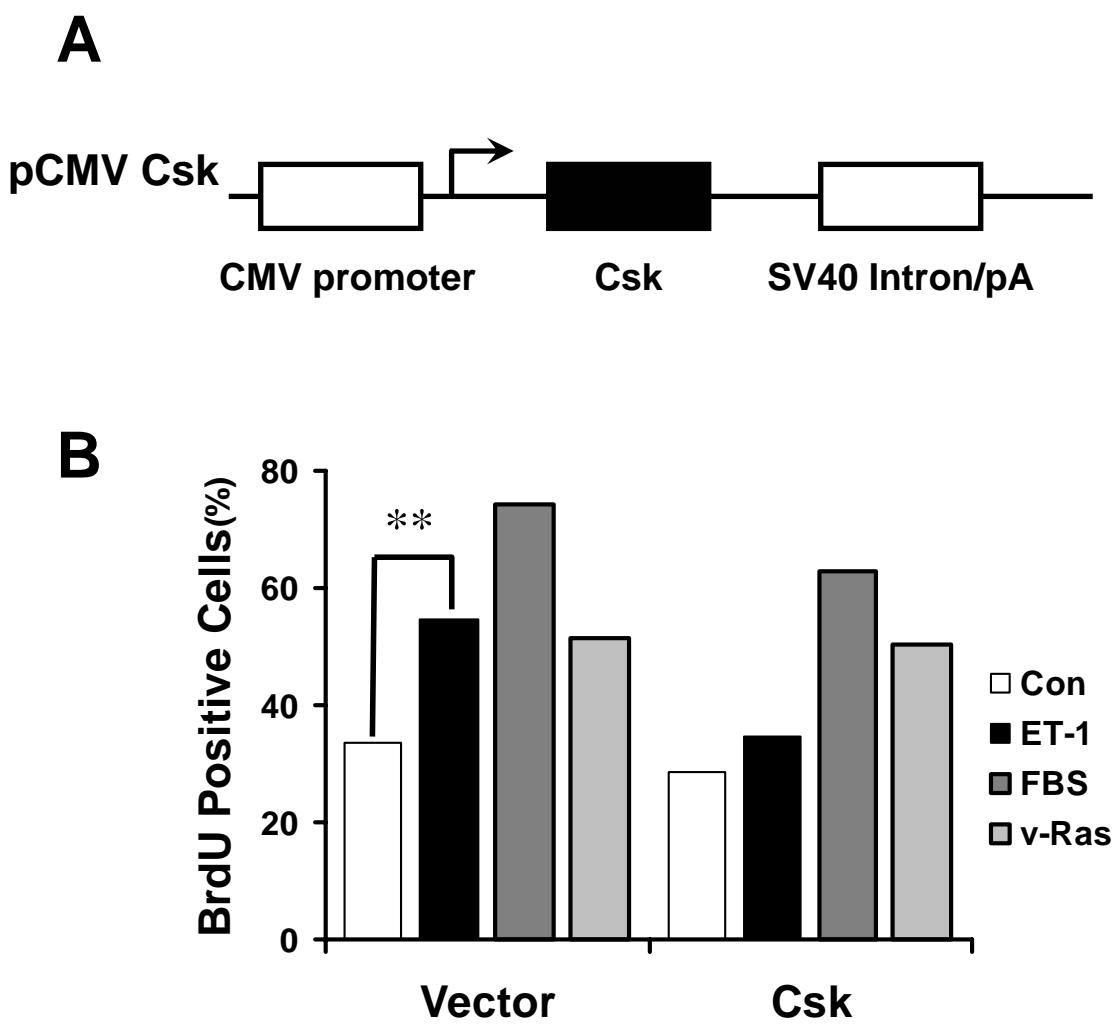


Figure 3

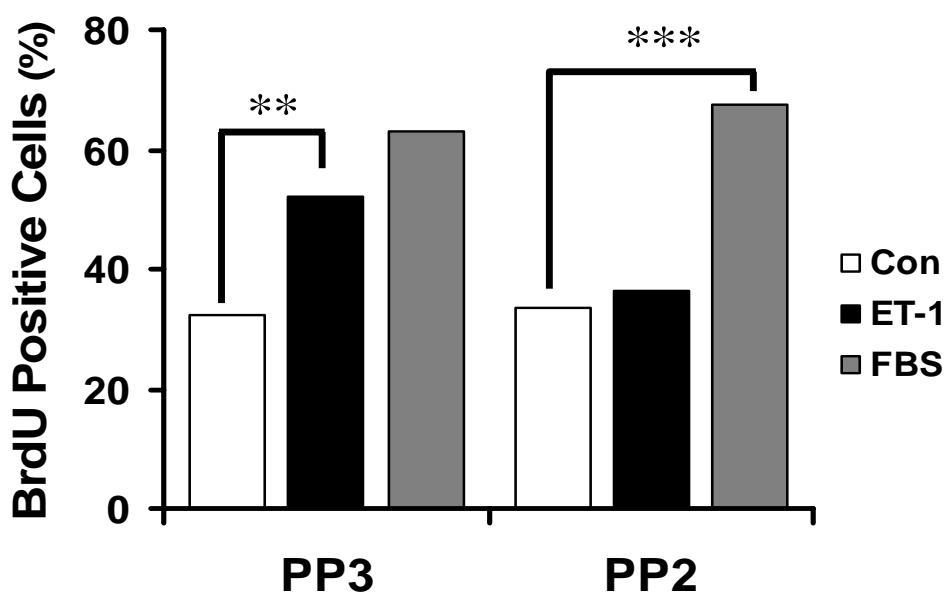


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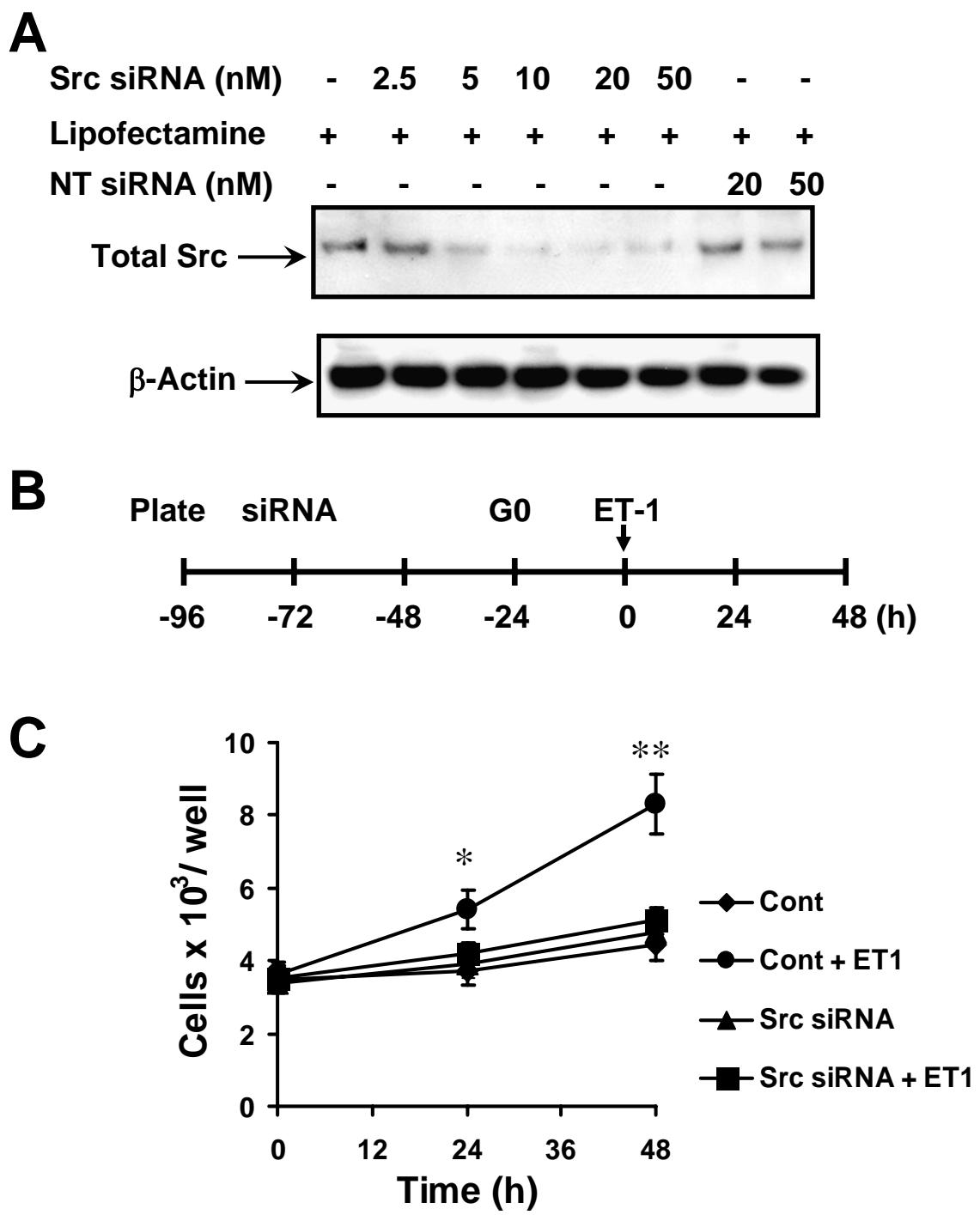


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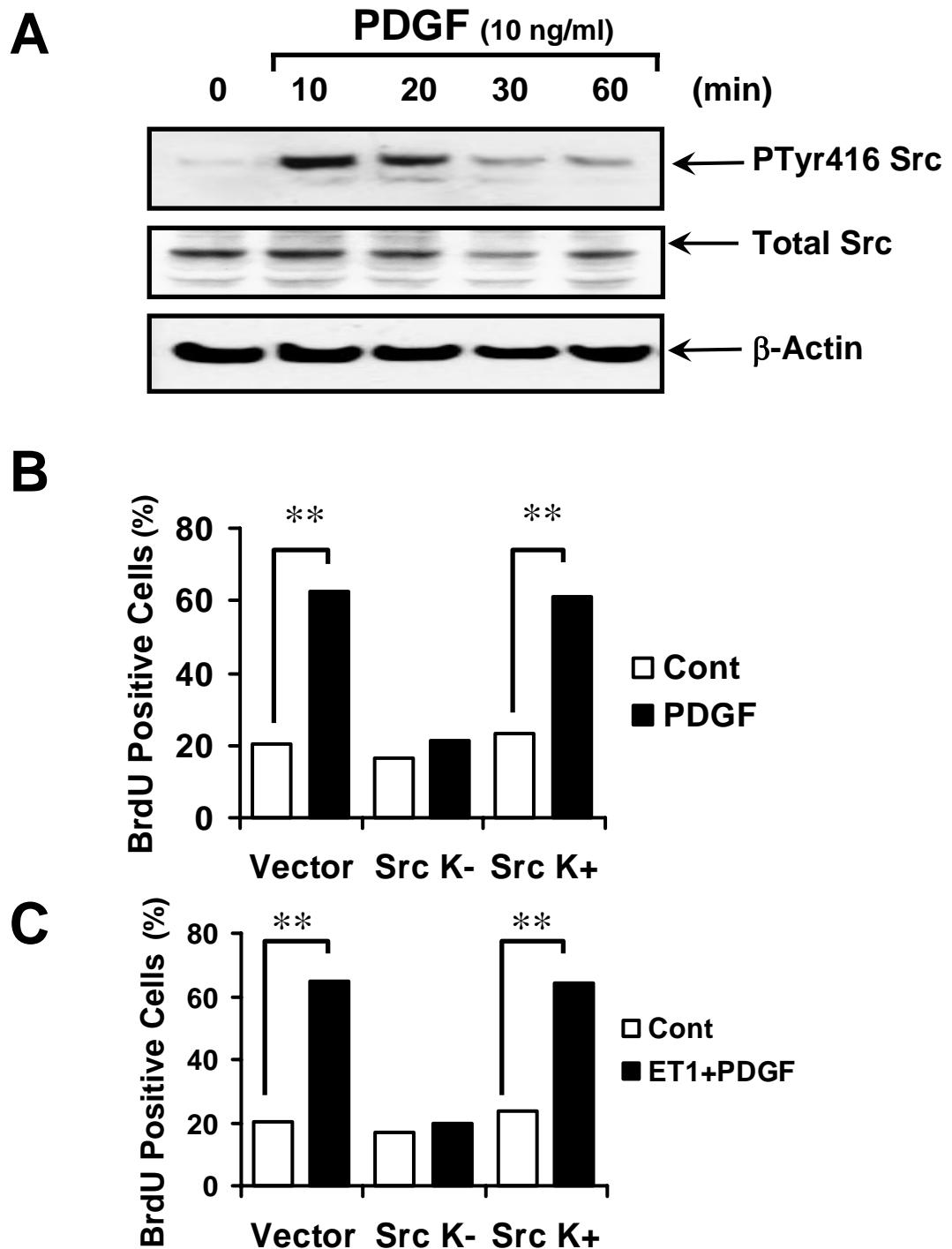


Figure 6

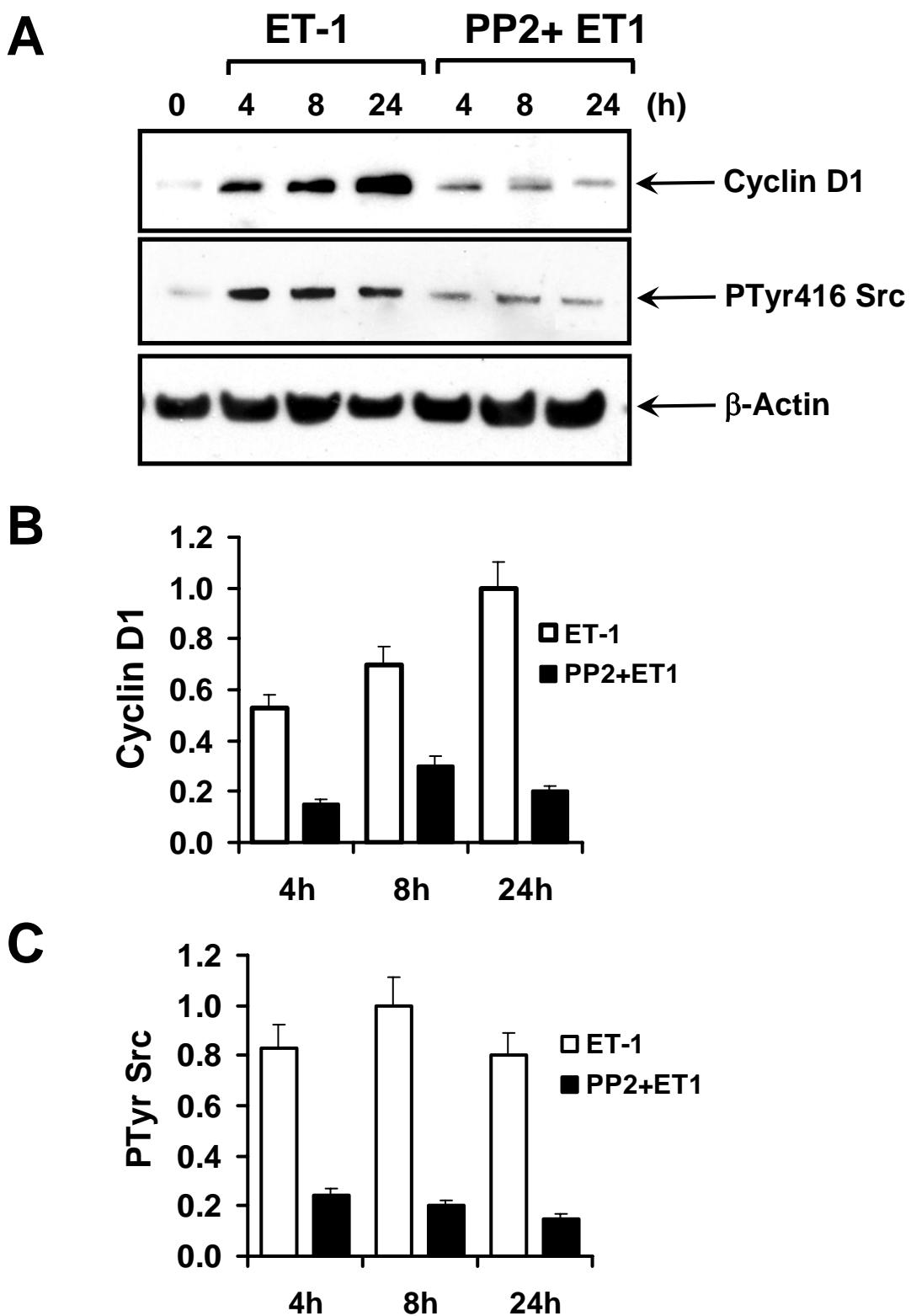


Figure 7

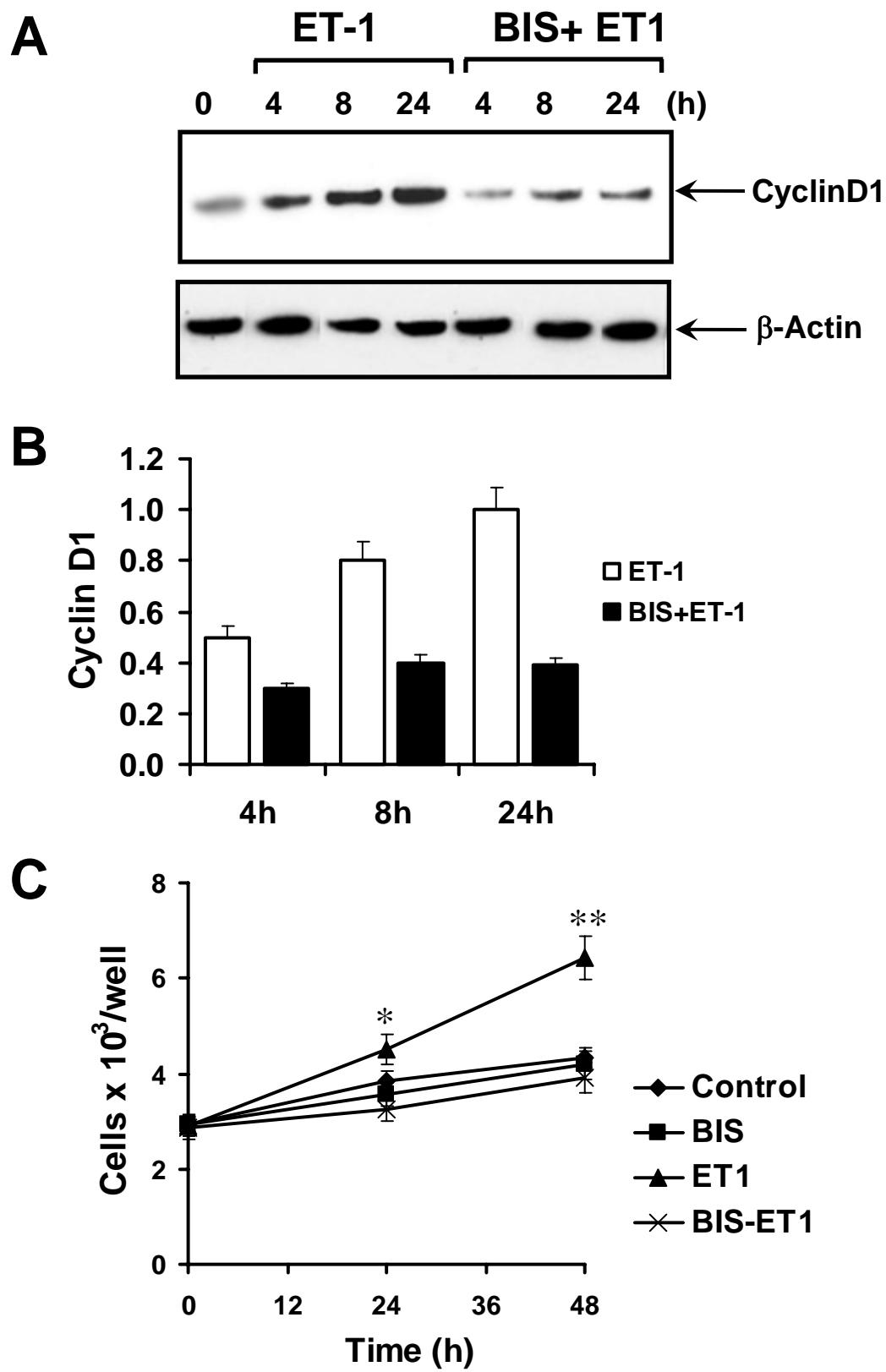


Figure 8

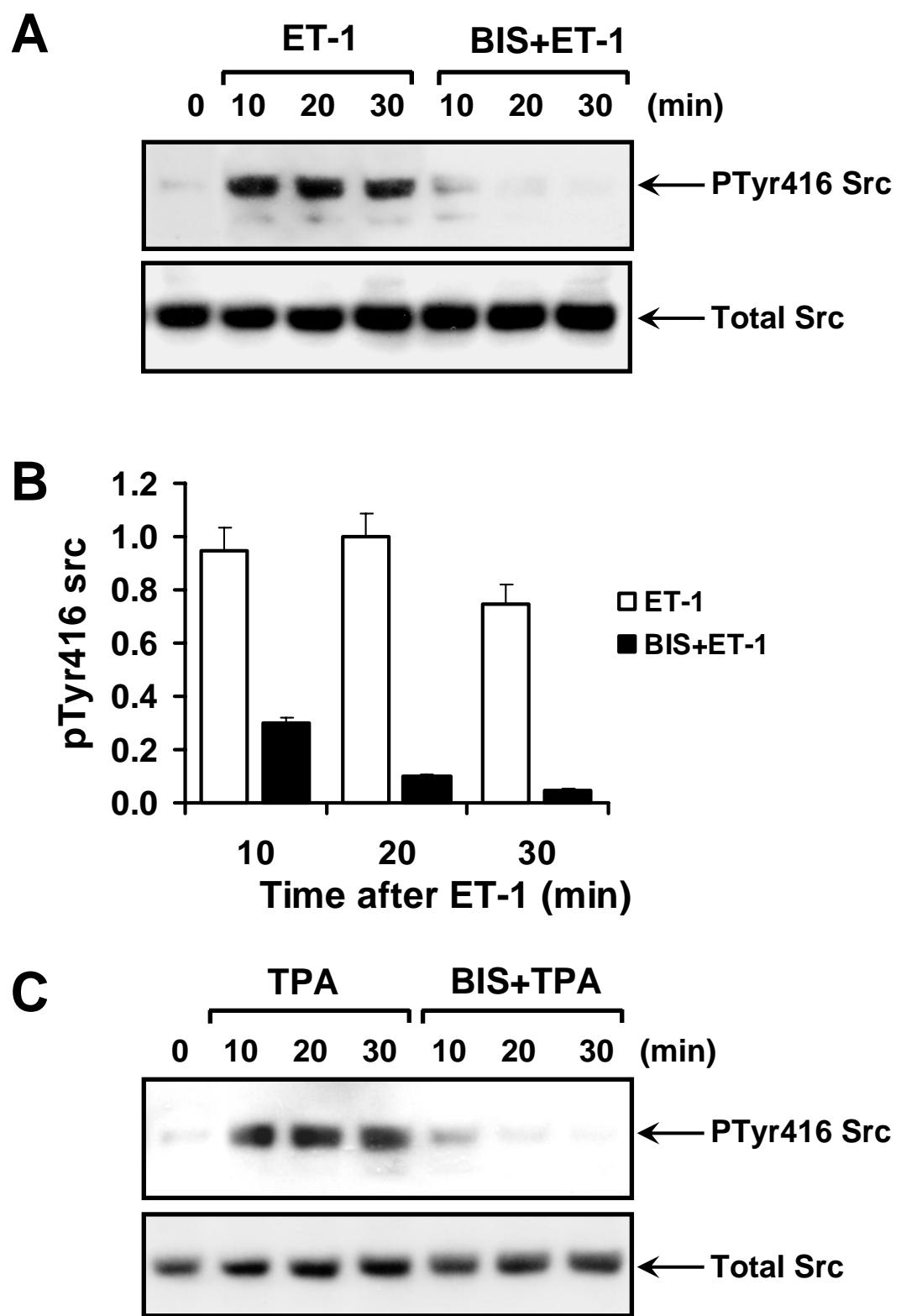


Figure 9