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Src is necessary and sufficient for human airway smooth muscle cell proliferation and migration

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ABSTRACT

Airway smooth muscle (ASM) hypertrophy and hyperplasia, important pathological features in chronic severe asthma, likely contribute to irreversible airflow obstruction. Despite considerable research effort, the precise cellular mechanisms that modulate ASM growth remain unknown. Src, a nonreceptor tyrosine kinase proto-oncogene, reportedly modulates cell proliferative responses to growth factors, contractile agonists, and inflammatory mediators. Here, we show that Src activation is required for human ASM mitogenesis and motility. Platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and thrombin induce rapid activation of Src, and inhibition of Src induces a concentration-dependent abrogation of PDGF-, EGF-, and thrombin-induced ASM cell proliferation. Src immunoprecipitates had associated phosphatidylinositol 3-kinase, or PI3K, activation in response to PDGF and thrombin but not EGF. Further, Src activation is both necessary and sufficient for the stimulation of DNA synthesis as demonstrated by dominant negative Src inhibition of PDGF-, EGF-, and thrombin-induced DNA synthesis. Human ASM cell migration was also attenuated by transfection of cells with dominant negative Src. Further, expression of constitutively active Src promoted cell migration. Collectively, these data demonstrate that Src modulates human ASM cell proliferation and migration, suggesting that Src may play an important role in promoting ASM cell growth and migration that occur in airway remodeling found in asthma and chronic obstructive pulmonary disease, or COPD.

Key words: asthma • airway remodeling • COPD

Increased airway smooth muscle (ASM) cell mass due, in part, to proliferation of ASM cells characterizes chronic severe asthma. Elucidation of the cellular and molecular mechanisms regulating ASM cell growth will advance our understanding of asthma pathogenesis and, potentially, the development of new therapeutic strategies to treat airway diseases. Ras and phosphatidylinositol 3-kinase (PI3K) signaling pathways are critical regulators of mitogen-induced ASM cell proliferation (1–7). In situ, however, smooth muscle cells are stimulated not

only by growth factors that activate receptor tyrosine kinases (RTK) but also contractile agonists that activate G protein-coupled receptors (GPCR) that modulate ASM growth. Importantly, cross-talk between GPCR and RTK signaling pathways likely promotes a synergy among growth factors and contractile agonists (8, 9). Src protein tyrosine kinase likely integrates GPCR- and RTK-induced signaling pathways that ultimately regulate cell proliferation, migration, differentiation, and gene transcription (10, 11). To date, however, little information is available concerning whether contractile agonists or growth factors activate Src and whether Src modulates human ASM cell proliferation and migration.

Src represents a ubiquitously expressed nonreceptor protein-tyrosine kinase that comprises nine family members (10). Src contains Src-homology 2 (SH2), Src-homology 3 (SH3), catalytic domains, and a short stretch of 20 amino acids in the C-terminal region with a conserved tyrosine residue (Tyr527) whose phosphorylation negatively regulates Src activation. In the inactive state, Src maintains a “closed” conformation, where the Tyr527-phosphorylated tail interacts with the SH2 domain and where kinase activity remains low, while the SH2 and SH3 domains remain inaccessible to intramolecular interactions (12). The phosphorylation of Tyr418 within the catalytic domain and the dissociation of the tail and SH2 domains induce an “open” conformation that leads to Src kinase activation and the availability of SH2 and SH3 domains for initiating interactions with other signaling molecules (13, 14).

Studies using pharmacological inhibitors of Src, PP1, or PP2 show a potential involvement of Src in regulating smooth muscle cell growth and migration. Inhibition of Src abrogated thrombin-induced DNA synthesis (15), potentially through inhibition of p42/p44 MAPK in guinea pig ASM (16, 17). In rat aortic vascular smooth muscle (VSM) cells, pharmacological inhibition of Src also inhibited angiotensin II-induced p42/p44 MAPK activation and cell proliferation (18). Angiotensin II- and PDGF-stimulated rat aortic or canine pulmonary VSM cell migration was sensitive to pharmacological inhibition of Src (19, 20). PP1 and PP2, while helpful tools in suggesting a potential role for Src in regulation of smooth muscle cell function, are not specific for Src inhibition, since PP1 and PP2 not only inhibit activation of all Src family members, but also attenuate kinase activities of JAK2, focal adhesion kinase (FAK), and EGFR (21, 22).

Although Src may contribute to both cell proliferation and migration, the role of Src in modulating human ASM cell proliferation and migration remains undefined. Here, we show, using molecular approaches, that platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and thrombin activate Src by phosphorylating Tyr418 within its activation loop and that inhibition of Src abrogates PDGF-, EGF-, and thrombin-induced DNA synthesis. Further, PDGF- and thrombin-induced stimulation of Src activated PI3K. Expression of constitutively active and dominant negative Src mutants also showed that Src is necessary and sufficient for human ASM cell proliferation and migration. Collectively, these studies identify Src as a potential therapeutic target to abrogate ASM cell growth and migration characteristically seen in the airways of subjects with chronic severe asthma and chronic obstructive pulmonary disease (COPD).

MATERIALS AND METHODS

Cell culture

Human trachea was obtained from lung transplant donors, in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings. A segment of trachea just proximal to the carina was removed under sterile conditions, and the trachealis muscle was isolated. Using this technique, ~0.5 g of wet tissue was obtained, minced, centrifuged, and resuspended in 10 ml of buffer containing 0.2 mM CaCl_2 , 640 U/ml collagenase, 1 mg/ml soybean trypsin inhibitor, and 10 U/ml elastase. Enzymatic dissociation of the tissue was performed for 90 min in a shaking water bath at 37°C. The cell suspension was filtered through 105 μm Nytex mesh, and the filtrate was washed with equal volumes of cold Ham's F12 medium supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT). Aliquots of the cell suspension were plated at a density of 1.0×10^4 cells/cm². The cells were cultured in Ham's F12 media supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin, and this was replaced every 72 h. Human ASM cells in subculture during the second through fifth cell passages were used because, during these cell passages, the cells retain native contractile protein expression, as demonstrated by immunocytochemical staining for smooth muscle actin and myosin (23). These cells also retain functional signaling pathways that are important in mediating ASM contraction as determined by fura-2 measurements of agonist-induced changes in cytosolic calcium (23, 24). All experiments were performed using a minimum of three different cell lines. Each human ASM cell line was established using tracheal tissue from a single human donor. Before each experiment, ASM cells were growth-arrested in serum-free Ham's F-12 media supplemented with 0.1% bovine serum albumin (BSA) for 48 h.

Microinjection and measurement of DNA synthesis

Near-confluent human ASM cells grown on two-well plastic chamber slides (Nunc, Naperville, IL) were growth-arrested. Microinjection pipettes (Femtotip I; Eppendorf, Hamburg, Germany) were either filled with Tris/EDTA buffer with pEGFP plasmid expressing green fluorescent protein (GFP) as a control to identify microinjected cells; comicroinjected with either Src wild-type (Src-WT), Src dominant negative (Src-DN), or Src constitutively active (Src-CA) plasmids with pEGFP plasmid; or comicroinjected with 1.5 mg/ml neutralizing Src (SRC 2) antibody against the C terminus of Src (Santa Cruz Biotechnology, Santa Cruz, CA) (25) with 5 mg/ml rabbit IgG, which served as a marker for microinjected cells.

Microinjection was performed as described previously (6). Briefly, ~2 h following microinjection, human ASM cells were treated with 10 ng/ml PDGF-BB, 10 ng/ml EGF, 1 U/ml thrombin, or diluent. Fifteen hours later, 10 μM of the thymidine analog, 5-bromo-2'-deoxyuridine (BrdU), was added to all wells. Twenty-four hours after the addition of BrdU, the cell monolayers were fixed with 3.7% paraformaldehyde (Polysciences, Warrington, PA) and then permeabilized with 0.1% Triton X-100. Following denaturation of DNA with 4N HCl (3 min at room temperature), the monolayers were incubated for 1 h at 37°C with 2 $\mu\text{g}/\text{ml}$ murine anti-BrdU antibody (Becton Dickinson, San Jose, CA) and then with 10 $\mu\text{g}/\text{ml}$ Texas Red conjugated anti-mouse antibody for 1 h at 37°C (Jackson ImmunoResearch Laboratories, West Grove, PA) to detect BrdU-positive cells. To identify microinjected cells, we incubated

monolayers with anti-GFP rabbit serum, and then with Alexa Fluor 594 goat anti-rabbit IgG conjugate (Molecular Probes, Eugene, OR). The slides were examined using a fluorescent microscope (Aristoplan: Leica, Wetzlar, Germany) under $\times 200$ magnification with the appropriate fluorescent filters. Results were presented as a mitotic index defined as the percentage of BrdU-positive nuclei/number of cells microinjected.

[^3H]-Thymidine incorporation assays

DNA synthesis was measured using a [^3H]-thymidine incorporation assay. Cells were growth-arrested by incubating the cultures at day 10 in serum-free media consisting of Ham's F-12 media with 0.1% BSA (3). Near-confluent, growth-arrested cells were used because cells can be synchronized in the G_0/G_1 phase of the cell cycle and, at this baseline, minimally incorporate [^3H]-thymidine. After 48 h in serum-free media, the cells were preincubated with PP2 (EMD Biosciences, La Jolla, CA) for 30 min, then stimulated with either 10 ng/ml PDGF, 10 ng/ml EGF, or 1 U/ml thrombin. After 16–18 h of stimulation, human ASM cells were labeled with 3 $\mu\text{Ci/ml}$ of [methyl- ^3H]-thymidine (40–60 Ci/mmol) (Amersham/Pharmacia, Arlington Heights, IL) for 24 h. The cells were then scraped and lysed, and the protein/DNA was precipitated with 10% TCA. The precipitant was aspirated onto glass filters and extensively washed, dried, and counted.

Cell migration assay

Cell motility was examined using a Boyden chamber apparatus (26). Confluent serum-deprived ASM cells were briefly trypsinized by 0.05% trypsin/0.53 mM EDTA, centrifuged at 900 rpm for 10 min, and resuspended in serum-free Ham's F-12 media supplemented with 0.1% BSA. Cells (5×10^4) were then placed into the upper wells of the Boyden chamber fitted with an 8- μm -pore membrane, coated with 100 $\mu\text{g/ml}$ Vitrogen (Cohesion, Palo Alto, CA). Agonists or vehicle in serum-free Ham's F-12 media with 0.1% BSA were added to either the lower chamber or both the lower and upper chambers. Cells in the Boyden chamber were incubated for 4 h at 37°C in a 5% CO_2 incubator. Nonmigrated cells were scraped off, and the membrane was fixed with methanol, stained with Hemacolor stain set, and scanned. Cell migration was analyzed using the Gel-Pro analyzer program (Media Cybernetics, Silver Spring, MD).

Transient transfection

Src cDNA allelic pack, containing Src-WT, Src-CA, and Src dominant negative (Src-DN) cDNA in pUSEamp vector were purchased from Upstate Biotechnology (Lake Placid, NY). Plasmids were prepared using the EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA). Transient transfection was performed using the Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. Briefly, cells were incubated with each plasmid at the ratio of 1 μg cDNA per 30 μl Effectene for 6 h, and then washed with PBS, and maintained for the next 24 h in medium supplemented with 10% FBS (27). The transfection efficiency of human ASM using Effectene transfection reagent was 21–38%. Cells were maintained for 48 h in serum-free media before the immunoblot or migration assays.

PI3K activity assay

PI3K activity assays were performed as described previously (3, 5). Briefly, serum-deprived ASM cells were stimulated with agonists; then cells were washed with ice-cold wash-buffer (137 mM NaCl, 20 mM Tris-HCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.2 mM vanadate [pH 7.5]) and were lysed in lysis buffer (wash buffer plus 10% glycerol, 1% Nonidet P-40 [NP-40], 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin) (28). Supernatants were incubated with anti-phosphotyrosine (4G10) or with anti-Src (Upstate Biotechnology). Protein A-Sepharose (60 µl; Amersham Biosciences, Piscataway, NJ) was then added to lysates for 2 h at 4°C. The immunoprecipitates were washed three times in PBS containing 1% NP-40; two times in 0.1 M Tris-HCl (pH 7.5) and 0.5 M LiCl; and two times in TNE (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA [pH 7.5]). All solutions contained 0.2 mM vanadate. Sonicated phosphatidylinositol (PtdIns) in Tris-HCl/EGTA (0.2 mg/ml, final concentration) was added to the immunoprecipitates; the phosphorylation reactions were started by the addition of MgCl₂, ATP, and [γ -³²P]-ATP (30 µCi/sample) (to achieve a final concentration of 4 mM MgCl₂, 50 mM ATP) for 10 min at room temperature. Reactions were stopped by the addition of 100 µl of 1 N HCl and extracted with 160 µl of chloroform-methanol (1:1). Lipids were separated on oxalate-coated thin-layer chromatography (TLC) plates (Silica Gel 60, Merck KGaA, Darmstadt, Germany) using a solvent system of chloroform-methanol-water-ammonium hydroxide (60:40:11.3:2). The lipids were then detected by autoradiography. The position of [³²P]-phosphatidylinositol-monophosphate (PIP) was determined by the position of a PIP standard subsequently separated on TLC in parallel and developed in iodine vapor.

Src activity assay

In vitro Src activity was measured by using the Src Assay Kit (Upstate Biotechnology) accordingly to the manufacturer's protocol. Briefly, serum-deprived cells were washed in ice-cold Tris-buffered saline (TBS), then lysed in TBS supplemented with 1% NP-40, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 0.2 mM vanadate (pH 7.5). Cell lysates were incubated with anti-Src (Upstate Biotechnology) antibody; then immunocomplexes were collected with Protein-A-Sepharose (Amersham Biosciences), followed by washes in lysis buffer and in TBS. The Src substrate peptide was added to Src immunoprecipitate, and the in vitro phosphorylation reaction was started by the addition of MgCl₂/ATP and [γ -³²P]-ATP (10 µCi/sample) for 10 min at room temperature. Reactions were stopped by the addition of 20 µl of 40% TCA, then transferred on phosphocellulose paper, which was washed in 0.75% phosphoric acid, dried in acetone, and counted on a scintillation counter.

Identification of proteins by immunoblot assay

Whole cell lysates were prepared from growth-arrested human ASM cells treated with agonists at 37°C for the times indicated. Cells were then washed with ice-cold PBS, and whole cell lysates were prepared. Protein contents were measured using a Bio-Rad (Hercules, CA) protein assay reagent kit. Equal amounts of lysate, adjusted to protein content, were subjected to SDS-PAGE and immunoblot analysis. The blots were exposed to either anti-phospho-tyrosine 418-Src (pY418), anti-phospho-tyrosine 529-Src (pY529) (Cell Signaling Technology, Beverly, MA), or anti-Src antibody (Upstate Biotechnology). All antibodies were in 20 mM Tris (pH 7.5), 150 mM

NaCl (TBS) plus 0.5% Tween 20 (TBST). After three washes in TBST, the nitrocellulose filters were exposed to horseradish peroxidase-conjugated secondary antibody (Boehringer-Mannheim, Indianapolis, IN). Filters were washed five times in TBST, incubated for 1 min in enhanced chemiluminescence (ECL) (Amersham) reagents, and visualized by autoradiography. To perform quantitative analysis of immunoblots, we normalized each sample in protein content before SDS-PAGE. X-ray film was then developed at different times, and the film with presaturation levels of chemiluminescence was scanned. We determined relative optical density of each protein and compared it with the optical density of the control sample using Gel-Pro analyzer software. Statistical analysis was performed using Excel software.

Data analysis

Data points from individual assays represent the mean values of triplicate measurements. Statistically significant differences among groups were assessed with the ANOVA (Bonferroni-Dunn test) or by Chi-square analysis, with values of $P < 0.05$ sufficient to reject the null hypothesis for all analyses. All experiments were designed such that matched control conditions existed within each experiment to enable statistical comparison of paired samples. Each set of experiments was performed with a minimum of three different human ASM cell lines.

RESULTS

Src modulates PDGF-, EGF-, and thrombin-induced proliferation of human ASM cells

Since Src regulation of growth factor-induced DNA synthesis is cell type-specific (10), we investigated whether Src mediated ASM cell growth induced by PDGF, EGF, or thrombin, using pharmacological and molecular approaches. In serum-deprived ASM cells, pretreatment with PP2, a Src inhibitor (21), followed by stimulation of cell monolayers with PDGF, EGF, or thrombin, PP2 completely abrogated PDGF-, thrombin-, and EGF-induced DNA synthesis as measured by [^{3} H]-thymidine incorporation in a concentration-dependent manner ([Fig. 1A](#)). In parallel experiments, PP2 also inhibited mitogen-induced cell proliferation as demonstrated by cell counting experiments (data not shown). The IC_{50} for PDGF-, EGF-, and thrombin-induced DNA synthesis was $\sim 0.3 \mu M$, $\sim 5.5 \mu M$, and $\sim 0.3 \mu M$, respectively, and correlates with the previously reported IC_{50} for the inhibition of Src kinase by PP2 in studies using other cell types (21, 22). Curiously, EGF-induced stimulation of DNA synthesis was somewhat less responsive to PP2 as compared with that induced by either PDGF or thrombin. To determine whether PP2 inhibition of Src kinase occurred at comparable concentrations in human ASM cells, we performed experiments to determine whether PP2 directly inhibits Src activation. Using an in vitro kinase assay in which Src was immunoprecipitated from ASM that was incubated with increasing concentrations of PP2, PP2 inhibited Src activation in a concentration-dependent manner with an $IC_{50} \sim 1 \mu M$, which correlates with the range of effective concentrations sufficient to inhibit 50% of mitogen-induced ASM cell DNA synthesis as shown in [Figure 1B](#). Together, these data suggest that Src modulates human ASM cell growth induced by agonists that activate either GPCRs or those that activate RTKs.

Src is necessary and sufficient for proliferation of human ASM cells

Our studies and others suggest that Src may play a role in the modulation of mitogen-induced ASM cell growth (15–17). The specificity of the pharmacological inhibitors and the experimental design prevented a definitive characterization of the role of Src in modulating ASM cell growth. To further address the role of Src in human ASM cell proliferation, we transiently transfected ASM cells with Src mutants, and DNA synthesis was characterized by incorporation of BrdU, a thymidine analog.

The Src-Y529F mutant, in which tyrosine-529 in the C terminus is replaced with phenylalanine, prevents the ability of Csk tyrosine kinase to phosphorylate this inhibitory site and thus renders this mutant a constitutively active Src (Src-CA). To determine whether Src-Y529F renders Src constitutively active, we measured phosphorylated Src using specific antibodies to phosphotyrosine-418-Src and phosphotyrosine-529-Src in cells transfected with Src-CA. Cells were also transfected with Src, which contains two point mutations that render a dominant negative form of Src (Src-DN). In cells transfected with Src-WT, phosphorylation levels of either the activation (Y418) or inhibitory (Y529) sites of Src was unaffected as shown in [Figure 2](#). These data suggest that overexpression of Src-WT does not have intrinsic kinase activity and that phosphorylation of the inhibitory site is comparable with that seen with endogenous Src. These data demonstrate the feasibility of transfection of human ASM cells with Src mutants that are either constitutively active or dominant negative depending on the target phosphorylation sites.

To determine whether Src is necessary and sufficient for human ASM DNA synthesis, we microinjected cells with the aforementioned Src mutants, and DNA synthesis was then measured by BrdU incorporation and immunocytochemical staining. Src plasmids were coinjected with GFP-expressing plasmids for supravital analysis and for immunostaining with anti-GFP antibodies to identify microinjected cells as shown in [Figure 3A](#). Overexpression of Src-WT or expression of Src-CA induced DNA synthesis of human ASM cells, as seen in [Figure 3B](#). Interestingly, Src-WT and Src-CA both promoted BrdU incorporation at comparable levels as compared with cells transfected with a GFP-containing vector alone. These experiments were performed on serum-deprived cells without mitogenic stimuli. Collectively, the data suggest that Src is sufficient to induce DNA synthesis in human ASM cells.

Expression of Src-DN, while having little effect on basal levels of BrdU incorporation, significantly inhibited PDGF-, EGF-, and thrombin-induced DNA synthesis, as shown in [Figure 3C](#). In cells microinjected with GFP-containing plasmid without Src-DN, there was no difference in microinjected cells stimulated with growth factors as compared with those that were microinjected with diluent. Interestingly, thrombin-induced DNA synthesis was markedly more sensitive to the Src-DN transfection as compared with that seen in PDGF- or EGF-stimulated cells. Additionally, we found that microinjection of neutralizing antibody directed against the C terminus of Src inhibited thrombin-induced BrdU incorporation by $40.7 \pm 21.7\%$ ($P=0.03$), while having little effect on EGF-induced DNA synthesis. Collectively, these data show that Src-CA or Src-WT is sufficient to induce DNA synthesis in human ASM cells, and Src-DN appears to inhibit both RTK- and GPCR-mediated DNA synthesis.

Src is necessary and sufficient for human ASM migration

In asthma, ASM mass is increased and myofibroblast numbers are markedly increased in the submucosa, which likely represents increased migration of myofibroblasts into the airways after repetitive injury and repair. Since Src regulates motility in cell type-specific manner and is activated by adhesion molecules (10), we next addressed whether transient expression of Src mutants modulates human ASM motility. As shown in [Figure 4A](#), expression of Src-DN attenuated chemokinesis by 0.38 ± 0.04 -fold ($P < 0.003$) in serum-deprived cells in the absence of any stimuli. Attenuation of cell migration was not associated with cell apoptosis or inhibition of cell attachment since equal numbers of viable cells were observed in the upper well of the Boyden chamber after the 4 h duration of the migration assay (data not shown).

Interestingly, overexpression of Src-WT or expression of Src-CA is sufficient to promote cell migration in the absence of PDGF stimulation, suggesting that Src is a critical modulator of cell motility ([Fig. 4A](#)). Importantly, while Src-DN attenuated PDGF-induced ASM cell motility, Src-WT or Src-CA synergistically enhanced cell migration in the presence of PDGF. PDGF alone induced a 4.84 ± 0.83 -fold ($P < 0.001$) increase, and Src-WT or Src-CA together with PDGF stimulated cell motility by 8.35 ± 0.52 -fold ($P < 0.01$) or 10.66 ± 0.162 -fold ($P < 0.008$), respectively. Importantly, PDGF, EGF, and thrombin have differential effects on human ASM cell migration. As seen in [Figure 4C](#), PDGF markedly stimulated cell migration by 4.57 ± 1.30 -fold; in contrast, EGF modestly increased cell motility by 1.78 ± 0.19 -fold. Interestingly, two different contractile agonists such as thrombin and bradykinin had little effect on cell migration ([Fig. 4C](#)). These data demonstrate that Src not only modulates cell proliferation but is necessary and sufficient to promote cell migration.

Mitogens activate Src in ASM cells

Although PP2 and Src mutants modulate human ASM cell growth and migration, parallel studies were performed to address whether mitogens directly activate Src. As shown in [Figure 5](#), confluent, serum-deprived cells stimulated with either PDGF, EGF, or thrombin at specified times promoted time-dependent Tyr-418 phosphorylation of the Src activation site. Interestingly, EGF and thrombin, which have little effect on ASM cell migration, promoted a rapid Tyr-418 phosphorylation of Src as early as 1–2 min of stimulation, followed by an abrupt return to basal levels of phosphorylation by 5 min as shown in [Figure 5B](#) and [5C](#). PDGF also induced a rapid Tyr-418 Src phosphorylation at 2 min; however, there was a sustained phosphorylation at 416 that remained elevated at 30 min and then returned to basal levels only after 60 min. Our data demonstrate that EGF and thrombin have little effect on cell migration while PDGF markedly induces cell proliferation (26). One potential mechanism by which PDGF can promote migration while other mitogens lack this ability may relate to the sustained activation of Src.

PI3K is activated by Src stimulation

Recent studies from our laboratories show that human ASM cell proliferation and migration are dependent on mitogen-induced PI3K activation (3, 5, 26). Although PI3K activation is critical for mitogen-induced cell proliferation, not all mitogens, such as thrombin, promote cell migration (26). Importantly, the cross-talk between PI3K and Src signaling is cell type-specific

(10, 29). We therefore examined whether there is cross-talk between Src and PI3K activation in ASM cells. PP2, a Src inhibitor, attenuated PI3K activity associated with tyrosine-phosphorylated proteins, suggesting a potential role of Src in PI3K activation (unpublished observation). To address whether Src and PI3K may physically associate to regulate their activation status, studies were performed in which confluent serum-deprived monolayers of ASM were stimulated with either PDGF, EGF, thrombin, or bradykinin at the times indicated; lysed and Src immunoprecipitates were assayed for PI3K activation. As shown in [Figure 6](#), PDGF and thrombin but not EGF or bradykinin induced PI3K activity associated with Src that reached a maximum level at 15 and 30 min for PDGF and thrombin, respectively, and then declined to basal levels. Bradykinin has little effect on DNA synthesis (3), or cell migration, but robustly induces increased calcium responses and forces generation of ASM cells (30). Interestingly, Src-associated PI3K activity in EGF-stimulated cells was not detected, suggesting that PI3K activation may be important for PDGF- and thrombin-induced Src signaling in human ASM cells. Our published study identified the signaling pathway of EGF-dependent PI3K activation in human ASM cells (4). Thus, upon EGF stimulation, autophosphorylated EGF receptor transactivates ErbB2 receptor, which, in turn, recruits and activates PI3K. Additionally, the differential sensitivity of EGF- and PDGF-induced DNA synthesis to Src inhibition is potentially due to differential PI3K activation by EGF and PDGF ([Fig. 1A](#)). Collectively, these data suggest that there is a differential regulation of Src and its associated PI3K activity in GPCR- and RTK-activated signaling pathways that potentially modulates cell proliferation and motility.

DISCUSSION

Src is activated by signaling events originating from activation of GPCRs, RTKs, and adhesion molecules and from cell adhesion and extracellular matrix interactions. Src is a proto-oncogene, whose mutations induce constitutive kinase activation and lead to malignant transformation. In human ASM PDGF, EGF, and thrombin activate Src, which is associated with the rapid Tyr418 phosphorylation within its activation loop. Concentration-dependent inhibition of Src with PP2, which correlates with the inhibitory effect of PP2 on mitogen-induced DNA synthesis, shows mitogen-induced human ASM mitogenesis in a Src-dependent manner. Transfection with Src-DN attenuates PDGF- and EGF-stimulated DNA synthesis, suggesting that Src modulates mitogenesis induced by these growth factors. Src also appears to be important for thrombin signaling, since thrombin-induced mitogenesis was also abrogated by Src-DN. Transient activation of Src is necessary and sufficient to induce human ASM DNA synthesis and migration, as determined by overexpression of Src.

Src kinases have a well-established role in the regulation of cell proliferation. The Src tyrosine kinase is the first tyrosine kinase to be identified and linked to the regulation of cell proliferation (10, 11). Because more than one mechanism is involved in Src activation, this enzyme is critical in transducing signals, which originate through GPCRs and RTKs and results in integrated cellular responses, such as DNA synthesis and migration. The cellular functions regulated by Src were identified by using predominantly overexpressed Src and its mutants in established immortalized cell lines and in vitro assays. However, the function of Src in physiologically relevant cell types, such as human ASM, remained to be determined. Using selective cell-permeable Src inhibitors PP1 and PP2, Src inhibition of DNA synthesis was demonstrated in

guinea pig ASM (15) and rat aortic smooth muscle cells (18). In our study, using pharmacological and molecular approaches, we demonstrate that Src is necessary and sufficient for human ASM DNA synthesis. Interestingly, PP2 had differential effects on EGF- vs. PDGF- and thrombin-induced DNA synthesis: IC_{50} for EGF was $\sim 5.5 \mu M$, which is a magnitude higher compared with the IC_{50} for PDGF and thrombin, which was $\sim 0.3 \mu M$ for both agonists. The high sensitivity of thrombin-induced DNA synthesis to Src inhibition is potentially due to a critical role of Src in modulating mitogenic signaling from activated GPCRs to its downstream targets (31, 32). We also found that the differential Src regulation of cell proliferation correlated with differential activation of Src-associated PI3K. Thus, upon stimulation of human ASM cells with PDGF or thrombin, PI3K activity was associated with Src but not in cells stimulated with EGF. Since PI3K activation is critical for cell proliferation, the lack of Src-associated PI3K activation may explain the insensitivity of EGF-induced ASM DNA synthesis to blocking Src. Importantly, our previously published study identified the signaling pathway of EGF-dependent PI3K activation in human ASM cells, which involved activation of ErbB2 receptor of EGF receptor family (4). Collectively, these data suggest a differential role of Src in growth promoting signaling stimulated by EGF, PDGF, and thrombin.

Microinjection of Src-WT and Src-CA demonstrated that Src is sufficient for human ASM cell proliferation. Importantly, both Src-WT and Src-CA increased DNA synthesis to comparable levels, while the Src-DN mutant showed that Src is necessary for PDGF-, EGF-, and thrombin-induced DNA synthesis. Interestingly, microinjection of Src-DN in thrombin-stimulated cells completely inhibited BrdU incorporation, suggesting a critical role of Src for thrombin-induced DNA synthesis, which correlates with the well-established role of Src in GPCR-induced mitogenesis (32, 33). Thrombin-induced mitogenesis involves activation of PI3K and Erk (3, 5, 34), suggesting a potential cross-talk between Src and PI3K as well as between Src and Erk. The mechanism by which this cross-talk occurs remains unknown. We now demonstrate that PI3K activation is associated with Src in response to stimulation of human ASM with thrombin.

In addition to increasing cell proliferation, Src promotes cell motility. By mediating signaling originated from RTKs, GPCRs, and integrins (10, 35), Src is important for focal complex formation that promotes directional cell motility (36). The role of Src in canine pulmonary artery and thoracic VSM cell migration was demonstrated by using pharmacological inhibition of Src (19, 20). By expressing either Src-DN or Src-CA, Src was found to be necessary and sufficient for human ASM cell migration. Importantly, our data demonstrate not all ASM mitogens are promigratory factors. Thus, only PDGF markedly induced human ASM cell migration, while EGF and thrombin had little effect on cell motility. Interestingly, these differences in cell motility may be associated with the differences in the kinetics of Src activation. Thus, stimulation of human ASM cells with PDGF induced sustained Src activation; in contrast, EGF and thrombin stimulated transient Src activation.

Our current findings provide new evidence that Src plays an important role in regulating human ASM cell proliferation and migration. Notably, Src is differentially regulated by growth factors and contractile agonists, which may be associated with the relative contribution of Src signaling in human ASM cell proliferation and migration. In diseases such as asthma and COPD, which are characterized by airway smooth muscle cell proliferation, Src may serve as a molecule of

potential importance in integrating complex signaling events and may serve as a potential therapeutic target in airway remodeling.

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Fig. 1

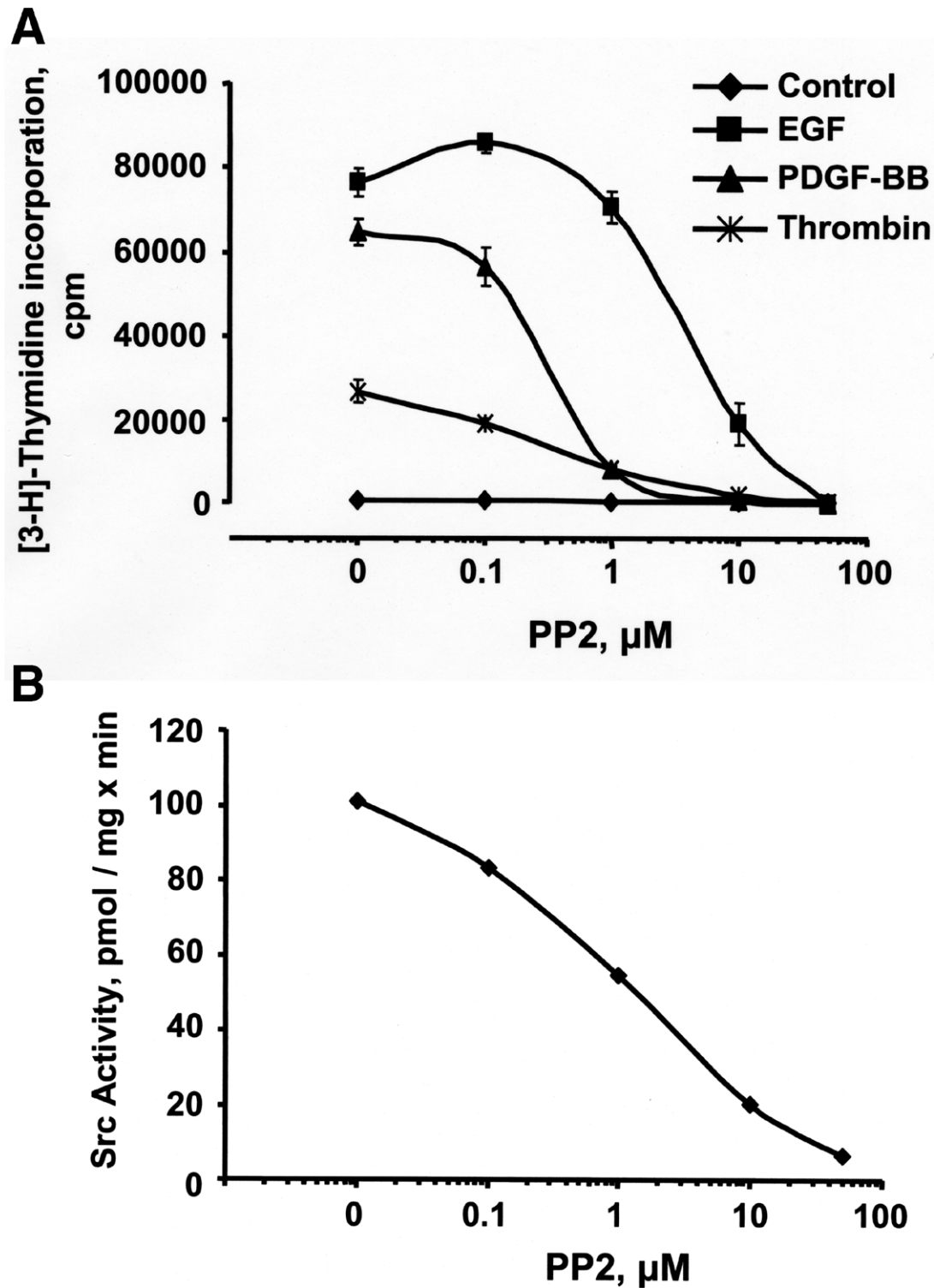


Figure 1. A) Src inhibition modulates PDGF-, EGF-, and thrombin-induced ASM cell growth. Confluent, growth-arrested ASM cells were pretreated with 0.1, 1.0, 10, or 100 μM PP2 for 30 min and stimulated with either 10 ng/ml PDGF, 1 U/ml thrombin, 10 ng/ml EGF, or diluent (control). [^3H]-thymidine incorporation was subsequently assessed as described in Materials and Methods. Data represent means $\pm$ SE from three experiments, each containing six replicates for each condition ($P < 0.01$ by one-way ANOVA [Bonferonni-Dunn test]). B) Src activity is inhibited by PP2. Cell lysates, equalized in protein contents, were prepared from growth-arrested human ASM cells and subjected to immunoprecipitations with anti-Src antibody. Src immunoprecipitates were pretreated with different concentrations of PP2 for 30 min followed by in vitro kinase activity assay as described in Materials and Methods.

Fig. 2

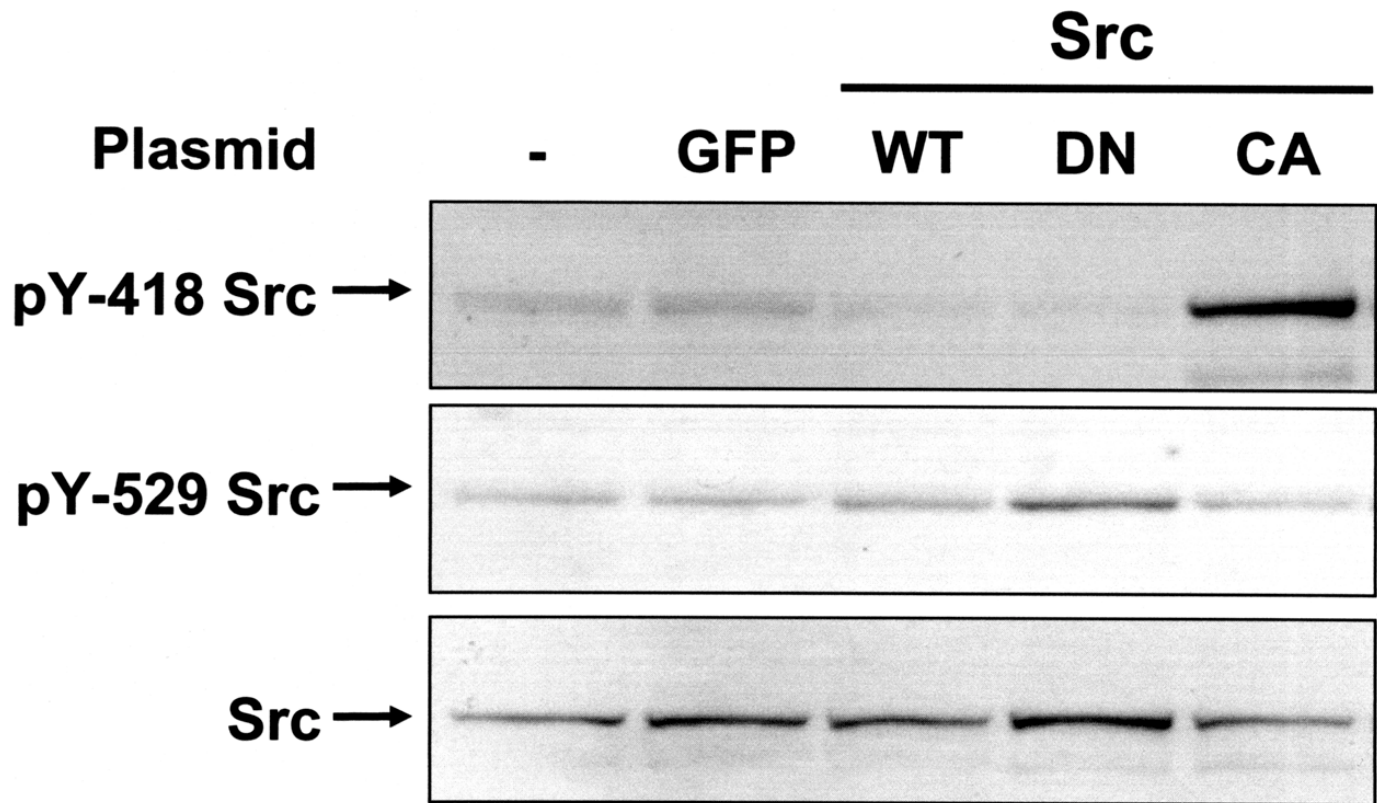


Figure 2. Expression of Src mutants in human ASM cells. Cells were transiently transfected with either Src-wild type (WT), Src constitutively activate (CA), Src dominant negative (DN) cDNA in pUSEamp vector, or pEGFP. Whole cell lysates were prepared followed by SDS-PAGE and immunoblot analysis using either anti-phospho-tyrosine 418-Src (pY418), anti-phospho-tyrosine 529-Src (pY529), or anti-Src antibody.

Fig. 3

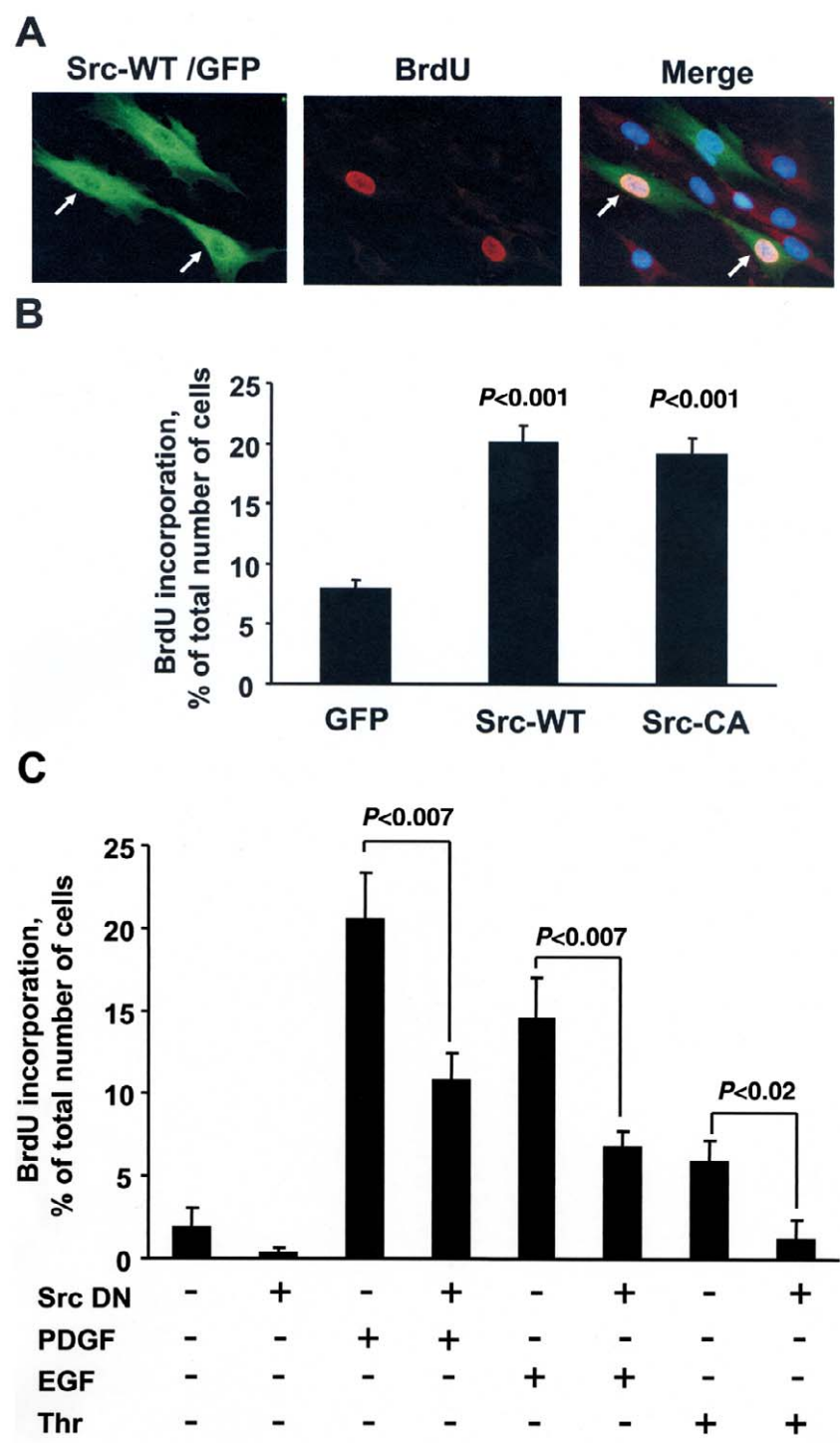


Figure 3. Src is necessary and sufficient for human ASM cell proliferation. Near-confluent, growth-arrested human ASM cells were co-microinjected with either Src-WT or Src-CA and GFP to identify microinjected cells, and compared with cells microinjected with GFP alone. DNA synthesis was measured using indirect anti-BrdU immunofluorescence. Results are expressed as the mitotic index (% BrdU-positive per total number of cells). **A**) Representative images of immunostaining with anti-BrdU antibody to identify DNA synthesis and with anti-GFP antibody to identify cells microinjected with Src mutants. Cells were either untreated (**B**); stimulated with PDGF, EGF, thrombin, or diluent for 16 h (**C**). Statistical analysis was performed by one-way ANOVA (Bonferonni-Dunn test); 472 of Src-CA-microinjected cells, 504 of Src-WT-microinjected cells, and 226 Src-DN-microinjected cells were analyzed from four separate experiments.

Fig. 4

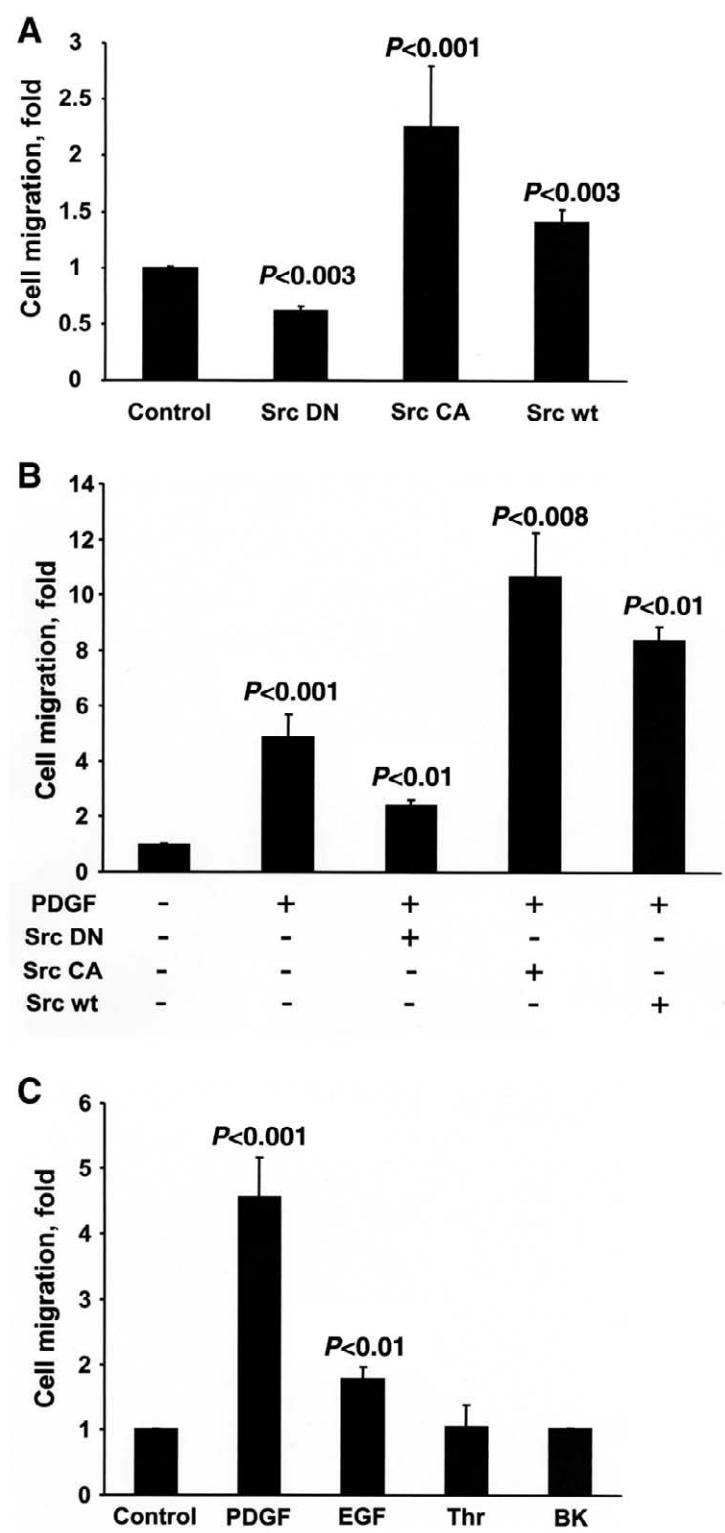


Figure 4. Modulation of human ASM cell migration by Src. Growth-arrested ASM, which were transfected with Src-DN or Src-CA, were harvested and transferred to a Boyden chamber for analysis of cell migration. Cells were either untreated (**A**); stimulated with either 10 ng/ml PDGF (**B**, **C**), 10 ng/ml EGF, 1 U/ml thrombin, or 1 μ M bradykinin (**C**); or stimulated with vehicle as described in Materials and Methods. Data represent mean values $\pm$ SE from three observations by ANOVA (Bonferroni-Dunn test).

Fig. 5

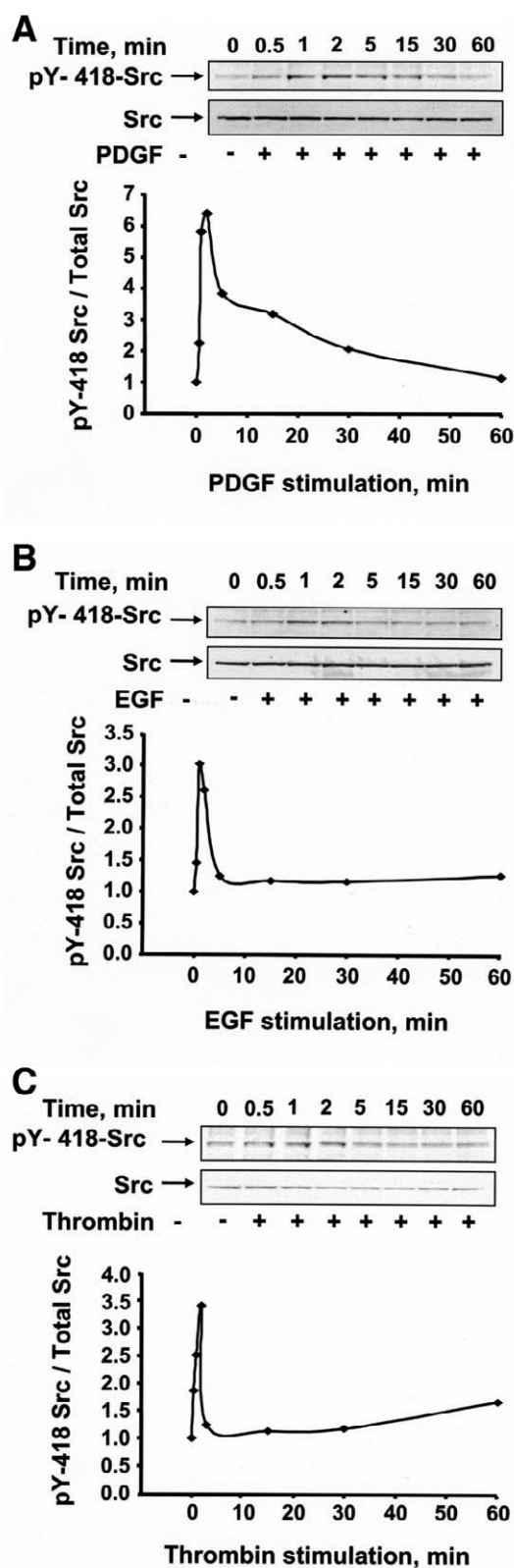


Figure 5. PDGF (A), EGF (B), and thrombin (C) stimulate Tyr-418 phosphorylation of the Src activation site. Immunoblot analysis using an anti-phospho-tyrosine 418-Src (pY418) or anti-Src antibody was performed on whole cell lysates from PDGF-, EGF-, and thrombin-stimulated cells at times indicated. Quantitative analysis of immunostaining was performed by using Gel-Pro Analyzer Software. Images are representative of three separate experiments for each condition that yielded similar results.

Fig. 6

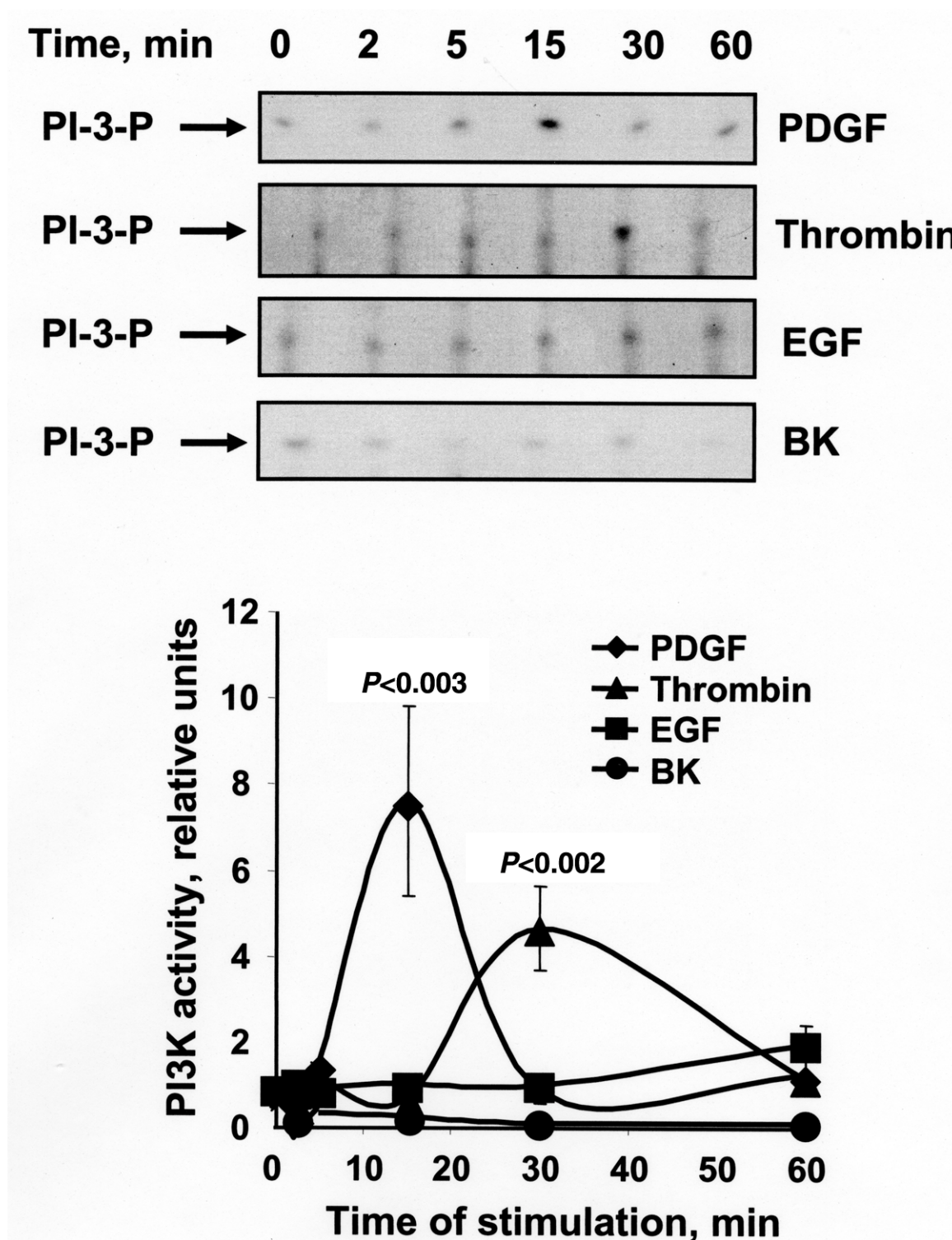


Figure 6. Mitogens activate PI3K in ASM cells. EGF and thrombin stimulate PI3K activation. Confluent growth-arrested monolayers were stimulated with either PDGF, EGF, thrombin, or bradykinin for 2, 5, 15, 30, and 60 min followed by immunoprecipitation with anti-Src antibody. PI3K activity was measured in anti-Src immunoprecipitates as described in Materials and Methods. Autoradiograph and graph are representative of three separate experiments that yielded similar results.