0026-895X/04/6504-832–841$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 65:832–841, 2004

Vol. 65, No. 4
3040/1136426
Printed in U.S.A.

Src and Cas Are Essentially but Differentially Involved in Angiotensin II-Stimulated Migration of Vascular Smooth Muscle Cells via Extracellular Signal-Regulated Kinase 1/2 and c-Jun NH_2-Terminal Kinase Activation

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Received October 17, 2003; accepted December 5, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Angiotensin II (Ang II) plays an important role in several cardiovascular diseases associated with vascular smooth muscle cell (VSMC) growth and migration. Src activity is known to be required for the migration of a number of cell types. p130Cas was reported to be essential for cell migration and actin filament reorganization. Mitogen-activated protein (MAP) kinases were also reported to be critical regulatory factors for growth and migration of VSMC. However, precise intracellular mechanisms involving c-Src, p130Cas, and MAP kinases in Ang II-stimulated migration of VSMC have not been well elucidated. Here we demonstrated that Ang II rapidly and significantly stimulated tyrosine phosphorylation of Src and Cas and their association in rat aortic smooth muscle cells (RASMC). Ang II-stimulated tyrosine phosphorylation of Src and Cas and activation of ERK1/2 and JNK, but not p38, were potently inhibited by Src family tyrosine kinase inhibitors, herbimycin A (HA) and PP2. Ang II-stimulated Src and Cas association, tyrosine phosphorylation of Cas, and activation of ERK1/2 and JNK were suppressed in kinase-inactive Src (KI Src)-overexpressed RASMC. Ang II-stimulated JNK activation but not ERK1/2 activation was blocked in substrate domain-deleted Cas (ΔSD Cas)-overexpressed RASMC. In addition, HA, PP2, ERK1/2 inhibitor, 2′-amino-3′-methoxyflavone (PD98059) and JNK inhibitor, and anthra[1,9-*cd*]pyrazol-6(2H)-one (SP600125) significantly inhibited Ang II-stimulated migration of RASMC. Ang II-induced colocalization of Src and Cas and migration were inhibited in both KI Src- and ΔSD Cas-overexpressed RASMC. These findings suggest that Src and Cas are essentially but differentially involved in Ang II-stimulated migration of VSMC through the activation of ERK1/2 and JNK.

Angiotensin II (Ang II) plays an important role in several cardiovascular diseases associated with vascular smooth muscle cell (VSMC) growth (Berk et al., 1989) and migration (Griendling et al., 1996), such as atherosclerosis, restenosis after angioplasty, and hypertension. It was indicated that tyrosine phosphorylation is a critical episode in several Ang II-stimulated signaling events (Marrero et al., 1996). Ang II induces tyrosine phosphorylation of proteins with apparent molecular masses of 42, 44, 70 to 80, 110 to 130, and 190 kDa in VSMC (Tsuda et al., 1991). The 130-, 60-, 44-, and 42-kDa proteins that were tyrosine-phosphorylated in response to Ang II in VSMC were identified as p130Cas [a CT-10–regulated kinase (Crk)-associated substrate] (Takahashi et al., 1998), 60-kDa c-Src, a nonreceptor tyrosine kinase (Ishida et al., 1995), and mitogen-activated protein (MAP) kinase isozymes (Tsuda et al., 1992), respectively.

Cell migration requires cytoskeletal reorganization involving phosphorylation of cytoskeleton-associated tyrosine kinases and formation of focal-adhesion complexes (Ilic et al.,

This work was supported in part by a Grant-in-Aid (14570078 to M.Y.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and (15500238 to K.T. and 13670016 to K.I.) from the Japan Society for the Promotion of Science.

ABBREVIATIONS: Ang II, angiotensin II; AT_1, angiotensin type 1; RASMC, rat aortic smooth muscle cells; VSMC, vascular smooth muscle cell; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; MAP, mitogen-activated protein; ERK1/2, extracellular signal-regulated kinase1/2; JNK, c-Jun NH_2-terminal kinase; p38, p38 mitogen-activated protein kinase; Crk, CT-10–regulated kinase; Cas, CT-10–regulated kinase-associated substrate; WT, wild-type; KI, kinase-inactive; ΔSD Cas, substrate domain-deleted CT-10–regulated kinase-associated substrate; HA, herbimycin A; Neo, neomycin-resistant gene; NC, normal RASMC; PD98059, 2′-amino-3′-methoxyflavone; SP600125, anthra[1,9-*cd*]pyrazol-6(2H)-one; ROS, reactive oxygen species.

1995). In VSMC, Ang II-stimulated focal-adhesion complex formation and cytoskeletal reorganization occur through AT_1 receptor and require c-Src (Ishida et al., 1999). Src family kinases and C-terminal Src kinase were reported to be localized to focal adhesions (Rohrschneider, 1980; Bergman et al., 1995). Under basal conditions, phosphorylation of Tyr527, one of the residues on the regulatory domain of c-Src by C-terminal Src kinase, results in the inhibition of c-Src activity (Klages et al., 1994), whereas activation is achieved by the dephosphorylation of this residue by a phosphotyrosine phosphatase (Fang et al., 1994). Autophosphorylation at Tyr416 in the catalytic domain may be another activating mechanism of c-Src (Clark and Brugge, 1993). It was reported that c-Src is the predominant tyrosine kinase responsible for Ang II- and thrombin-stimulated tyrosine phosphorylation of the focal-adhesion proteins Cas, paxillin, and tensin in VSMC (Ishida et al., 1999).

p130Cas was reported to be essential for cell migration (Klemke et al., 1998) and actin filament reorganization (Honda et al., 1998). p130Cas was initially characterized as a phosphotyrosine-containing protein in v-Crk– and v-Src–transformed cells (Matsuda et al., 1990). p130Cas is an adaptor family protein consisting of an N-terminal SH3 domain, a proline-rich domain, a central "substrate" domain composed of a cluster of 15 potential SH2-binding motifs, and a C-terminal domain containing both consensus SH3 and SH2 binding sites for Src (Sakai et al., 1994). It was reported that 9 of the 15 tyrosine phosphorylation sites present in the substrate domain of Cas conform to the SH2-binding motif for Crk, the primary docking protein for Cas (Klemke et al., 1998). The formation of the Cas/Crk adaptor protein complex serves as a molecular switch facilitating Rac-dependent cell migration response on the extracellular matrix (Klemke et al., 1998). p130Cas localizes to focal adhesions, where it seems to recruit focal-adhesion proteins and cytoskeletal-signaling molecules such as focal-adhesion kinase, paxillin, and tensin to the focal adhesions (Nojima et al., 1995; Polte and Hanks, 1995; Rozengurt, 1995; Salgia et al., 1996).

Ang II activates MAP kinase family members, extracellular signal-regulated kinases 1/2 (ERK1/2) (Tsuda et al., 1992), c-Jun *N*-terminal kinase (JNK) (Viedt et al., 2000), and p38 (Ushio-Fukai et al., 1998) in VSMC. MAP kinases were reported to be critical regulatory factors for growth and migration of VSMC and various cell types (Rousseau et al., 1997; Xi et al., 1999; Denes et al., 2002; Iijima et al., 2002; Huang et al., 2003). Activation of ERK1/2 by Ang II was reported to be primarily dependent on c-Src in VSMC (Ishida et al., 1998; Touyz et al., 2001). In a previous study, we demonstrated that Src and Cas mediated reactive oxygen species (ROS)-stimulated JNK activation but not ERK1/2 and p38 in VSMC (Yoshizumi et al., 2000).

Nevertheless, precise cellular signaling mechanisms and molecular interactions involving c-Src, p130Cas, and MAP kinases in Ang II-stimulated migration of VSMC have not been well elucidated. To clarify those mechanisms, we used Src family tyrosine kinase inhibitors and rat aortic smooth muscle cells (RASMC) stably transfected and overexpressed with kinase-inactive Src (KI Src) or substrate domain-deleted Cas (ΔSD Cas). In the present study, we determined the effect of KI Src- and ΔSD Cas overexpression on Ang II-induced Src and Cas association, their colocalization at focal adhesion, ERK1/2, JNK activation and migration of VSMC.

Materials and Methods

Chemicals. Herbimycin A (HA) and PP2 were purchased from Calbiochem (San Diego, CA). PD98059 was from Wako Pure Chemicals (Osaka, Japan). SP600125 was from BIOMOL Research Laboratories (Plymouth Meeting, PA). Geneticin (G418 sulfate) was from Invitrogen (Carlsbad, CA). Phospho-tyrosine antibody (P-Tyr-100), phospho-ERK 1/2 antibody (Thr202/Tyr204), phospho-p38 MAP kinase antibody (Thr180/Tyr182), and a stress-activated protein kinase/JNK assay kit were purchased from New England Biolabs (Beverly, MA). Anti-Src antibody and anti-p130Cas antibody were from Upstate Biotechnology (Lake Placid, NY). Anti-Src phosphor-specific antibody (Tyr418), which recognizes the activated form of Src, was from Biosource International (Camarillo, CA). Anti-vinculin antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Fluorescein isothiocyanate-conjugated donkey anti-mouse IgG, Cy3-conjugated donkey anti-rabbit IgG, and Cy5-conjugated donkey anti-goat IgG were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). For transfection of wild-type (WT) or kinase-inactive (KI) Src, commercially available pUSE mammalian expression vectors encoding pp60c-Src (WT) or catalytically inactive Src (K297R) were purchased from Upstate Biotechnology, Inc. pSSRα mammalian cell expression vectors encoding p130Cas or ΔSD Cas were provided by Dr. Vuori (La Jolla Cancer Research Center, La Jolla, CA). pRc/CMV2, which introduces a neomycin phosphotransferase gene, was from Invitrogen. For transfection, the Nucleofector kit with human aortic smooth muscle cells (Amaxa Biosystems, Köln, Germany) was used. All other chemicals were of reagent grade, were obtained from commercial sources, and were used without further purification.

Cell Culture and Stable Transfection. RASMC were isolated from male Sprague-Dawley rats weighing 200 to 250 g and maintained in 10% calf serum and Dulbecco's modified Eagle's medium (DMEM) as described previously (Duff et al., 1993). For transfection of WT or KI Src, commercially available pUSE mammalian expression vectors encoding pp60c-Src (WT) or catalytically inactive Src (K297R) were used. For transfection of wild-type Cas (WT Cas) or ΔSD Cas, pSSRα mammalian cell expression vectors encoding p130Cas or ΔSD Cas were used. Transfections of the cells were performed using the Nucleofector kit with aortic smooth muscle cells (Amaxa Biosystems). Passage 2 to 3 RASMCs ($0.5–1 \times 10^6$ cells/sample) were transfected with pRc/CMV2 alone or cotransfected with pRc/CMV2 plus WT Src or pRc/CMV2 plus KI Src or pRc/CMV2 plus WT Cas or pRc/CMV2 plus ΔSD Cas using the selected program U-25 of Nucleofector (Amaxa Biosystems) and performing the procedure according to the manufacturer's protocol. Transfected cells were plated onto 35-mm collagen-coated dishes (IWAKI, Osaka, Japan) and cultured in DMEM containing 10% fetal bovine serum at 37°C. After reaching confluence in 35-mm collagen-coated dishes, cells were harvested by brief trypsinization and grown in 100-mm culture dishes (IWAKI). When reaching confluence in 100-mm dishes, stably transfected cells were selected in the presence of the neomycin analog Geneticin (G418 sulfate) at 0.5 mg/ml (Invitrogen). Stably transfected cells survived and were allowed to grow into small subcolonies. These subcloned cells were picked up using a 10-mm cloning ring (IWAKI) with brief trypsinization. Then, cells were cultured and allowed to develop subsequent passages in larger dishes and flasks. Expression of the WT Src, KI Src, WT Cas, and ΔSD Cas were confirmed by Src kinase assay (Wakatsuki et al., 1999), immunoprecipitation, and immunoblotting analysis using anti-Src antibody and anti-Cas antibody (Upstate Biotechnology). To avoid clonal variability, experiments were performed with three different subcloned cells in each set of construct.

Preparation of Cell Lysates for Immunoprecipitation, Immunoblotting, and JNK Activity Assay. Either normal RASMC (NC) or stably transfected RASMCs were seeded ($1–2 \times 10^4$ cells/cm^2) and grown to subconfluence in 60-mm culture dishes (IWAKI) in DMEM/10% fetal bovine serum. Cells were made quiescent for

48 h in serum-free DMEM. Normal cells were preincubated with or without herbimycin A (10 μM) or PP2 (100 μM) for 2 h followed by stimulation with or without Ang II (100 nM) at the indicated time points. Stably transfected cells were treated with or without Ang II (100 nM) at the indicated time points. Then, incubation medium was discarded, and cell monolayers were washed once with ice-cold PBS containing sodium orthovanadate (1 mM). Cells were lysed (30–60 min at 4°C) with cell lysis buffer consisting of 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. After freeze-thawing, lysed cells were sonicated (Handy Sonic UR-20 P; Tomy Seiko, Tokyo, Japan) on ice for 1 min, transferred to microcentrifuge tubes, and centrifuged at 16,000*g* for 20 min at 4°C. The protein concentrations of the supernatants were measured with a protein assay kit (Pierce Chemical, Rockford, IL) and stored at −80°C until immunoprecipitation, immunoblotting, and JNK activity assay were performed.

Immunoprecipitation and Immunoblotting. For immunoprecipitation, cell lysates were incubated with rabbit anti-Cas antibody (Upstate Biotechnology) overnight at 4°C and then incubated with 30 μl of protein A/G plus agarose (Santa Cruz Biotechnology) for 2 h in a roller system at 4°C. The beads were washed twice with 1 ml of lysis buffer, twice with 1 ml of LiCl wash buffer (500 mM LiCl, 100 mM Tris-Cl, pH 7.6, 0.1% Triton X-100, and 1 mM dithiothreitol), and twice in 1 ml of washing buffer (20 mM HEPES, pH 7.2, 2 mM EGTA, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1% Triton X-100). For immunoblot analysis, cell lysates or immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were transferred to nitrocellulose membranes (Hybond-ECL; Amersham Biosciences Inc., Piscataway, NJ) as described previously (Yoshizumi et al., 2000). The membranes were blocked for 1 h at room temperature with a commercial blocking buffer (Amersham Biosciences). The blots were then incubated for 12 h with anti-Src (Upstate Biotechnology), anti-p130Cas (Upstate Biotechnology), anti-Src phosphospecific antibody (Tyr418; Biosource), anti-phosphotyrosine (P-Tyr-100) (New England Biolabs), anti-phosphospecific ERK1/2 (New England Biolabs), and anti-phosphospecific p38 MAP kinase antibodies (New England Biolabs), followed by incubation for 1 h with a secondary antibody (horseradish peroxidase conjugated). Immunoreactive bands were visualized using enhanced chemiluminescence (Amersham Biosciences) and were quantified by densitometry in the linear range of film exposure using an Astra 2200 scanner (UMAX Technologies, Dallas, TX) and NIH Image Software 1.60.

JNK Activity Assay. JNK activity was measured with a commercially available kit from the phosphorylation of recombinant c-Jun (New England Biolabs). After treatment, cells were rinsed twice with ice-cold PBS, scraped off the plates into lysis buffer (included in the kit), and sonicated three times on ice. After removing the cell debris by centrifugation (16,000*g* for 20 min at 4°C), the protein content in the supernatant was measured using a protein assay kit (Pierce). Equal amounts of protein (300 μg) were then immunoprecipitated with c-Jun (1–89) fusion protein beads overnight. After washing the beads, kinase assays were performed according to the manufacturer's instructions. Beads were loaded on a 10% SDS-polyacrylamide gel, and immunoblotting was performed with the use of an antibody against phosphospecific c-Jun (Yoshizumi et al., 2000, 2001).

Migration. Cell migration was performed using a modification of the assay as described previously (Keely et al., 1995). Migration assay was examined using Transwell chambers (6.5 mm, model 3422; Costar, Cambridge, MA) with an 8-μm pore polycarbonate membrane. The underside of the polycarbonate membrane was coated with 5 μg/ml collagen I rat tail (3.78 mg/ml, model 35-4236; Collaborative Biochemical Products, Bedford, MA) overnight at 4°C. Then, the lower chamber was blocked with DMEM/0.1% bovine serum albumin for 30 min at room temperature. Growth-arrested cells were harvested, washed, and suspended in serum-free DMEM. Cells were added to the upper chamber of the Transwell at 5×10^5 cells in 100 μl/well. A total of 600 μl of serum-free DMEM was added to the lower chamber and then pretreated with or without herbimycin A (10 μM), PP2 (100 μM), PD98059 (30 μM), or SP600125 (30 μM) in the lower chamber for 1 h at 37°C followed by treatment with or without Ang II (1 μM) in the lower chamber, and cells were allowed to migrate at 37°C. After 6 h of incubation, the nonmigratory cells were removed from the upper surface of the membrane by scraping off with cotton swabs. Membrane was fixed with methanol, stained with Diff-Quik solution (Baxter, McGaw Park, IL) and allowed to air-dry at room temperature. Then, the membrane was excised from the plastic supports with a sharp scalpel and mounted on a glass slide using Permount (Sigma Chemical, St. Louis, MO). Migrated cells were counted at 400× magnification in 10 microscope fields per filter.

Immunofluorescence. For multiple fluorolabeling, cells were plated onto 35-mm glass base dishes (IWAKI) and cultured overnight. After treatment with Ang II (100 nM), cells were rinsed in ice-cold PBS and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 30 min at room temperature. After being incubated in 1% bovine serum albumin (Sigma) for 1 h, the cells were incubated in a mixture of mouse monoclonal anti-Src (1:125; clone GD11, Upstate Biotechnology), rabbit polyclonal anti-p130Cas (1:500; Upstate Biotechnology), and goat polyclonal anti-vinculin (C-20) (1:100; sc-7648, Santa Cruz Biotechnology) antibodies overnight at 4°C. Then, the cells were rinsed in PBS several times and incubated in a mixture of species-specific secondary antibodies, fluorescein isothiocyanate-conjugated donkey anti-mouse IgG (1:200), Cy3-conjugated donkey anti-rabbit IgG (1:200), and Cy5-conjugated donkey anti-goat IgG (1:200) (all from Jackson ImmunoResearch) for 1 h at room temperature. After rinsing several times in PBS, cells were mounted in Vectashield (Vector Laboratories, Burlingame, CA). Cells, triple-stained with the above-mentioned combinations of fluoroprobes, were examined with a confocal laser scanning light microscope (Leica TCS-NT, mounted on a Leica DMRB light microscope; Leica, Mannheim, Germany) using laser beams of 488, 568, and 647 nm wavelengths, respectively, for excitation with appropriate filter sets. Serial optical sections were recorded using an oil-immersion objective lens of ×63 (numerical aperture = 1.4). Pseudocolored images were created from triple fluorolabeling and analyzed using a personal computer (PowerMac G4; Apple Computer, Cupertino, CA) with Photoshop 3.03-J (Adobe Japan, Inc., Tokyo, Japan). These multiple fluorolabeling methods generally followed our previous study (Toida et al., 2000).

Statistical Analysis. Values are reported as the means ± S.D. from experiments done in triplicate. Two-way analysis of variance was used to determine the significance among groups, after which the modified *t* test with the Bonferroni's post hoc test was used for comparison between individual groups. A value of $P < 0.05$ was considered to be statistically significant.

Results

Ang II Stimulates Tyrosine Phosphorylation of Src and Cas in RASMC. To evaluate the relative magnitude of Ang II on tyrosine phosphorylation of Src and Cas, growth-arrested RASMC were treated with 100 nM Ang II for the indicated period of time up to 60 min. The tyrosine phosphorylations of Src and Cas in the cell lysate were determined as described under *Materials and Methods*. Src was tyrosine-phosphorylated by Ang II very rapidly and reached maximal response within 1 min and to the greatest extent (2.64-fold) compared with tyrosine phosphorylation of Src at 0 time (untreated control) (Fig. 1, A and C). Phosphorylation of Tyr418 represents the activation of Src kinase. Cas was also tyrosine-phosphorylated by Ang II similar to Src and reached maximal response within 1 min and to the greatest extent (2.66-fold) compared with tyrosine phosphorylation of Cas at

0 time (untreated control) (Fig. 1, B and C). Then, both Src and Cas tyrosine phosphorylation gradually returned to the basal level within 60 min. These results suggest that Src and Cas are very prompt responsive proteins for tyrosine phosphorylation in Ang II-stimulated signaling events in RASMC.

Src Associates with Cas in Response to Ang II Stimulation in RASMC. It was suggested that Ang II-stimulated focal-adhesion complex formation and cytoskeletal reorganization occur through AT_1 receptor and require c-Src (Ishida et al., 1999). Therefore, we examined the effect of Ang II on molecular association between Src and Cas in RASMC. Growth-arrested RASMC were stimulated with 100 nM Ang II for the indicated period of time up to 60 min, and we determined the association of Src and Cas using immunoprecipitation and immunoblotting as described under *Materials and Methods*. Ang II stimulation caused rapid increases in the association of Src and Cas within 1 min and peaked at 5 min to the greatest extent (7.46-fold) compared with Src and Cas association at 0 time (untreated control) (Fig. 2, A and B). We observed that their association starts to decrease at 10 min; however, it remained above the basal level until 60 min of Ang II stimulation. These findings show that Src and Cas rapidly and significantly associate each other in response to Ang II stimulation in RASMC.

Ang II-Stimulated Association of Src and Cas, and Tyrosine Phosphorylation of Cas Are Dependent on Src Kinase Activity. It has been reported that integrin-mediated tyrosine phosphorylation of Cas is largely dependent on the presence of c-Src (Hamasaki et al., 1996). To determine whether Ang II-stimulated tyrosine phosphorylation of Cas is dependent on Src kinase activity, we used the Src family tyrosine kinase inhibitors HA and PP2. We first confirmed their effects on Ang II-stimulated tyrosine phosphorylation of Src (pY418) in RASMC and found that both HA (10 μM) and PP2 (100 μM) significantly abolished tyrosine phosphorylation of Src (Fig. 3, A and B). We next investigated the effects of these Src kinase inhibitors on Ang II-stimulated tyrosine phosphorylation of Cas in RASMC.

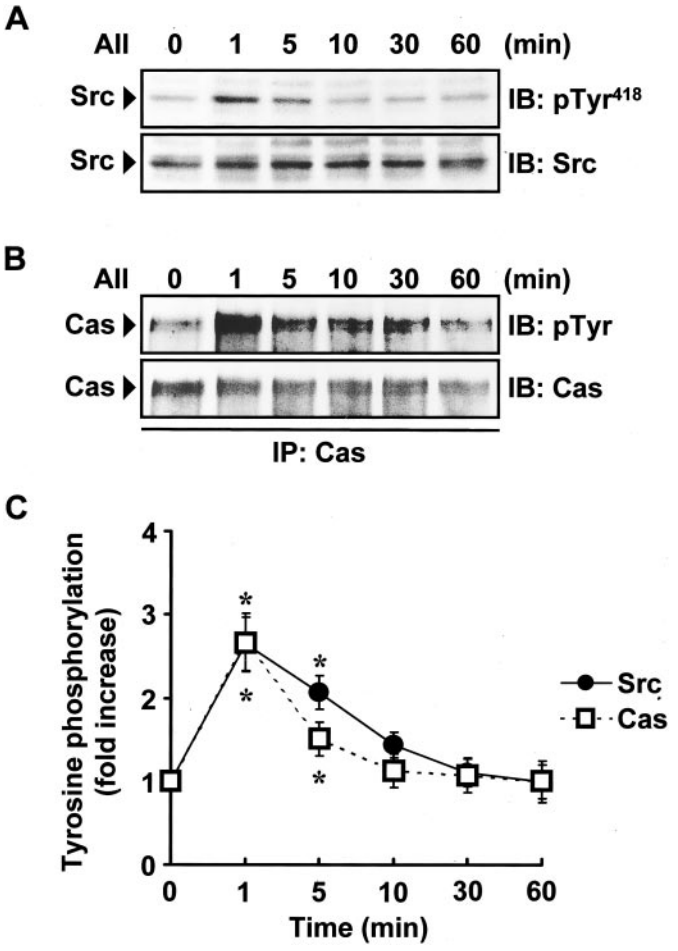

Fig. 1. Ang II-stimulated tyrosine phosphorylation of Src and Cas in RASMC. Cells were stimulated with Ang II (100 nM) for the indicated periods. A, the tyrosine phosphorylation of Src was determined by immunoblotting with anti-Src phosphospecific antibody (pTyr^{418}) as described under *Materials and Methods* (top). No significant difference in the amount of Src was observed in lysates by immunoblotting with anti-Src antibody (bottom). B, the tyrosine phosphorylation of Cas was determined by immunoprecipitation with anti-Cas antibody and immunoblotting with phosphotyrosine antibody (pTyr) as described under *Materials and Methods* (top). No significant difference in the amount of Cas was observed in lysates by immunoblotting with anti-Cas antibody (bottom). C, densitometric analysis of tyrosine phosphorylation of Src and Cas. Values (mean ± S.D., $n = 3$) were normalized by arbitrarily setting the densitometry of control cells (time = 0) to 1.0. ⋆, significant differences compared with the values of unstimulated control at $P < 0.05$.

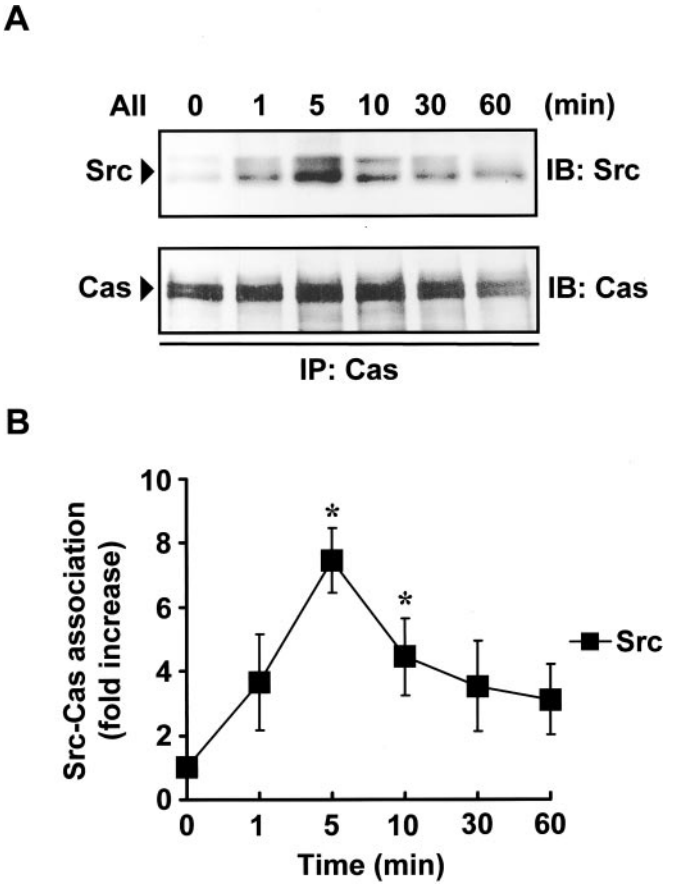

Fig. 2. Ang II-stimulated association of Src and Cas in RASMC. Cells were stimulated with Ang II (100 nM) for the indicated periods. A, the association of Src and Cas was determined by immunoprecipitation with anti-Cas antibody and immunoblotting with anti-Src antibody as described under *Materials and Methods* (top). No significant difference in the amount of Cas was observed in lysates by immunoblotting with anti-Cas antibody (bottom). B, densitometric analysis of Src that bound to Cas. Values (mean ± S.D., $n = 3$) were normalized by arbitrarily setting the densitometry of control cells (time = 0) to 1.0. ⋆, significant differences compared with the values of unstimulated control at $P < 0.05$.

Ang II-stimulated tyrosine phosphorylation of Cas was significantly inhibited by either HA (10 μM) or PP2 (100 μM) (Fig. 3, C and D). These results suggest that c-Src is involved in Ang II-stimulated tyrosine phosphorylation of Cas. However, in this study, the possible inhibitory effects of HA and PP2 on other tyrosine kinases in addition to Src tyrosine kinase cannot be excluded. Therefore, we generated and used stably transfected, WT Src- and KI Src-overexpressed RASMC to investigate the specific role of Src in Ang II-stimulated Src and Cas association and tyrosine phosphorylation of Cas. As shown in Fig. 4 A, Ang II treatment caused increases in the association of Src and Cas in normal cells and in neomycin-resistant gene- and WT Src-overexpressed cells. However, KI Src-overexpressed cells failed to respond to Ang II stimulation. Furthermore, we found that tyrosine phosphorylation of Cas by Ang II stimulation occurred in normal cells and in neomycin-resistant gene- and WT Src-overexpressed cells, whereas it did not occur in KI Src-overexpressed cells (Fig. 4B). These findings revealed that Src serves as an important mediator in Ang II-stimulated molecular interaction between Src and Cas as well as tyrosine phosphorylation of Cas.

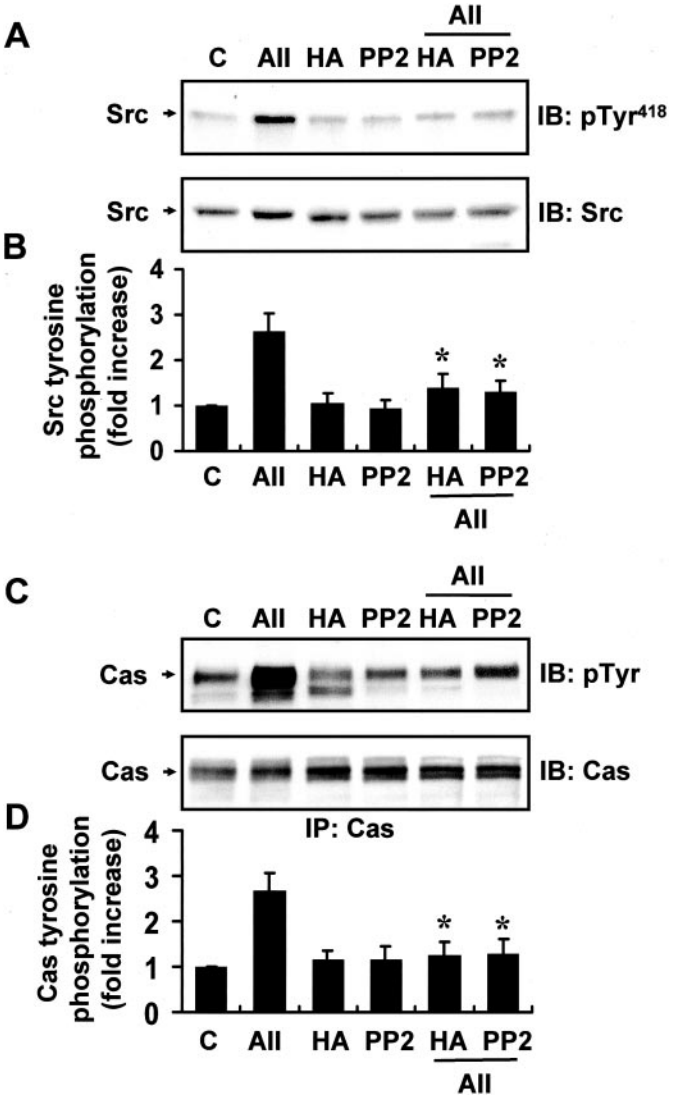

Fig. 3. HA and PP2 inhibited Ang II-stimulated tyrosine phosphorylation of Src and Cas in RASMC. Cells were pretreated with or without HA (10 μM) or PP2 (100 μM) for 2 h followed by stimulation with or without Ang II (100 nM) for 2 min. A, the tyrosine phosphorylation of Src was determined by immunoblotting with anti-Src phosphospecific antibody (pTyr^{418}) as described under *Materials and Methods* (top). No significant difference in the amount of Src was observed in lysates by immunoblotting with anti-Src antibody (bottom). B, densitometric analysis of tyrosine phosphorylation of Src. C, the tyrosine phosphorylation of Cas was determined by immunoprecipitation with anti-Cas antibody and immunoblotting with phosphotyrosine antibody (pTyr) as described under *Materials and Methods* (top). No significant difference in the amount of Cas was observed in lysates by immunoblotting with anti-Cas antibody (bottom). D, densitometric analysis of tyrosine phosphorylation of Cas. Values (mean ± S.D., $n = 3$) were normalized by arbitrarily setting the densitometry of control cells (without Ang II) to 1.0. ⋆, significant differences compared with the values of Ang II stimulation at $P < 0.05$.

Src Is Required for Activation of MAP Kinase Family Members ERK1/2 and JNK by Ang II Stimulation. Ang II activates MAP kinase family members, ERK1/2 (Tsuda et al., 1992), JNK (Viedt et al., 2000), and p38 (Ushio-Fukai et al., 1998) in VSMC. In the previous study, we demonstrated that Src and Cas mediated ROS-stimulated JNK activation but not ERK1/2 and p38 in VSMC (Yoshizumi et al., 2000). To clarify the role of c-Src in Ang II-stimulated activation of three MAP kinase family members, ERK1/2, JNK, and p38, we examined the effects of the Src family tyrosine kinase inhibitors HA and PP2 on Ang II-stimulated activation of ERK1/2, JNK, and p38 using immunoblot analysis and JNK

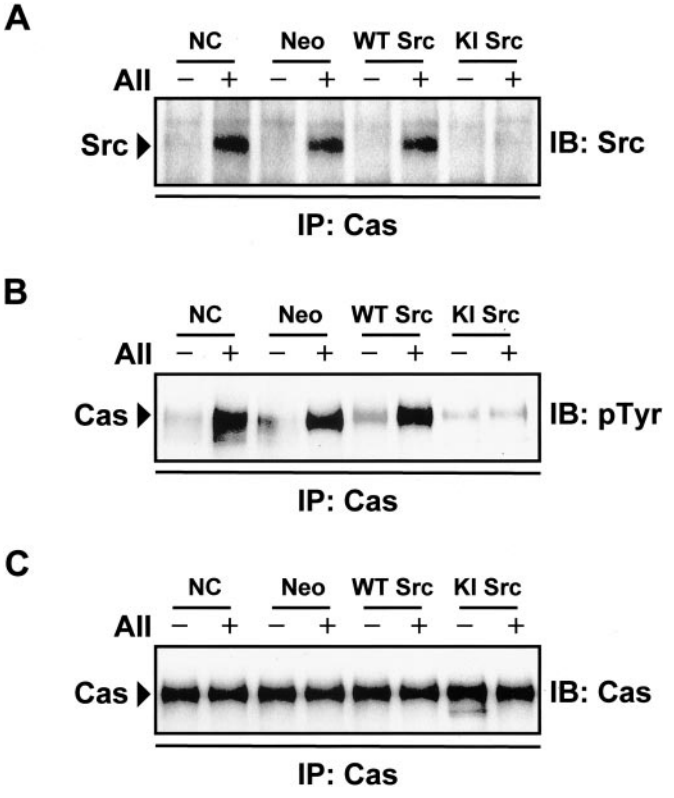

Fig. 4. Ang II-stimulated association of Src and Cas and tyrosine phosphorylation of Cas were inhibited in KI Src-overexpressed RASMC. NC and neomycin-resistant gene (Neo)-, WT Src-, and KI Src-overexpressed RASMC were used. Cells were stimulated with (+) or without (−) Ang II (100 nM) for 2 min. A, the association of Src and Cas was determined by immunoprecipitation with anti-Cas antibody and immunoblotting with anti-Src antibody as described under *Materials and Methods*. B, the tyrosine phosphorylation of Cas was determined by immunoprecipitation with anti-Cas antibody and immunoblotting with phospho-tyrosine antibody (pTyr) as described under *Materials and Methods*. C, no significant difference in the amount of Cas was observed in lysates by immunoblotting with anti-Cas antibody.

assay as described under *Materials and Methods*. We observed that HA (10 μM) or PP2 (100 μM) significantly inhibited the activation of ERK1/2 and JNK but not p38 by Ang II stimulation (Fig. 5, A and B). It seems, therefore, that Src is required for the Ang II-stimulated signaling pathway for ERK1/2 and JNK activation but not for p38 in RASMC.

Src, ERK1/2, and JNK Are Involved in Ang II-Stimulated Migration of RASMC. It has been demonstrated that Ang II plays an important role in several cardiovascular diseases associated with VSMC migration (Griendling et al., 1996). Therefore, we investigated whether Src, ERK1/2, and JNK are involved in Ang II-stimulated migration of RASMC using the Transwell migration assay as described under *Materials and Methods*. We observed that Ang II (1 μM) induced significant increases in migration of RASMC (2.23-fold) and was potently inhibited by the Src family tyrosine kinase inhibitors HA (10 μM) or PP2 (100 μM) (Fig. 6A). Furthermore, we also verified that the ERK1/2 inhibitor PD98059 (30 μM) and the JNK inhibitor SP600125 (30 μM) could strongly reduce Ang II-stimulated migration of RASMC (Fig. 6B). Hence, these results suggest that Src, ERK1/2, and JNK are important signaling molecules in Ang II-stimulated migration of RASMC.

Ang II-Stimulated ERK1/2 Activation Depends on Src Kinase but Not Cas. Next, to determine whether Src and Cas specifically mediates Ang II-stimulated ERK1/2 activation, we generated and used stably transfected WT Src-, KI Src-, WT Cas-, and ΔSD Cas-overexpressed RASMC. We examined Ang II-stimulated ERK1/2 activation by immunoblotting as described under *Materials and Methods*. Ang II produced marked increases in activation of ERK1/2 in normal cells and in neomycin-resistant gene- and WT Src-overexpressed cells. However, Ang II stimulation had no noticeable effect on the phosphorylation of ERK1/2 in KI Src-overexpressed cells (Fig. 7, A and C). In contrast, Ang II stimulation resulted in an increase in activation of ERK1/2 in ΔSD Cas-overexpressed cells to a similar extent as in normal cells and in neomycin-resistant gene- and WT Cas-overexpressed cells (Fig. 7, B and C). These results strongly suggest that Src but not Cas is essential for Ang II-stimulated signaling pathway for ERK1/2 activation in RASMC.

Ang II-Stimulated JNK Activation Depends on Src Kinase and Cas. To determine whether Src and Cas specifically mediate Ang II-stimulated JNK activation, we used

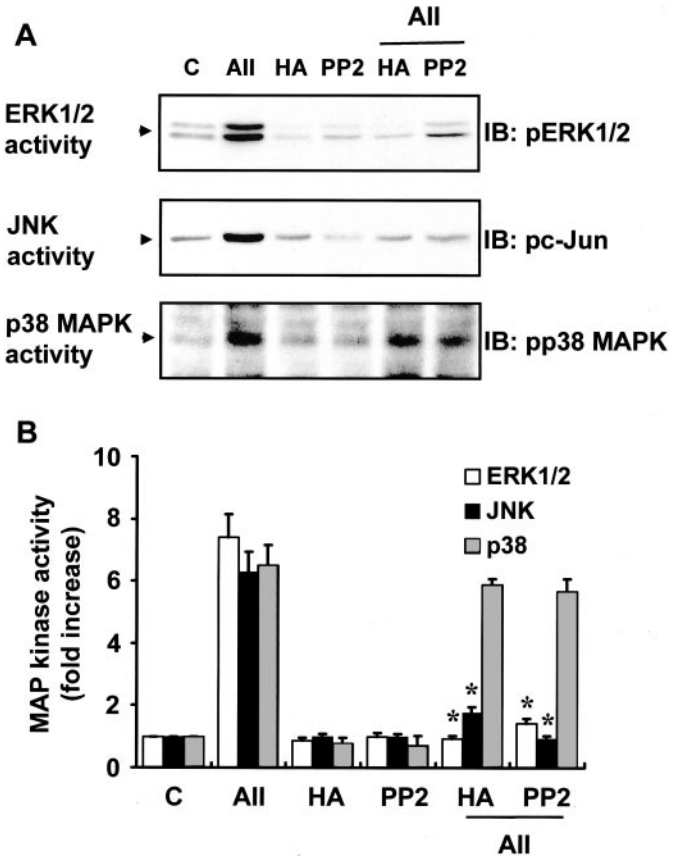

Fig. 5. HA and PP2 inhibited Ang II-stimulated ERK1/2 and JNK activation in RASMC. Cells were pretreated with or without HA (10 μM) or PP2 (100 μM) for 2 h followed by stimulation with or without Ang II (100 nM) for 5 min. A, the activities of ERK1/2, JNK, and p38 were measured as described under *Materials and Methods*. No significant difference in the amount of ERK1/2, c-Jun, and p38 were observed in lysates by immunoblotting with anti-ERK1/2, anti–c-Jun, and anti-p38 antibodies (data not shown). B, densitometric analysis of the activity of ERK1/2, JNK, and p38. Values (mean ± S.D., $n = 3$) were normalized by arbitrarily setting the densitometry of control cells (without Ang II) to 1.0. ⋆, significant differences compared with the values of Ang II stimulation at $P < 0.05$.

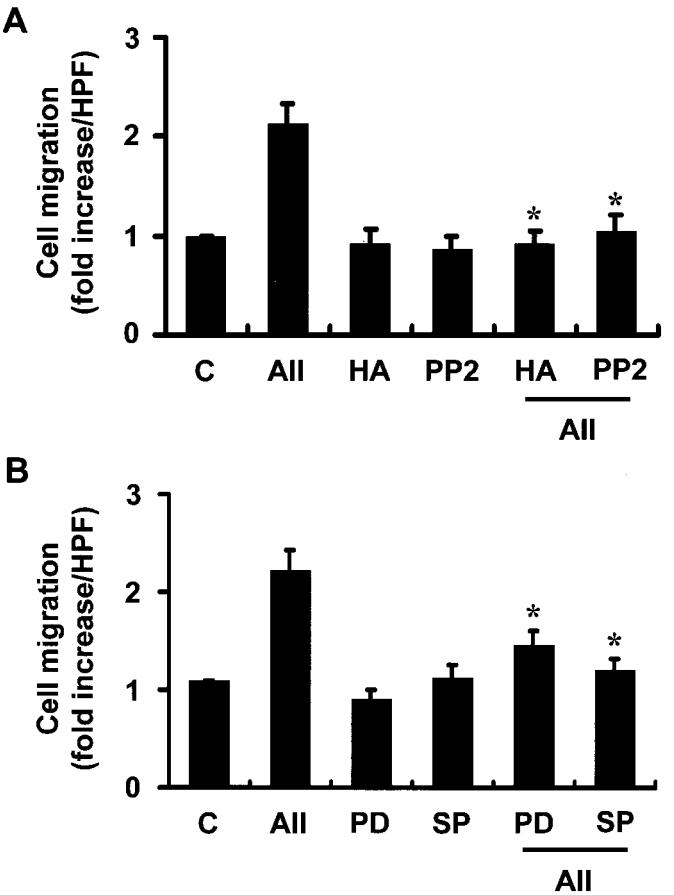

Fig. 6. HA, PP2, PD98059, and SP600125 inhibited Ang II-stimulated migration of RASMC. Cells were pretreated with or without HA (10 μM), PP2 (100 μM), PD98059 (30 μM), or SP600125 (30 μM) for 1 h at 37°C followed by treated with or without Ang II (1 μM) and were allowed to migrate for 6 h at 37°C. After 6 h of incubation, migration assay was performed as described under *Materials and Methods*. Values (mean ± S.D., $n = 3$) were normalized by arbitrarily setting the fold increases in the migration of control cells (without Ang II) to 1.0. ⋆, significant differences compared with the values of Ang II stimulation at $P < 0.05$.

stably transfected WT Src-, KI Src-, WT Cas-, and ΔSD Cas-overexpressed RASMC. Then, we determined Ang II-stimulated JNK activation by JNK assay as described under *Materials and Methods*. Ang II produced significant increases in activation of JNK in normal cells and in neomycin-resistant gene-, WT Src-, and WT Cas-overexpressed cells, whereas Ang II stimulation did not show significant effects on the activation of JNK in both KI Src- and ΔSD Cas-overexpressed cells (Fig. 8, A-C). These results suggest that both Src and Cas are critically involved in Ang II-stimulated signaling pathway for JNK activation in RASMC.

Ang II Induces Colocalization of Src, Cas, and Vinculin at the Focal Adhesion. Cell migration requires cytoskeletal reorganization involving phosphorylation of cytoskeleton-associated tyrosine kinases and formation of focal-adhesion complexes (Ilic et al., 1995). Paxillin and vinculin are cytoskeletal focal-adhesion proteins that link the actin network to the plasma membrane (Wood et al., 1994). To determine whether Src, Cas, and vinculin are colocalized at focal adhesion in response to Ang II stimulation, we treated the cells with Ang II (100 nM) for 5 min and performed triple fluorolabeling with high-resolution confocal laser scanning light microscope as described under *Materials and Methods*. Generally, there were no remarkable differences in immunointensity of Src, Cas, and vinculin between Ang II-stimulated and -nonstimulated samples. Ang II stimulation resulted in colocalization of Src, Cas, and vinculin in normal cells and in neomycin-resistant gene-, WT Src-, and WT Cas-overexpressed cells, whereas neither KI Src- nor ΔSD Cas-overexpressed cells responded to the stimulation (Fig. 9). We also observed that Ang II-stimulated colocalization of tyrosine phosphorylated Src with Cas in normal cells and in neomycin-resistant gene-, WT Src-, and WT Cas-overexpressed cells; however, it did not occur in KI Src- and ΔSD Cas-overexpressed cells (data not shown). These results suggest that Src and Cas are actively involved in the formation of focal-adhesion complexes, which is the essential step for cell migration.

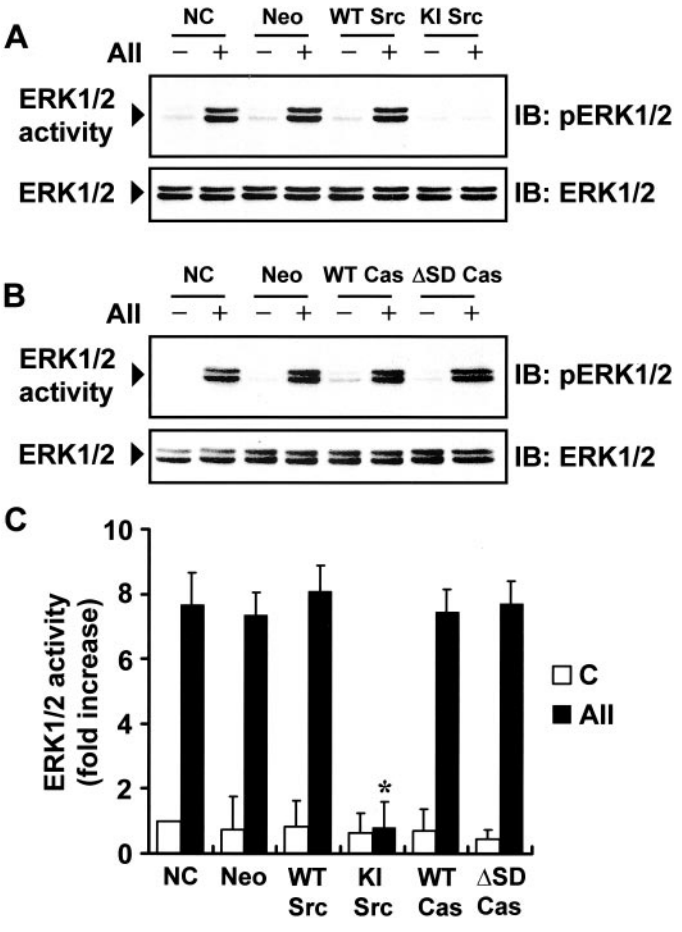

Fig. 7. Ang II-stimulated ERK1/2 activation was inhibited in KI Src-overexpressed RASMC, whereas it was not inhibited in ΔSD Cas-overexpressed RASMC. A, NC and Neo-, WT Src-, and KI Src-overexpressed RASMC were used. B, NC and Neo-, WT Cas-, and ΔSD Cas-overexpressed RASMC were used. Cells were stimulated with (+) or without (−) Ang II (100 nM) for 5 min. The activity of ERK1/2 was measured as described under *Materials and Methods* (A and B top). No significant difference in the amount of ERK1/2 was observed in lysates by immunoblotting with anti-ERK1/2 antibody (A and B bottom). C, densitometric analysis of the activity of ERK1/2. Values (mean ± S.D., $n = 3$) were normalized by arbitrarily setting the densitometry of control normal RASMC (without Ang II) to 1.0. ⋆, significant differences compared with the values of Ang II stimulation in normal RASMC at $P < 0.05$.

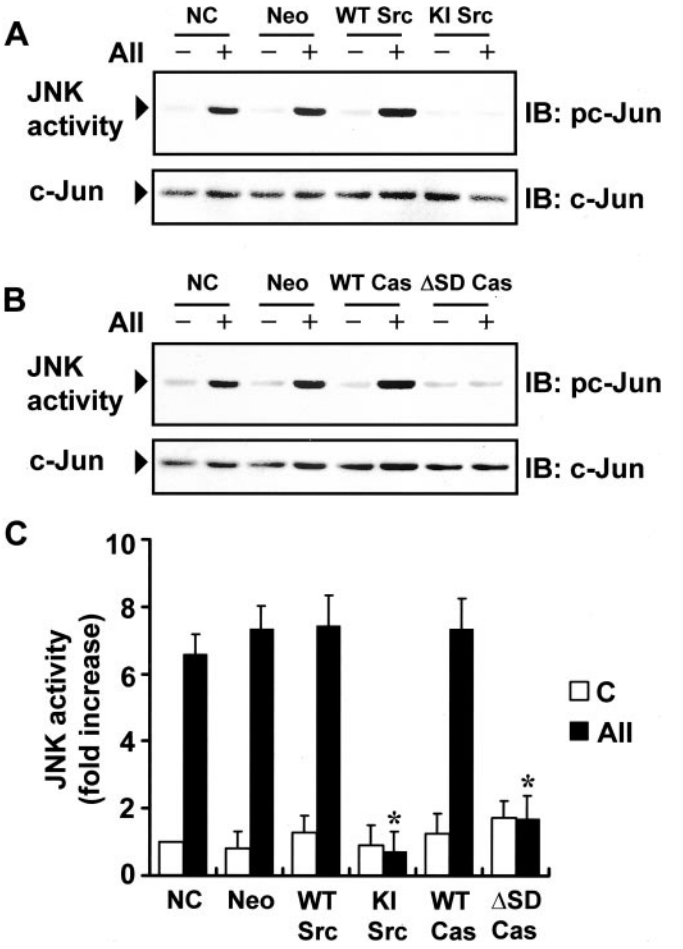

Fig. 8. Ang II-stimulated JNK activation was inhibited in both KI Src- and ΔSD Cas-overexpressed RASMC. A, NC and Neo-, WT Src-, and KI Src-overexpressed RASMC were used. B, NC and Neo-, WT Cas-, and ΔSD Cas-overexpressed RASMC were used. Cells were stimulated with (+) or without (−) Ang II (100 nM) for 5 min. The activity of JNK was measured as described under *Materials and Methods* (A and B, top). No significant difference in the amount of c-Jun was observed in lysates by immunoblotting with anti-c-Jun antibody (A and B, bottom). C, densitometric analysis of the activity of JNK. Values (mean ± S.D., $n = 3$) were normalized by arbitrarily setting the densitometry of control normal RASMC (without Ang II) to 1.0. ⋆, significant differences compared with the values of Ang II stimulation in normal RASMC at $P < 0.05$.

Src and Cas Play an Important Role in Ang II-Stimulated Migration of RASMC. We further examined the specific role of Src and Cas in Ang II-stimulated migration of RASMC using KI Src- and ΔSD Cas-overexpressed cells. Although we observed that Ang II (1 μM) evoked significant migratory effect in normal cells and in neomycin-resistant gene-, WT Src-, and WT Cas-overexpressed cells, it was suppressed in both KI Src- and ΔSD Cas-overexpressed cells (Fig. 10). These results strongly suggest that Src and Cas play a critical role in Ang II-stimulated migration of RASMC.

Discussion

Although Ang II has been shown to stimulate VSMC migration (Griendling et al., 1996), the responsible intracellular molecular mechanisms have not been well clarified. Recently, tyrosine phosphorylation was reported to be an important signaling event in several Ang II-stimulated signaling pathways (Marrero et al., 1996). Among the Ang II-induced tyrosine-phosphorylated proteins, p130Cas (Takahashi et al., 1998), c-Src (Ishida et al., 1995) and MAP kinase isozymes (Tsuda et al., 1992) are well identified. In the present study, we first demonstrated that Ang II stimulation resulted in rapid and significant increases in tyrosine phosphorylation of both Src and Cas in a similar time course (Fig. 1). Touyz et al. (2001) also reported that Ang II rapidly increased c-Src phosphorylation in VSMC. Another study demonstrated that the effect of Ang II on tyrosine phosphorylation of p130Cas was detected within 1 min after the addition of Ang II and reached maximum at ~2 min and then declined rapidly (Takahashi et al., 1998). Consistent with these previous findings, the present results indicate that Src and Cas are very prompt responsive proteins for tyrosine phosphorylation in Ang II-stimulated signaling events in RASMC.

Next, we found that Ang II stimulation caused rapid increases in the association of Src with Cas within 1 min and peaked at 5 min to the greatest extent (Fig. 2). However, we observed in the present study that the time course of Src and Cas association (Fig. 2) is different from that of Src and Cas tyrosine phosphorylation (Fig. 1). Because Src and Cas have been reported to participate in multiple signaling molecules containing focal-adhesion complexes, one possible explanation for the difference of time courses is that other signaling mechanisms in addition to Src and Cas tyrosine phosphorylation-dependent mechanism may also partly regulate and enhance the association of Src with Cas in response to Ang II stimulation. It was also demonstrated that the C-terminal portion of Cas directly binds to SH2 and SH3 domains of Src kinase (Nakamoto et al., 1996). We next observed that Src family tyrosine kinase inhibitors significantly abrogated Ang II-stimulated tyrosine phosphorylation of Cas (Fig. 3). Furthermore, we found that Ang II failed to increase the association of Src and Cas in addition to tyrosine phosphorylation of Cas in KI Src-overexpressed cells (Fig. 4). These findings are consistent with the previous observation that c-Src predominantly mediated Ang II-stimulated tyrosine phosphorylation of the focal-adhesion proteins Cas, paxillin, and tensin in VSMC (Ishida et al., 1999). It has also been demonstrated

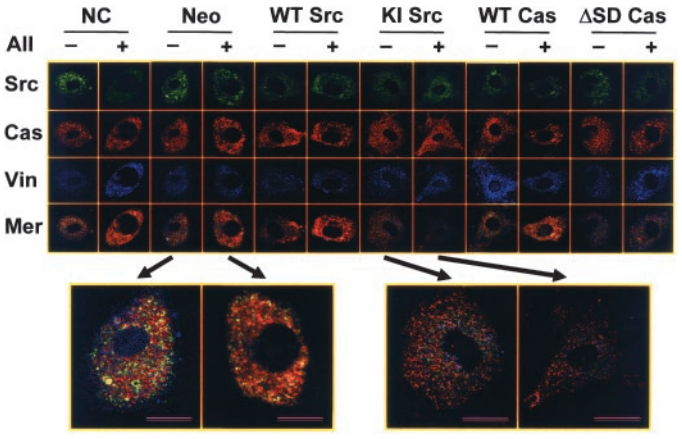

Fig. 9. Ang II-stimulated colocalization of Src, Cas, and vinculin in normal RASMC, which was inhibited in both KI Src- and ΔSD Cas-overexpressed RASMC. NC and Neo-, WT Src-, KI Src-, WT Cas-, and ΔSD Cas-overexpressed RASMC were used. Confocal laser scanning light microscopic images showing triple immunostaining for Src (green), Cas (red), vinculin (Vin; blue), and merged images with triple pseudocolors (Mer) with (+) or without (−) Ang II (100 nM) stimulation for 5 min (top). Typical images as examples of colocalization (Neo) and noncolocalization (KI Src) in response to Ang II stimulation are magnified (bottom). In the image from Neo-overexpressed RASMC, Src (green), Cas (red), and Vin (blue) are differentially localized before Ang II stimulation so that three pseudocolors are differentially visualized. After Ang II stimulation, however, the three pseudocolors are rather overlapped, showing colocalization pattern of Src, Cas, and Vin. On the other hand, in the image from KI Src-overexpressed RASMC, differential localization of Src (green), Cas (red), and Vin (blue) exhibited no change after Ang II stimulation. Bars, 50 μm.

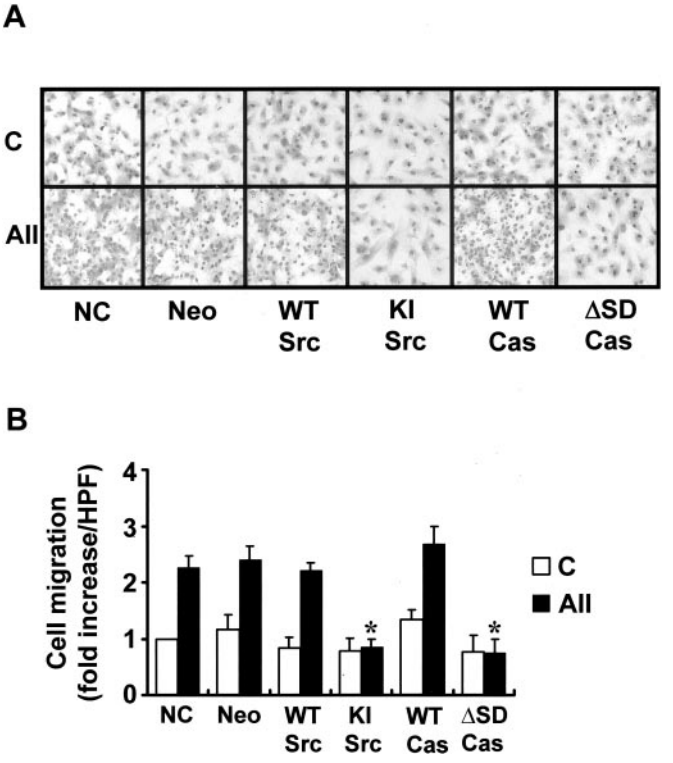

Fig. 10. Ang II-stimulated cell migration was inhibited in both KI Src- and ΔSD Cas-overexpressed RASMC. NC and Neo-, WT Src-, KI Src-, WT Cas-, and ΔSD Cas-overexpressed RASMC were used. Cells were treated with or without Ang II (1 μM) and were allowed to migrate for 6 h at 37°C. After 6 h of incubation, migration assay was performed as described under *Materials and Methods*. A, representative images of three independent experiments are shown. B, Values (mean ± S.D., $n = 3$) were normalized by arbitrarily setting the fold increases in the migration of normal RASMC (without Ang II) to 1.0. ⋆, significant differences compared with the values of Ang II stimulation in normal RASMC at $P < 0.05$.

that p130Cas, an adaptor protein in Ang II-induced signal transduction, binds catalytically active molecules such as c-Src and protein kinase Cα (Sayeski et al., 1998). It was also reported that Ang II-stimulated tyrosine phosphorylation of p130Cas was mediated via G_q-coupled AT_1 receptor (Takahashi et al., 1998) and was dependent on c-Src in VSMC (Sayeski et al., 1998). Our present findings as well as others strongly suggest that Src acts as an important regulator in Ang II-stimulated association of Src and Cas, in addition to tyrosine phosphorylation of Cas.

Ang II was reported to activate MAP kinase family members ERK1/2 (Tsuda et al., 1992), JNK (Viedt et al., 2000), and p38 (Ushio-Fukai et al., 1998) in VSMC. In the present study, we demonstrated that Src activation is required for Ang II-induced ERK1/2 and JNK activity but not for p38 in RASMC (Fig. 5). It was also reported that Ang II-stimulated activation of ERK1/2 was primarily dependent on c-Src in VSMC (Ishida et al., 1998; Touyz et al., 2001). On the other hand, it was also reported that Ang II-stimulated p38 activation was shown to be mediated in a tyrosine kinase-independent manner in VSMC (Viedt et al., 2000). In addition, we observed that Ang II stimulation showed no detectable effect on the activation of ERK1/2 or JNK in KI Src-overexpressed cells (Figs. 7A and 8A). These results further support the finding that Src is essential for Ang II-stimulated signaling pathway for ERK1/2 and JNK activation in RASMC. Because the substrate domain of Cas contains most of the putative tyrosine phosphorylation sites (tyrosines 377 to 414) and accounts for a significant portion of its tyrosine phosphorylation (Sakai et al., 1994), we examined the specific role of Cas in Ang II-stimulated ERK and JNK activation using stably transfected ΔSD Cas-overexpressed RASMC. Although we observed the stimulatory effect of Ang II on ERK1/2 activation in ΔSD Cas-overexpressed cells (Fig. 7B), Ang II did not show significant effects on the activation of JNK in ΔSD Cas-overexpressed cells (Fig. 8B). These results are consistent with our previous findings that Src and Cas mediated ROS-stimulated JNK activation but not ERK1/2 and p38 in VSMC (Yoshizumi et al., 2000). Dolfi et al. (1998) also reported that expression of Cas induced JNK activation, which was blocked by the SH2-mutant of Crk.

Cytoskeletal reorganization involving the phosphorylation of cytoskeleton-associated tyrosine kinases and formation of focal-adhesion complexes were known to be required for cell migration (Ilic et al., 1995). It was reported that a focal-adhesion complex is a highly organized site at which the actin cytoskeleton indirectly links to aggregates of transmembrane heterodimeric α- and β-integrins through the proteins vinculin, paxillin, and talin (Giancotti and Ruoslahti, 1999). In our present study, Ang II stimulation resulted in a colocalization of Src, Cas, and vinculin in normal RASMC (Fig. 9). It was also supported by the findings that tyrosine containing the proteins c-Src (Rohrschneider, 1980) and p130Cas (Harte et al., 1996) participate in focal-adhesion complexes and are essential proteins in cell migration (Klemke et al., 1998; Rahimi et al., 1998; Verbeek et al., 1999). Furthermore, our present findings indicate that Src kinase activity and Cas tyrosine phosphorylation were important for their colocalization at focal adhesion because Ang II-stimulated colocalization did not occur in both KI Src- and ΔSD Cas-overexpressed cells (Fig. 9). We also observed that Ang II failed to induce colocalization of tyrosine-phosphorylated Src with Cas in both KI Src- and ΔSD Cas-overexpressed cells (data not shown). These findings are consistent with the report that Ang II-stimulated focal-adhesion complex formation and cytoskeletal reorganization occur through AT_1 receptor and require c-Src in VSMC (Ishida et al., 1999). It was reported that p130Cas localizes to focal adhesions and recruits focal-adhesion proteins and cytoskeletal-signaling molecules such as focal-adhesion kinase, paxillin, and tensin to the focal adhesions (Nojima et al., 1995; Polte and Hanks, 1995; Rozengurt, 1995; Salgia et al., 1996). Accordingly, our present findings suggest that Src and Cas seem to be colocalized at focal adhesions and may further provide signal transduction leading to VSMC migration in response to Ang II stimulation.

In this study, we observed that the Src family tyrosine kinase inhibitors ERK1/2 and JNK significantly suppressed the migratory effect of Ang II (Fig. 6, A and B), which indicates the important role of Src, ERK1/2, and JNK in Ang II-stimulated migration of VSMC. Previously, Xi et al. (1999) demonstrated that MAP kinase antisense oligodeoxynucleotides, which deplete ERK1/2, and a synthetic inhibitor of ERK1/2, PD98059, inhibited Ang II-stimulated migration of VSMC. Recent data reported that JNK phosphorylates paxillin and was essential for rapid cell migration (Huang et al., 2003). Furthermore, we found that the migratory effect of Ang II was suppressed in both KI Src- and ΔSD Cas-overexpressed cells (Fig. 10). These results suggest that Src and Cas are critically required for Ang II-stimulated migration of RASMC. It was also reported that Src activity was required for the migration of a number of cell types (Rahimi et al., 1998; Verbeek et al., 1999). Klemke et al. (1998) also reported that Cas/Crk coupling serves as a "molecular switch" for the induction of cell migration.

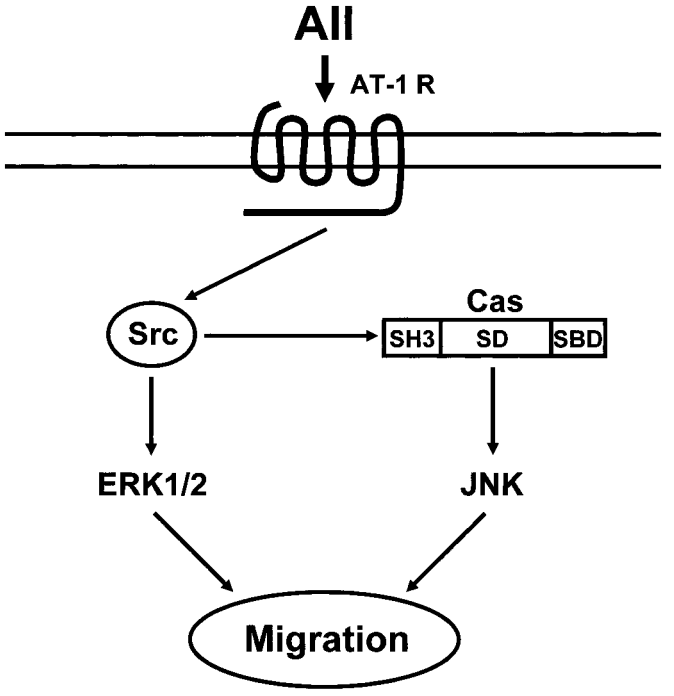

Fig. 11. Diagram of signaling pathways for Src- and Cas-mediated Ang II-induced migration of RASMC. Ang II (AII) stimulates migration of RASMC through Src- and Cas-mediated ERK1/2 and JNK signaling pathway. SH3, SH3 domain; SD, substrate domain; SBD, Src binding domain in Cas adaptor protein.

Finally, the present findings demonstrated that Ang II stimulation caused Src and Cas association, colocalization at focal adhesion, and induction of tyrosine phosphorylation of Cas in a Src-dependent manner. Furthermore, it was also revealed that Src and Cas differentially regulate the activity of ERK1/2 and JNK, which are required for Ang II-stimulated migration of RASMC. These findings strongly suggest that Src and Src-dependent tyrosine-phosphorylated Cas are essentially but differentially involved in Ang II-stimulated migration of VSMC via ERK1/2 and JNK activation (Fig. 11). Our present study proposes that Src and Cas may serve as therapeutic target molecules for atherosclerotic cardiovascular diseases relevant to VSMC migration.

Acknowledgments

We thank Masayuki Shono at Support Center for Advanced Medical Sciences for technical assistance of confocal laser microscopy.

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