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The Role of Src Kinase in Insulin-like Growth Factor-dependent Mitogenic Signaling in Vascular Smooth Muscle Cells*

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Activation of the MAPK pathway mediates insulin-like growth factor-I (IGF-I)-dependent proliferation in vascular smooth muscle cells (SMC). Our previous studies have shown that IGF-I-induced Shc phosphorylation is necessary for sustained activation of MAPK and increased cell proliferation of SMCs, and both Shc and the tyrosine phosphatase SHP-2 must be recruited to the membrane protein SHPS-1 in order for Shc to be phosphorylated. These studies were undertaken to determine whether Src kinase activity is required to phosphorylate Shc in response to IGF-I in SMC and because SHP-2 binds to Src whether their interaction was also required for IGF-I-stimulated mitogenesis. Our results show that IGF-I induces activation of Src kinase and that is required for Shc phosphorylation and for optimal MAPK activation. We tested whether Shc is a substrate of c-Src in SMC by disrupting Src/Shc association using a peptide containing a *YXXL* (Tyr^{328}) motif sequence derived from Src. The peptide blocked the binding of Src and Shc *in vitro* and *in vivo*. Cells expressing a mutant Src (Src-FF) that had Tyr^{328}/Tyr^{358} substituted with phenylalanines (Src-FF) showed defective Src/Shc binding, impaired IGF-I-dependent Shc phosphorylation, and impaired mitogenesis. This supports the conclusion that Src phosphorylates Shc. IGF-I induced both Src/SHP-2 and Src/SHPS-1 association. SMCs expressing an SHP-2 mutant that had the polyproline-rich region of SH2 deleted (SHP-2Δ10) had disrupted SHP-2/Src association, impaired IGF-I-dependent Shc phosphorylation, and an attenuated mitogenic response. IGF-I-induced association of Src and SHPS-1 was also impaired in SHP-2Δ10-expressing cells, although SHP-2/SHPS-1 association was unaffected. Upon IGF-I stimulation, a complex assembles on SHPS-1 that contains SHP-2, c-Src, and Shc wherein Src phosphorylates Shc, a signaling step that is necessary for an optimal mitogenic response.

IGF-I[2] stimulation of vascular smooth muscle cells (SMC) leads to activation of two major signaling pathways, *e.g.* the MAP kinase (MAPK) and PI 3-kinase pathways (1). Activation of the MAPK pathway is required for IGF-I-dependent proliferation, whereas activation of the PI 3-kinase pathway is the predominant determinant of IGF-I-dependent SMC migration. Binding of IGF-I to the IGF-I receptor leads to receptor autophosphorylation followed by tyrosine phosphorylation of substrates such as IRS-1 and Shc (2). These adaptor proteins bind Grb2/SOS and activate the Ras/MAPK pathway (3). Previous studies in SMC have shown that IGF-I-induced Shc phosphorylation and its association with Grb-2 are necessary for sustained phosphorylation of Erk1/2 MAPK and IGF-I-dependent cell proliferation (4). The requirement of Shc phosphorylation for growth factor-dependent mitogenesis has been demonstrated in other cell types as well (5, 6).

Src family kinases (SFK) have been implicated in mediating the mitogenic effect of several growth factors (7, 8); however, the mechanism by which SFK function is not completely understood. Src has also been implicated in IGF-I action. The requirement of Src kinase activity for IGF-I-dependent MAP kinase activation has been demonstrated in 3T3 preadipocytes (9). *In vitro* studies have shown that the IGF-I receptor is a substrate for v-Src (10). Src activation stimulates IGF-I-dependent proliferation in pancreatic carcinoma cells by increasing the number of IGF-I receptors (11). c-Src consists of several domains as follows: N-terminal Src homology (SH) 4 domain containing a myristoylation and membrane localization signal, followed by a unique domain and then SH3 and SH2 domains, the catalytic SH1 domain, and the C-terminal tail. An important mechanism of c-Src regulation is the phosphorylation and dephosphorylation of two critical tyrosine residues. c-Src is positively regulated by activation loop autophosphorylation (Tyr^{416} in mammalian Src), which occurs following SH2 and SH3 domain-mediated interactions. Src activity is negatively controlled by C-terminal tyrosyl phosphorylation (Tyr^{529} in mammalian Src), which creates a binding site for the SH2 domain of c-Src, locking the molecule in an inactive state (8). Another important factor that determines Src function is its

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[2] The abbreviations used are: IGF-I, insulin-like growth factor-I; SHP-2, SRC homology-2 domain tyrosine phosphatase; MAPK, mitogen-activated protein kinase; PI 3-kinase, phosphatidylinositol 3-kinase; SHPS-1, Src homology 2 domain containing protein-tyrosine phosphatase substrate-1; pSMC, porcine smooth muscle cell; Shc, Src homology collagen; siRNA, short interfering RNA; DMEM, Dulbecco's modified Eagle's medium; HA, hemagglutinin; MAP, mitogen-activated protein; SFK, Src family kinases; WT, wild type; RNAi, RNA interference; SH, Src homology.

differential subcellular localization. When present at plasma membrane, Src is thought to be involved in signal transduction events regulating cell growth and proliferation via activated growth factor receptors (8).

SHP-2, an Src homology containing protein-tyrosine phosphatase, plays an important role in mediating growth factor-dependent mitogenic signaling (12). Intact phosphatase activity of SHP-2 and its proper recruitment to other signaling molecules are necessary for full activation of the MAPK pathway following stimulation by epidermal growth factor and insulin (13–15). SHP-2 has also been implicated in the regulation of c-Src tyrosine kinase activity by distinct mechanisms that are both dependent and independent of its tyrosine phosphatase activity (16, 17). Our prior studies have shown that SHP-2 transfer to the plasma membrane via activation of $\alpha V\beta 3$ is required for IGF-I to initiate sustained MAPK activation and optimal mitogenic signaling (18). Phosphorylation of the $\beta 3$ subunit of $\alpha V\beta 3$ is stimulated by ligand occupancy, and this results in recruitment of SHP-2 (19). Following its recruitment to $\alpha V\beta 3$, SHP-2 is transferred to the transmembrane protein SHPS-1 in response to IGF-I. This transfer requires the phosphorylation of at least two tyrosines contained within the SHPS-1 cytoplasmic domain. IGF-I stimulates phosphorylation of these tyrosines that results in recruitment of SHP-2 to SHPS-1 and blocking SHP-2 transfer inhibits IGF-I-stimulated mitogenic signaling (20). Recently, we have shown that Shc association with SHPS-1 is necessary for Shc phosphorylation in response to IGF-I and that Shc/SHPS-1 association is dependent on SHP-2 recruitment to SHPS-1 (4). This requirement of SHP-2 recruitment to SHPS-1 suggests that it could mediate the localization of signaling components (*e.g.* a kinase) that are necessary for Shc phosphorylation and subsequently MAPK activation. These studies were undertaken to determine the role of Src kinase in IGF-I-induced Shc activation and whether SHP-2 plays a role in mediating Src transfer.

MATERIALS AND METHODS

Human IGF-I was a gift from Genentech (South San Francisco, CA). Immobilon-P membranes were purchased from Millipore Corp. (Bedford, MA). DMEM containing 4500 mg of glucose/liter (DMEM-H) was purchased from Invitrogen. Streptomycin and penicillin were purchased from Invitrogen. The Src family kinase inhibitor, SU6656, was purchased from Calbiochem. Two different anti-Src antibodies (*i.e.* Ab327 and Src-B12) were used in this study. Ab327 was the kind gift from J. S. Brugge. Src-B12 and anti-phosphotyrosine (PY99) monoclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The polyclonal antibody anti-Src (Tyr(P)418) recognizing activated Src was from BIOSOURCE. To detect SHP-2 protein, we used a polyclonal serum prepared in our laboratory. Three peptides containing the sequences between amino acid positions 367–368, 486–505, and 569–591 from human SHP-2 were synthesized by the University of North Carolina Peptide Synthesis Facility. They were purified and then linked to keyhole limpet hemocyanin and used for immunization of a rabbit as described previously (21). The hemagglutinin epitope (HA) polyclonal antibodies were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). The anti-phospho-Erk1/2 (p44/p42 MAPK) antibody and anti-β-actin antibody were obtained from Cell Signaling Technology (Beverly, MA). Monoclonal anti-Erk1/2 and monoclonal and polyclonal anti-Shc antibodies were from BD Transduction Laboratories. The plasmids, pcDNA3.1 containing mouse wild type Src cDNA or catalytically inactive Src (K295R), were kindly provided by J. S. Brugge. Three synthetic peptides were prepared that contained the TAT sequence that confers cell permeability (22) followed by either the proline-rich region of the SHP-2 sequence (underline indicates positions 558–570) YARAAARQARASPLPPCTPTPPCA or the sequence homologous to Src that contains Tyr^{328} (underline indicates positions 325–334) YARAAARQARAVQLYAVVSEE, or sequence from the N-terminal region of p52 Shc (underline indicates positions 29–39) YARAAARQARASFVNKPTRGWL, respectively. The peptides were synthesized by the Protein Chemistry Core Facility, University of North Carolina, Chapel Hill. Purity and sequence confirmation were determined by mass spectrometry.

Cell Culture—pSMCs were prepared from porcine aortas as described previously (23). The cells were maintained in DMEM-H with 10% fetal bovine serum (Hyclone, Logan, UT), streptomycin (100 ng/ml), and penicillin (100 units/ml). The smooth muscle cells that were used in these experiments were between passages 4 and 16.

Generation of pLenti Expression Vectors pLenti-Src-WT, pLenti-SrcK295R, and pLenti-Src-FF Mutants—The mouse cDNAs encoding the wild type (WT) Src and the kinase-dead Src (K295R) were kindly provided by Dr. J. S. Brugge. The kinase-dead Src (K295R) has a substitution for lysine 295, which is the ATP-binding site within the catalytic domain of Src kinase (24). Both c-Src cDNAs were amplified by PCR using the following forward and reverse primers, respectively: 5′-CACCATGGGCAGCAACAAGAGCAAGCCC-3′ and 5′-TTAAGCGTAATCTGGAACATCGTATGGGTATAGGTTCTCCCCGGGCTGGTAC-3′. The reverse primer contains a sequence encoding the HA epitope (underlined). The PCR products were cloned into the pLenti6/V5-D-TOPO expression vector (Invitrogen). The complete sequence was verified by DNA sequencing. To generate the Src-FF mutant, the tyrosines at positions 328 and 358 (potential binding sites for SH2 domains) were substituted with phenylalanines using double-stranded mutagenesis. The PCR product bearing the wild type Src cDNA sequence was cloned into the pEntr/D-TOPO Gateway entry vector according to the manufacturer's instructions (Invitrogen). The following forward and reverse oligonucleotides were used to generate PCR product ("megaprimer"), respectively, 5′-TGCAGCTGTTTGCTGTGGTGTCGGA-3′ and 5′-TAGCCGCAAAAATTTGCCCGTTTCCC-3′, and the underlined bases indicate the substitutions that change Tyr to Phe. The PCR product was then used as a primer for double-stranded mutagenesis, and the product was cloned into pEntr/D-TOPO. The Src-Y328F/Y358F sequence was excised from pEntr/D-TOPO cell ligated into pLentiCMV Gateway according to the manufacturer's instructions (Invitrogen). The complete sequence was verified by DNA sequencing.

pLenti-SHP-2/WT and pLenti-SHP-2Δ10 Mutant—The human cDNA for wild type SHP-2 was derived from a human fibroblast cell line GM10 (Coriell Institute, Camden, NJ) (20).

Full-length SHP-2 was PCR-amplified using the following forward and reverse primers, respectively, 5′-CACCATGACATCGCGGAGATGGTTTCACCC-3′ and 5′-TTAAGCGTAATCTGGAACATCGTATGGGTATCTGAAACTTTTCTGCTGTTGCATCAG-3′. The reverse primer contains the sequence encoding the HA epitope (underlined). The PCR product was cloned into the pEntr/D-TOPO Gateway entry vector according to the manufacturer's instructions (Invitrogen). To generate the SHP-2Δ10 mutant the amino acids ^{559}PLPPCTPTPP^{568} were deleted from SHP-2 by double-stranded mutagenesis of pEntr/SHP-2. The complementary oligonucleotides (100 ng) were annealed to 50 ng of supercoiled plasmid and extended by linear PCR amplification using *Pfx* polymerase (Invitrogen). The following forward and reverse oligonucleotides were used: 5′-AGACGAGTGGAGATCAGAGCTGTGCAGAAATGAGAGAAGACA-3′ and 5′-TGTCTTCTCTCATTTCTGCACAGCTCTGATCTCCACTCGTCT-3′; the underlined bases indicate the deletion border. The resulting plasmids were digested using DpnI (New England Biolabs, Beverly, MA) for 1 h 37 °C and transformed into TOP10 competent cells (Invitrogen). Deletion of amino acids 559–568 was confirmed by sequencing (University of North Carolina Genome Analysis Facility, Chapel Hill). Both the SHP-2/WT and SHP-2Δ10 sequences were then excised from pEntr/D-TOPO and ligated into pLentiCMV Gateway using the LR clonase reaction according to the manufacturer's instruction (Invitrogen).

pLenti-U6 siRNA-Src Silencing c-Src Gene Expression—The siRNA plasmids were generated using BLOCK-iT U6 RNAi system (Invitrogen) according to the manufacturer's instructions. Two target sequences from the c-*src* gene sequence were chosen: (bp 47–63) 5′-GCAACAAGAGCAAGCCC-3′ and (bp 200–220) 5′-AAGCTGTTTGGTGGCTTCAAC-3′. These sequences were shown previously to effectively silence *src* gene expression (25). Two single-stranded oligonucleotides corresponding to each sequence (forward, 5′-CACCGCAACAAGAGCAAGCCCAACGAATTGGGCTTGCTCTTGTTGC-3′, and reverse, 5′-AAAAGCAACAAGAGCAAGCCCAATTCGTTGGGCTTGCTCTTGTTGC-3′, for bp 8–22, and forward, 5′-CACCGAAGCTGTTTGGTGGCTTCAACAACGGTTGAAGCCACCAAACAGC-3′, and reverse, 5′-AAAAGCTGTTTGGTGGCTTCAACCGTTGTTGAAGCCACCAAACAGCTTC-3′, for bp 200–220) were obtained and annealed to generate a double-stranded oligonucleotide that was cloned into the pENTR/U6 vector. Constructs were confirmed by DNA sequencing and then excised from pEntr/U6 and ligated into pLentiU6 Gateway using the LR clonase reaction according manufacturer's instruction (Invitrogen).

siRNA-Src cells were generated as described below except that two different viral stocks were combined when transducing SMC (one corresponding to pLentiU6 Src bp 8–22 and the another corresponding to pLentiU6 bp 200–220).

Generation of Virus Stocks—293FT cells (Invitrogen) were prepared for generation of virus stocks of each individual pLenti-construct. Cells were plated at 5×10^6 per 75-cm^2 flask (Corning Inc., Corning, NY) the day before transfection in the growth medium (DMEM-H with 10% fetal bovine serum with streptomycin at 100 ng/ml and penicillin at 100 units/ml). On the day of transfection, the culture medium was replaced with 5 ml of Opti-MEM-I (Invitrogen) without antibiotics or serum. The DNA-Lipofectamine^{TM} 2000 complexes for each transfection were prepared and added along with total 8 ml of Opti-MEM-I medium according to the manufacturer's protocol (Invitrogen). The next day the medium containing the DNA-Lipofectamine^{TM} 2000 complexes was removed and replaced with 12 ml of growth medium. The virus-containing supernatants were harvested 48 h post-transfection, filtered through a 0.2-μm filter, and stored as 1-ml aliquots at −80 °C.

Establishment of SMCs Expressing pLenti Constructs—pSMCs (passage 4–5) were seeded at 3×10^5/well in 6-well plates the day before transduction. The viral stocks were thawed, and the viral complexes were precipitated as follows. For each 1 ml of virus stock, 1 μl of an 80 mg/ml solution of chondroitin sulfate (C4384, Sigma) was added and then mixed gently and incubated at 37 °C for 10 min. 1 μl of 80 mg/ml Polybrene (Sigma, H9286) was subsequently added and incubated at 37 °C for 10 min. The mixture was centrifuged at 10,000 rpm for 5 min to pellet the virus, and the supernatant was removed. For transduction, the pellet was resuspended in 1 ml of growth medium, and 1 μl of Polybrene (40 mg/ml) was added. The mixture was then incubated with the cells for 24 h. The virus-containing medium was removed and changed to 2 ml of growth medium for another 24 h and then replaced with selection medium (growth medium containing 4 μg/ml blasticidin; Invitrogen). The cultures were grown until they reached confluent density. The expression of the HA-tagged Src or SHP-2 proteins was detected by immunoblotting with an anti-HA antibody (1:1000) followed by a horseradish peroxidase-conjugated anti-rabbit secondary antibody.

Immunoprecipitation and Immunoblotting—Cells were seeded at 1×10^6 cells per 10-cm plate (BD Biosciences) and grown for 7 days to reach confluency. The cultures were incubated in serum-free DMEM-H for 16–20 h prior to the addition of IGF-I (100 ng/ml). In experiments in which the Src family kinase inhibitor, SU6656, was used, it was dissolved in dimethyl sulfoxide (Me_2SO) and added to confluent cultures for 1 h at a final concentration 3 μM. Control cultures were exposed to Me_2SO alone. For the experiments in which the cell-permeable peptides were added, 10 μg/ml of each peptide was added directly to the serum-free medium for 1 h prior to adding IGF-I. The cell monolayers were lysed in a modified RIPA buffer (1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 150 mM NaCl, and 50 mM Tris-HCl (pH 7.5)) in the presence of protease inhibitors (10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin) and phosphatase inhibitors (25 mM sodium fluoride and 2 mM sodium orthovanadate). The cell lysates were centrifuged at 14,000 × *g* for 10 min at 4 °C. The supernatants were exposed to a 1:330 dilution of anti-Src, anti-SHP-2, or anti-SHC antibody overnight at 4 °C. The immunoprecipitates were immobilized using protein-A-Sepharose beads for 2 h at 4 °C and washed three times with the same buffer. The precipitated proteins were eluted in 40 μl of 2× Laemmli sample buffer, boiled for 5 min, and separated using either 7.5 or 8% SDS-PAGE. The proteins were then transferred to Immobilon-P membranes, which were blocked for 1 h in Tris/saline buffer containing 0.2% Tween 20 and 1% bovine serum albumin. The blots were incubated overnight at 4 °C with the indicated antibodies (1:500 for

SHP-2 or 1:1000 for antibodies against Src, activated Src-Tyr(P)^{418}, Shc, or Tyr(P)). To detect phosphorylation of Erk1/2 MAPK, 30 μl of cell lysate was removed prior to immunoprecipitation and mixed with 30 μl of 2× Laemmli sample buffer and then separated by SDS-PAGE using an 8% gel. Anti-phospho-Erk1/2 MAPK antibody (1:1000) was used to detect activated MAPK. The total amount of Erk protein was detected using a monoclonal anti-Erk1/2 antibody at a dilution of 1:1000. The proteins were visualized using enhanced chemiluminescence (Pierce), and their abundance was analyzed using the GeneGenome CCD image system (Syngene, Ltd., Cambridge, UK). The images obtained were also scanned using an Agfa scanner. Densitometric analyses of the images were undertaken using NIH Image, version 1.61. All experiments were conducted at least three times unless stated otherwise.

Src Kinase Assay—In vitro Src kinase activity was determined by Src assay kit (Upstate Biotechnology, Inc.). The experiments were performed according to the manufacturer's instruction. Briefly, Src protein was immunoprecipitated from cell lysates with anti-Src antibody (M327 Ab). The Src-specific peptide (KVEKIGEGTYGWYK) was used as a substrate in the assay, and its phosphorylation was measured. Phosphorylation of peptide reflects the activity of Src kinase.

Cell Proliferation Assay—Assessment of SMC proliferation was performed as described previously (26). Cells expressing wild type or mutated proteins were incubated with or without IGF-I (50 ng/ml) for 48 h, and the cell number in each well was counted. Each group was analyzed in triplicate within a single experiment, and the results represent mean values of the three independent experiments.

Statistical Analysis—Student's *t* test was used to compare the differences between control and treatment groups or control cells and cells expressing mutant proteins. $p \leq 0.05$ was considered statistically significant.

RESULTS

IGF-I Induces Src Activation in pSMC—To examine whether IGF-I could induce activation of c-Src tyrosine kinase in pSMC, we analyzed c-Src immunoprecipitates following IGF-I stimulation by Western blotting using an antibody that detects activated c-Src-Tyr(P)^{416}. Src tyrosine phosphorylation was minimally detectable without IGF-I stimulation, and upon stimulation it increased 2.4-fold at 1 min and then returned to base line by 5 min (Fig. 1*A*) (2.4 ± 1.1 (mean ± S.D.)-fold increase at 1 min, $p < 0.05$; 1.4 ± 0.3-fold increase at 2 min, $n = 3$, $p < 0.05$). To confirm that phosphorylation of Tyr^{416} accurately reflects the Src kinase activity, we tested the Src immunoprecipitates in Src kinase assay *in vitro* using a synthetic peptide that is known to be an Src substrate. IGF-I induced transient increase in Src activity at 1 min which decreased to the basal level after 5 min (Fig. 1*B*). Thus, the phosphorylation of Src Tyr^{416} in SMC correlates with Src activity measured by the *in vitro* kinase assay.

To determine whether SFKs play a role in IGF-I-mediated MAPK activation, we examined MAPK (*e.g.* Erk1/2) activation by IGF-I in the presence or absence of the Src family kinase inhibitor SU-6656. This inhibitor has been shown to be specific for SFK (27). pSMC were incubated with 3 μM of SU-6656 for 1 h and then stimulated with 100 ng/ml IGF-I for the indicated times. IGF-I-induced Erk1/2 phosphorylation was inhibited in the presence of SU-6656 at all time points when compared with control samples (*e.g.* 79 ± 2% decrease at 10 min, $n = 3$, $p < 0.05$, and 30 ± 3% decrease at 20 min) (Fig. 1*C*). This result suggests that SFK activity is required for optimal MAPK activation by IGF-I. To show that Src kinase activity was inhibited, Src activity was analyzed in the presence or absence of SU-6656. As shown in Fig. 1*D*, SU-6656 efficiently inhibited Src activity. (The 2.27 ± 0.83-fold increase was reduced to a 1.09 ± 0.14-fold increase at 1 min, $p < 0.05$, $n = 3$). Src activity was also inhibited by SU6656 when assessed in the *in vitro* kinase assay (data not shown).

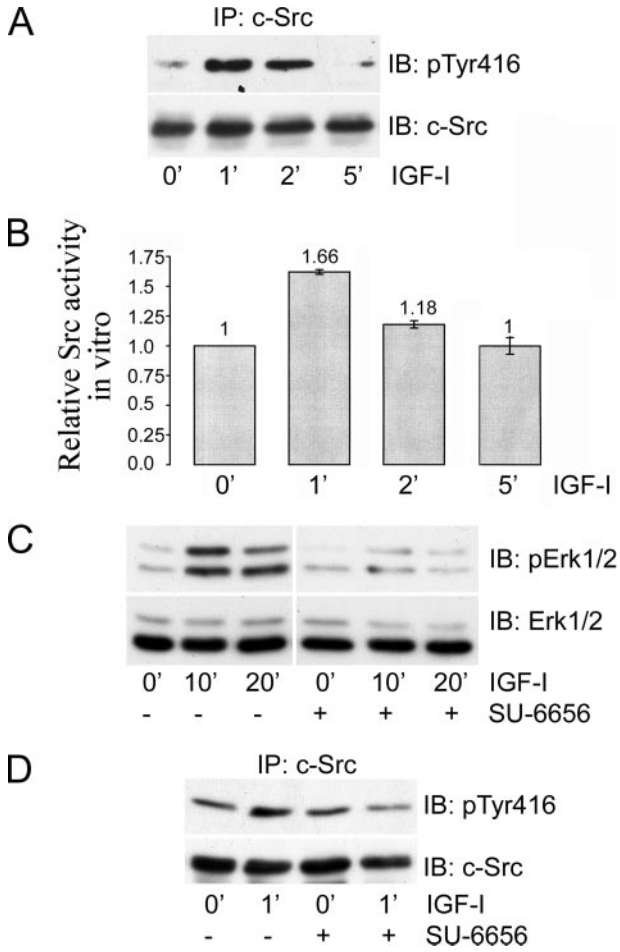

FIGURE 1. **IGF-I-stimulated Src activation and its role in MAPK activation.** *A,* Tyr^{416} phosphorylation after IGF-I stimulation. pSMC cultures were treated with 100 ng/ml IGF-I for the times indicated. Cell lysates were immunoprecipitated (*IP*) with an anti-Src antibody (mAb327) and immunoblotted (*IB*) with an antibody that detects Tyr(P)^{416}. The total immunoprecipitated Src protein is shown in the *lower panel. B,* activation of Src kinase activity. Src immunoprecipitates were tested in an *in vitro* Src kinase assay using an Src-specific peptide as a substrate. *C,* effect of the Src family kinase inhibitor (SU-6656) on IGF-I-dependent Erk1/2 MAPK activation. Cultures were treated with SU-6656 (3 μM) for 1 h followed by IGF-I (100 ng/ml) for the times indicated. The level of Erk1/2 MAPK phosphorylation was determined by immunoblotting with phospho-specific MAPK antibody (*upper panel*). The total amount of MAPK was determined by reprobing the membrane with a MAPK antibody (*lower panel*). *D,* effect of Src inhibitor SU-6656 on Src activity. pSMC cultures were treated with SU-6656 (3 μM) for 1 h followed by IGF-I (100 ng/ml) for 1 min. Src activity was determined by immunoblotting for Tyr(P)^{416}. The amount of total immunoprecipitated Src protein in each lane is shown in the *lower panel.* All experiments in *A–D* were repeated three times.

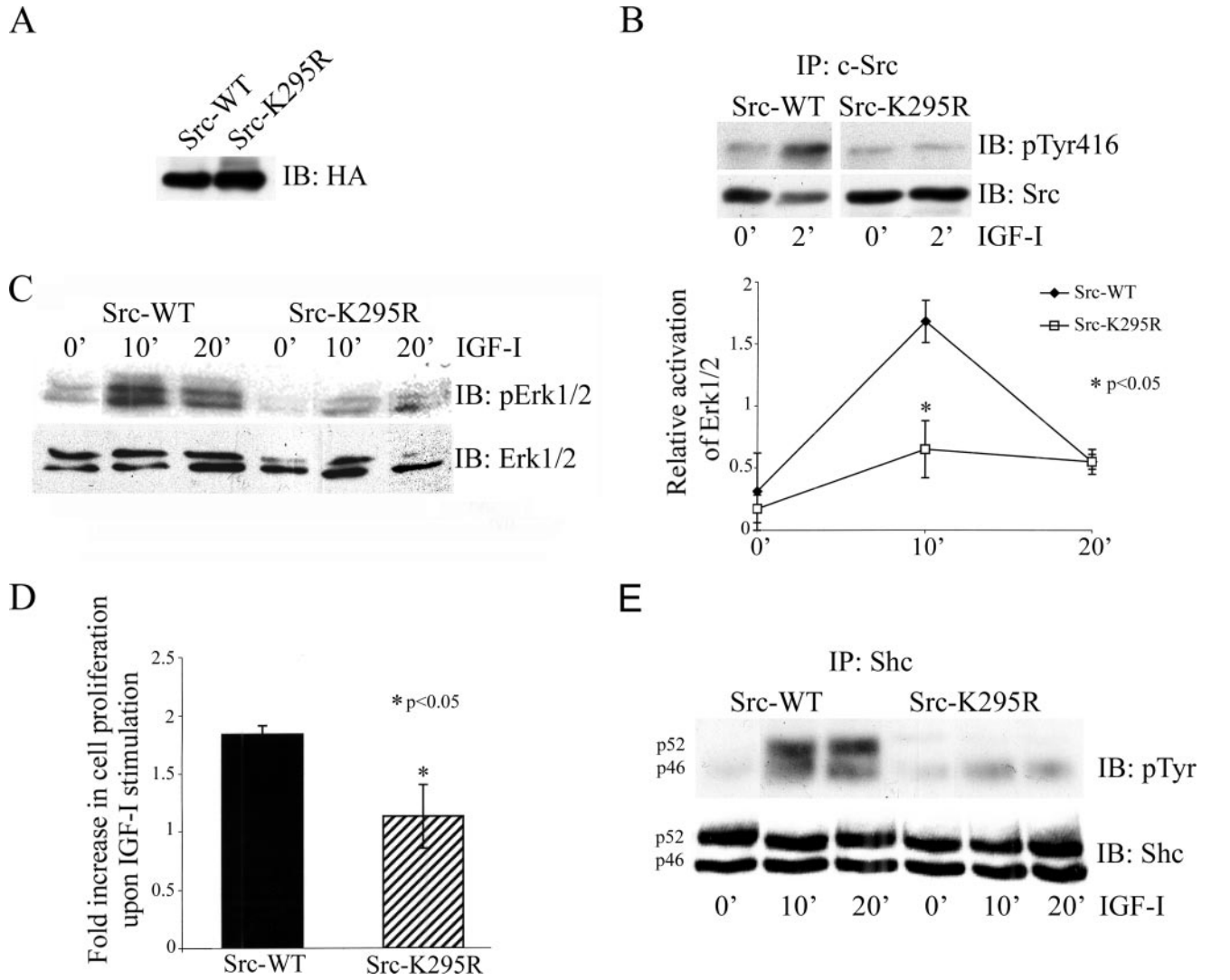

FIGURE 2. **Characterization of cells expressing wild type Src (Src-WT) or kinase-inactive Src (Src-K295R).** *A,* expression of Src-WT and Src-K295R in pSMC. Cells expressing Src-WT or Src-K295R were generated as described under "Materials and Methods." The expression of the HA-tagged protein was determined by immunoblotting (*IB*) with anti-HA antibody. *B,* activation of c-Src in Src-WT and Src-K295R cells upon IGF-I stimulation. Cultures were treated with 100 ng/ml of IGF-I as indicated. Cell lysates were immunoprecipitated (*IP*) with an anti-Src antibody (mAb327), and c-Src activity was determined by immunoblotting for Src-Tyr(P)^{416}. The membrane was reprobed with antibody against Src (*lower panel*). *C,* MAPK activation in Src-WT and c-Src-K295R cells. Cultures were treated with IGF-I (100 ng/ml) for the indicated times. Cells were lysed and analyzed for total Erk1/2 activation. Representative immunoblots of phosphorylated Erk1/2 and total Erk1/2 protein are shown. The phosphorylation of Erk1/2 was quantified using scanning densitometry and normalized by the total Erk1/2 protein level. The graph on *right* illustrates the mean ± S.E. of three separate experiments. *D,* proliferation of Src-WT and Src-K295R cells after IGF-I. Cell proliferation was assessed as described under "Materials and Methods." Cells were exposed to serum-free medium alone or stimulated with 50 ng/ml IGF-I for 48 h, and the final cell number was determined. The results represent a mean value of the fold increase (±S.E.) of triplicate determinations from three independent experiments. *E,* Shc phosphorylation in Src-WT and Src-K295R cells. Cultures were treated with 100 ng/ml of IGF-I as indicated. Cell lysates were immunoprecipitated with an anti-Shc antibody, and phosphorylated Shc was determined by immunoblotting. Total immunoprecipitated Shc is shown in the *lower panel.* Each experiment in *B–E* was repeated three times. and a representative blot is shown.

Dominant-negative Src Inhibits IGF-I-stimulated Mitogenic Signaling in pSMC—To further confirm that c-Src activation was required for optimal IGF-I-stimulated MAPK activation, we generated pSMC-expressing wild type c-Src (Src-WT) or catalytically inactive c-Src (Src-K295R). This substitution of lysine 295 with arginine causes inactivation of the ATP-binding site within the c-Src catalytic domain. As illustrated in Fig. 2*A*, Src-WT cells and Src-K295R cells expressed comparable levels of HA-tagged c-Src protein as measured by Western blotting. To confirm that Src-K295R functioned in a dominant-negative manner, we analyzed IGF- induced c-Src activation. IGF-I stimulated activation of c-Src kinase (Tyr^{416} phosphorylation) in Src-WT-expressing cells but failed to do so in cells expressing the kinase-dead Src-K295R mutant (Fig. 2*B*). To determine the consequences of expression of the kinase-dead form of c-Src on IGF-I-mediated MAPK activation, we analyzed Erk1/2 activation in Src-WT and Src-K295R cells. As shown in Fig. 2*C* the amount of IGF-I-induced Erk1/2 activation was significantly reduced in cells expressing the mutant form of c-Src compared with Src-WT-expressing cells (at 10 min 60 ± 2% decrease in Src-K295R cells, $n = 3$, $p < 0.05$). We also measured cell proliferation in cells expressing either c-Src-WT or c-Src-K295R after IGF-I stimulation. IGF-I stimulated a 1.84 ± 0.07-fold increase in proliferation in control, c-Src-WT-expressing cells but failed to stimulate an increase in c-Src-K295R-expressing cells (1.13 ± 0.27-fold increase; $n = 6$, $p < 0.05$; see Fig. 2*D*).

These results confirm that in pSMC c-Src activity is required for MAPK activation and increased cell proliferation in response to IGF-I.

IGF-I-induced Shc Phosphorylation Is Dependent on Src Activation—Previously we have shown that sustained Erk1/2 activation is required for an optimal mitogenic response to IGF-I in pSMCs and that this activation requires Shc phosphorylation (4). To determine whether Shc phosphorylation is altered by inhibiting c-Src kinase, we measured Shc phosphorylation in response to IGF-I in the cells expressing kinase-inactive Src (Src-K295R) and compared them to cells expressing wild type c-Src. Following IGF-I stimulation, Shc phosphorylation was increased in c-Src-WT cells (Fig. 2*E*). In contrast, no activation of Shc was seen in cells expressing c-Src-K295R, confirming that Src activity is required for Shc phosphorylation (3.4 ± 1.4-fold increase in Src-WT cells at 10 min compared with 1.3 ± 0.91-fold increase in Src-K295 cells, $p < 0.05$; 4.2 ± 1.1-fold at 20 min compared with 1.5 ± 0.2-fold at 20 min, $p < 0.05$).

To directly prove that Src kinase is necessary for IGF-I-dependent Shc phosphorylation, we used RNA interference to decrease the Src protein level. Cells expressing Src-targeted siRNA (siRNA-Src) were generated as described under "Materials and Methods." As a control, we used cells transfected with an empty vector. First we tested whether we achieved silencing of Src expression. Fig. 3*A* shows that cells expressing siRNA-Src have a 75 ± 13% reduction in level of detectable Src. Western blot analysis with anti-β-actin antibody indicated that the same amount of total protein was loaded in each lane. IGF-I induced a 3.1 ± 0.15-fold increase in Shc phosphorylation in control cells; however, in cells expressing siRNA-Src, Shc phosphorylation was significantly impaired (1.0 ± 0.24-fold, $p < 0.01$) (Fig. 3*B*). Moreover, IGF-I-dependent MAPK activation was also decreased in siRNA-Src cells when compared with control cells (Fig. 3*C*). More importantly, the proliferative response to IGF-I in siRNA-Src cells was impaired in comparison with control cells. (There was a 1.62 ± 0.05-fold increase in control cells that was decreased to 1.07 ± 0.07-fold in siRNA-Src cells, $p < 0.01$) (Fig. 3*D*). Thus, by employing two different methods of inhibiting Src kinase (either by overexpression of dominant-negative Src or by targeting Src with small interfering RNA), we observed impairment in IGF-I-dependent Shc phosphorylation and cellular proliferation.

Src Association with Shc Is Required for Shc Activation and IGF-I-stimulated Mitogenesis in SMCs—Although c-Src activation is required for Shc phosphorylation, this could be either an indirect consequence of phosphorylation of other signaling molecules that are required for Shc phosphorylation or, alternatively, it could reflect the fact that c-Src is the kinase that directly phosphorylates Shc. We tested the possibility that Shc is a substrate of c-Src by disrupting the Src/Shc association using a cell-permeable peptide that contained a region of Src with a *YXXL* motif (Tyr^{328}) and thus could potentially bind to the SH2 domain of Shc (hereafter referred to as an Src blocking peptide). IGF-I-induced association of Src and Shc was decreased in the presence of the peptide when compared with either control cells that were not exposed to either peptide or cells that were preincubated with a control peptide that contained a different sequence from the N-terminal part of the Shc (Fig. 4*A*) (1.8 ± 0.5-fold increase in control compared with a 0.63 ± 0.1-fold increase in cultures exposed to the Src blocking peptide, $p < 0.01$). To confirm that this Src blocking peptide was directly inhibiting the Src/Shc interaction, a phosphoryla-

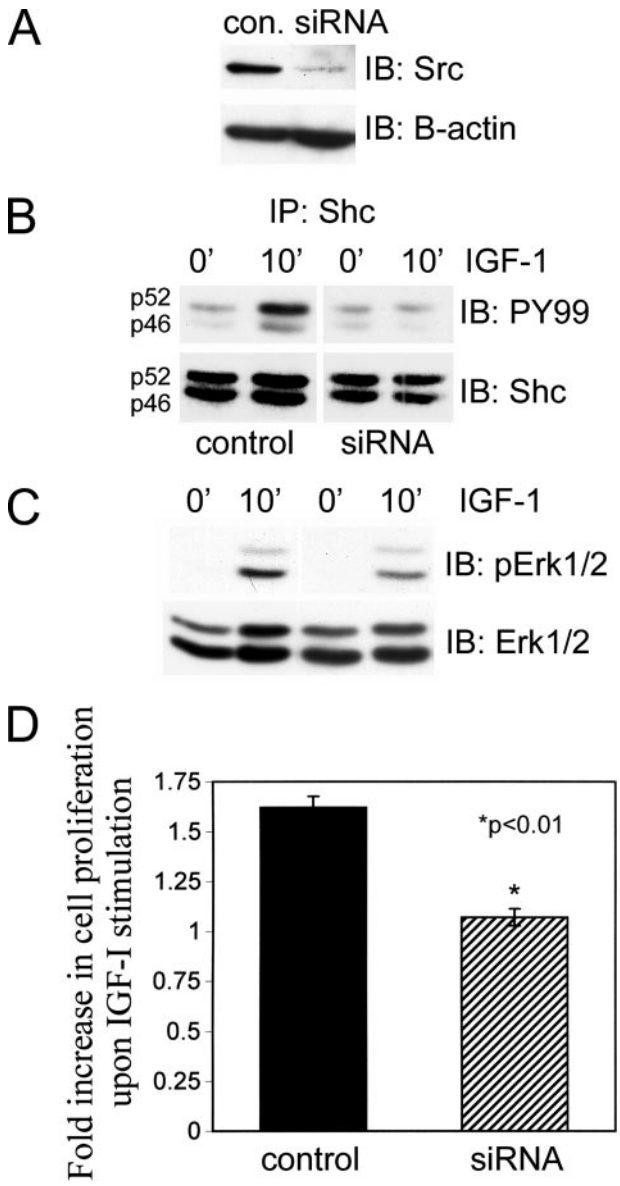

FIGURE 3. **siRNA-mediated reduction of Src expression and the changes in IGF-I-stimulated Shc phosphorylation and cell proliferation.** *A,* expression of Src protein in control and siRNA-Src cells. Cells expressing either Src-targeted siRNA (siRNA-Src) or empty vector were generated as described under "Materials and Methods." Western blotting was performed using anti-Src antibody mAb327. The membrane was stripped and probed with an anti-β-actin antibody. *con*, control. *B,* Shc phosphorylation in siRNA-Src cells. Cultures were treated with 100 ng/ml IGF-I for 10 min. Cell lysates were immunoprecipitated (*IP*) with an anti-Shc antibody and immunoblotted (*IB*) for phosphotyrosine. The membrane was stripped and immunoblotted with anti-Shc antibody. *C,* MAPK activation in siRNA-Src cells. Cultures were treated with 100 ng/ml of IGF-I for 10 min. Western blot analysis was performed for phosphorylated Erk1/2, and the stripped membrane was reprobed with anti-Erk1/2. Each experiment in *A–C* was repeated twice. *D,* proliferation of siRNA-Src and control cells after addition of IGF-I. Cell proliferation was assessed as described under "Materials and Methods." Cells were exposed to serum-free medium alone or stimulated with 50 ng/ml IGF-I for 48 h, and the final cell number was determined. The results represent a mean value of the fold increase (±S.E.) of six determinations from two independent experiments.

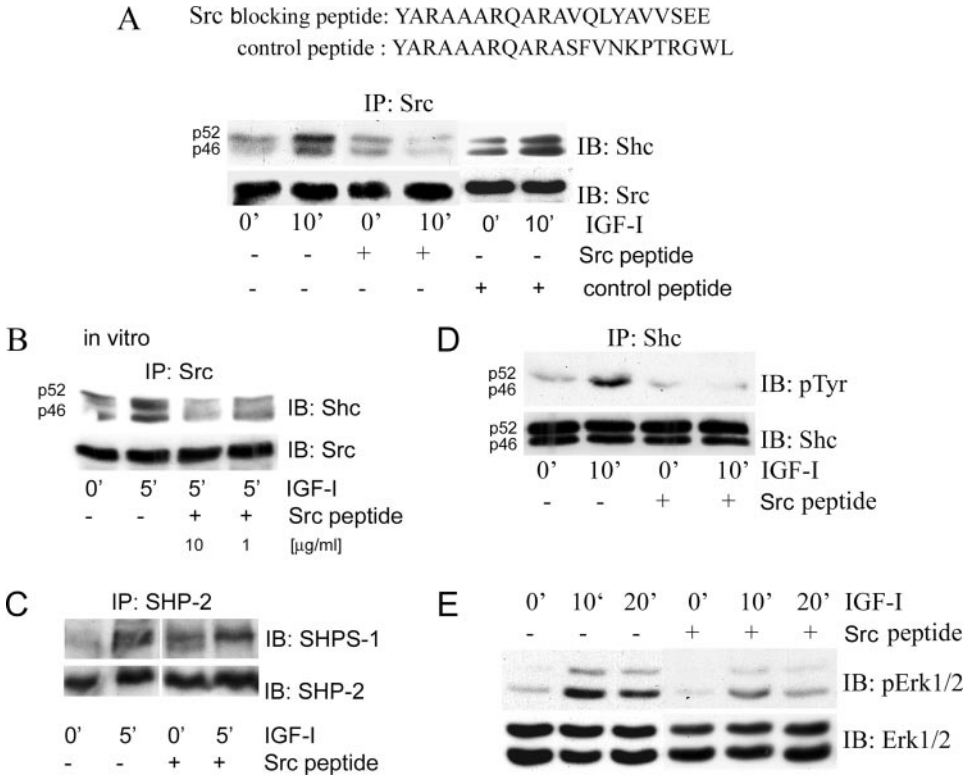

FIGURE 4. **The effect of Src/Shc disruption on Shc phosphorylation and MAPK activation in response to IGF-I.** *A,* disruption of IGF-I-induced Src association with Shc. The sequences of the Src blocking peptide and the control peptide are shown. pSMCs were incubated for 1 h in the presence or absence of 10 μg/ml of the synthetic Src peptide and then stimulated with 100 ng/ml IGF-I for the indicated times. Cell lysates were immunoprecipitated (*IP*) with an anti-Src antibody and immunoblotted (*IB*) for Shc. The immunoprecipitated Src is shown in the *lower panel.* The experiment was repeated three times. *B,* disruption of IGF-I-induced Src association with Shc *in vitro*. A phosphorylated form of the Src peptide was added to cell lysates for 30 min prior precipitation with anti-Src antibody. The amount of Shc that was coprecipitated Src was detected by immunoblotting. The immunoprecipitated Src is shown in the *lower panel. C,* the effect of Src peptide on the SHP-2/SHPS-1 interaction. SHP-2 was precipitated from the cell lysate and associated SHPS-1 was determined by immunoblotting. The immunoprecipitated SHP-2 is shown in the *lower panel.* The experiments in *B* and *C* were repeated twice. *D,* Shc phosphorylation following disruption of Src/Shc association. Cell lysates were immunoprecipitated with an anti-Shc antibody and then immunoblotted for phosphotyrosine. The immunoprecipitated Shc is shown in the *lower panel.* The experiment was repeated three times. *E,* MAPK activation following disruption of Src/Shc association. Cell lysates were immunoblotted for phosphorylated Erk1/2. The total Erk protein is shown in the *lower panel.* IGF-I-induced phosphorylation of Erk1/2 that had been normalized for total Erk protein was decreased by 28 $\pm$ 3% at 10 min and 40 $\pm$ 11% at 20 min, $n = 3$, both $p < 0.05$ compared with control cells that were not exposed to the peptide.

ted form of the peptide was added to cell lysates, and Src/Shc association was quantified. Src/Shc association was decreased in a concentration-dependent manner (Fig. 4*B*). (The 1.98 $\pm$ 0.29-fold increase was reduced to 1.09 $\pm$ 0.43 when 1 μg of the test peptide was added, $p < 0.05$, and to a 0.78 $\pm$ 0.2-fold increase when 10 μg/ml was added, $p < 0.01$.) To show that the Src peptide specifically blocked Src/Shc association and did not affect other SH2 domain/Y*XX*L motif interactions, we tested its ability to inhibit SHPS-1/SHP-2 association. IGF-I induced an increase in SHPS-1/SHP-2 association that was comparable in the presence or absence of the peptide (Fig. 4*C*). To determine the consequences of decreased Src and Shc association, we analyzed IGF-I-induced Shc phosphorylation and MAPK activation. IGF-I stimulated a 2.92 $\pm$ 0.73-fold increase in Shc phosphorylation but failed to do so in the presence of the peptide (0.91 $\pm$ 0.18-fold, $n = 3$, $p < 0.05$) (Fig. 4*D*). Erk1/2 phosphorylation was also significantly decreased in the presence of the peptide compared with control cells (28 $\pm$ 3% decrease at 10 min and 40 $\pm$ 11% decrease at 20 min, $n = 3$, both $p < 0.05$) (Fig. 4*E*). These results suggest that the association of Src and Shc is mediated through the Y*XX*L motif (Tyr^{328}) of Src and that their association is required for optimal Shc and MAPK activation following IGF-I stimulation.

To confirm the involvement of Src kinase in Shc phosphorylation, we generated cells expressing a Src mutant wherein tyrosines 328 and 358 (both sites that could potentially bind to the SH2 domains in Shc) were substituted with phenylalanine. As a control we used cells expressing the wild type Src. Fig. 5*A* shows that Src-WT cells and Src-FF cells expressed comparable levels of HA-tagged Src protein as measured by Western blotting. To determine whether the Src-FF mutant could bind to Shc, we immunoprecipitated c-Src from Src-WT and Src-FF cells and immunoblotted for Shc. The association of Shc and c-Src observed in control Src-WT cells was eliminated in cells expressing Src-FF (Fig. 5*B*) (1.8 $\pm$ 0.23-fold increase in Src-WT cells compared with a 0.85 $\pm$ 0.08-fold increase in Src-FF cells at 10 min, $p < 0.01$). This result further confirms that the phosphorylated tyrosines (*e.g.* Tyr^{328}/Tyr^{358}) are involved in binding of c-Src to Shc and that expression of the Src-FF mutant functions in a dominant-negative manner. To exclude the possibility that substitution of tyrosines to phenylalanines in Src-FF mutant had altered IGF-I-dependent Src activation, Src activity was analyzed. The ability of IGF-I to induce tyrosine 416 phosphorylation in Src-FF cells was preserved (Fig. 5*C*) (1.73 $\pm$ 0.4-fold increase). To determine consequences of the loss of association between Shc and Src, we analyzed Shc phosphorylation. As expected, Shc phosphorylation was increased in Src-WT cells following IGF-I stimulation (Fig. 5*D*). However, IGF-I-induced Shc phosphorylation in Src-FF cells was completely eliminated (4 $\pm$ 0.38-fold increase in control compared with a 1.3 $\pm$ 0.39-fold increase in Src-FF cells, $p < 0.01$). Thus, defective binding of Src to Shc resulted in impairment of Shc phosphorylation. We also determined the effect of the loss of Src and Shc association on Erk1/2 activation and cell proliferation following IGF-I stimulation in Src-WT and Src-FF cells. Cells transduced with a vector containing the *lacZ* gene were used as a control to show that overexpression of Src (Src-WT) did not enhance IGF-I-dependent Erk1/2 activation. IGF-I stimulated an increase in Erk1/2 phosphorylation in both types of control cells. In

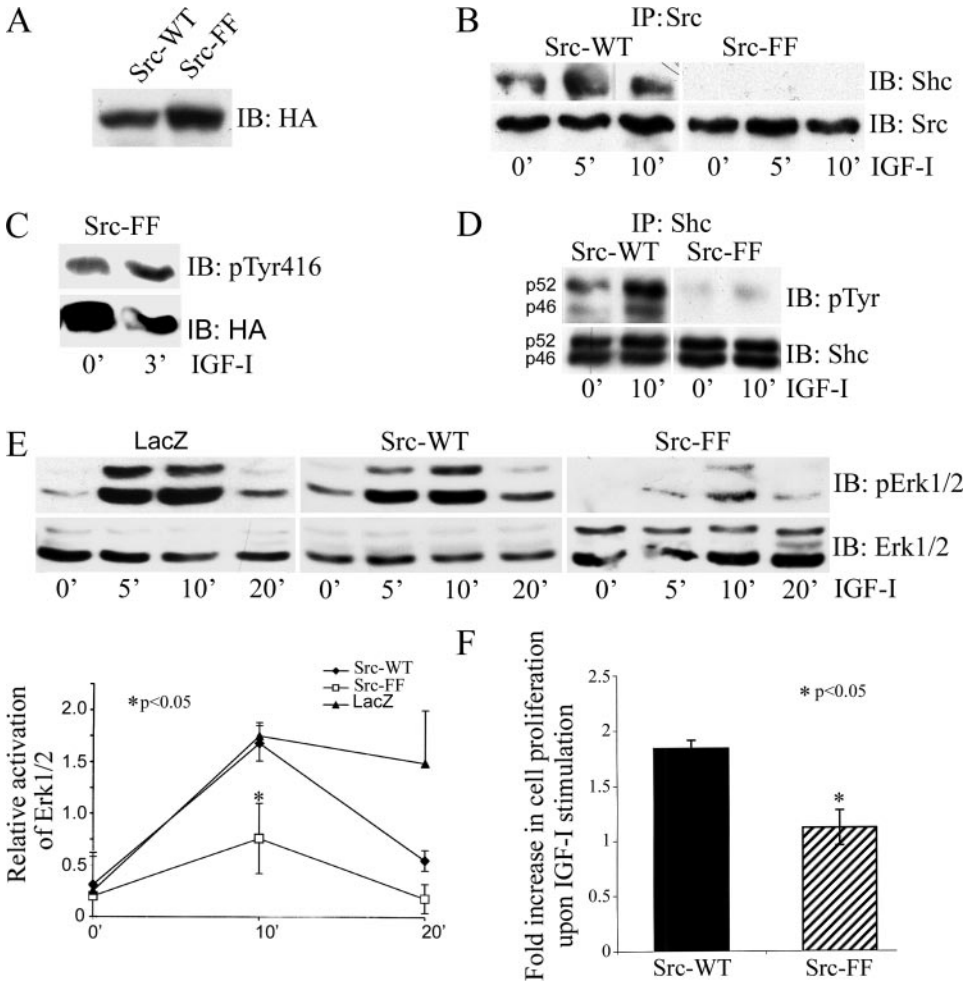

FIGURE 5. **Effect of disrupting Shc and c-Src association on IGF-I actions.** *A,* expression of wild type Src (Src-WT) and mutated form of Src (Src-FF) in pSMC. Cells expressing Src-WT or Src-FF were generated as described under "Materials and Methods." Cells were lysed, and the expression of HA-tagged protein was determined by immunoblotting (*IB*). *B,* disruption of Shc association with c-Src in Src-WT and Src-FF cells. Cultures were stimulated with IGF-I. Cell lysates were exposed to an anti-Src antibody, and the amount of coprecipitated Shc was visualized by immunoblotting for Shc. The immunoprecipitated Src is shown in the *lower panel.* The experiment was repeated three times. *C,* Src activation in Src-FF cells. Cultures were treated with 100 ng/ml IGF-I. Cell lysates were immunoblotted for the activated form of Src. The membrane was stripped and reprobed with an anti-HA antibody. The experiment was repeated twice. *D,* Shc phosphorylation in Src-WT and Src-FF. Cultures were treated with 100 ng/ml IGF-I. Cell lysates were immunoprecipitated with an anti-Shc antibody and immunoblotted for phosphotyrosine. The immunoprecipitated Shc is shown in the *lower panel.* The experiment was repeated three times. *E,* MAPK activation in LacZ, Src-WT, and Src-FF cells. Cultures were treated with 100 ng/ml IGF-I. Cells were lysed and analyzed for Erk1/2 MAPK activation. Representative immunoblots of phosphorylated and total Erk1/2 protein are shown. The phosphorylation of Erk1/2 was quantified using scanning densitometry and normalized by total Erk1/2. Each point is the mean ± S.E. of three separate experiments. *F,* proliferation of Src-WT and Src-FF cells upon IGF-I stimulation. Cells were exposed to serum- free medium or stimulated with 50 ng/ml IGF-I for 48 h, and final cell number was determined. The results represent a mean value of a fold increase of three replicates (±S.E.) from two independent experiments.

contrast, Erk1/2 activation was severely impaired in cells expressing Src-FF mutant at all time points analyzed (decreased 56 ± 17% at 10 min and 67 ± 22% at 20 min, $n = 3$, both $p < 0.05$ compared with cells expressing Src-WT; Fig. 5*E*). When cell proliferation was assessed, IGF-I induced 1.84 ± 0.07-fold increase in cell proliferation in the Src-WT cells, whereas IGF-I-dependent cell proliferation was suppressed in cells expressing the Src-FF mutant (1.12 ± 0.16-fold increase, $n = 9$, $p < 0.05$) (Fig. 5*F*). Taken together, our results suggest that the association of Src and Shc is essential for IGF-I-induced Shc and Erk1/2 activation.

Src Associates with SHPS-1 and Mediates Shc Localization—Previously, we have shown that IGF-I-dependent Shc phosphorylation requires Shc association with SHPS-1 (4). As demonstrated in Figs. 4 and 5, the binding of Src to Shc is also required for Shc phosphorylation. Taken together these findings suggest that Shc recruitment to SHPS-1 may be Src-dependent. To test this hypothesis, we first determined whether Src associates with SHPS-1. Fig. 6*A* shows that SHPS-1/Src association is enhanced upon IGF-I stimulation (Fig. 6*A*) (3.05 ± 0.59-fold increase). To determine whether Src played a role in Shc transfer to SHPS-1, cells expressing the Src-FF mutant were utilized. A control experiment showed that this substitution had no effect on Src/SHPS-1 association, which was comparable with cells expressing Src-WT (Fig. 6*B*) (3.05 ± 1.76-fold increase in Src-WT cells compared with a 2.0 ± 0.53-fold increase in Src-FF cells, p value is not significant). When SHPS-1/Shc association was analyzed, it was significantly impaired in cells expressing Src-FF suggesting that Src mediates Shc association with SHPS-1 (Fig. 6*C*) (3.57 ± 1.07-fold increase in Src-WT cells compared with a 0.82 ± 0.15-fold increase in Src-FF cells, $p < 0.01$).

Src Association with SHPS-1 Is Mediated by SHP-2—Our previous studies showed that SHP-2 transfer to SHPS-1 was required for Shc localization on SHPS-1 following IGF-I stimulation (4, 20). Therefore, we wished to determine whether c-Src association with SHPS-1 is SHP-2-dependent. First we analyzed whether SHP-2 associated with c-Src in pSMC. Following IGF-I stimulation there was a 3.87 ± 1.32-fold increase in c-Src association with Shp-2 at 5 min (Fig. 7*A*). *In vitro* studies had shown that SHP-2 binds to the SH3 domain of c-Src (16); thus we used a cell-permeable peptide that contained the polyproline-rich region of SHP-2 (YARAAARQARASPLPPCTPTPPCA) to disrupt the association of SHP-2 and c-Src (hereafter referred to as a SHP-2 blocking peptide). pSMC were treated with 10 μg/ml of this peptide for 1 h and then stimulated with IGF-I for the indicated times. The IGF-I-induced increase in Src/Shp-2 association was

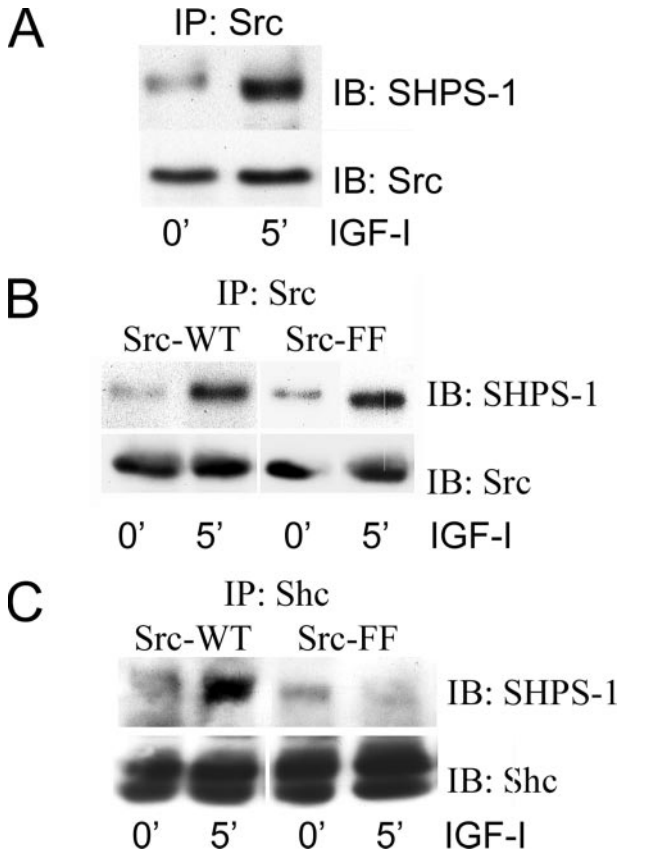

FIGURE 6. **Src association with SHPS-1 and effect of Src and Shc disruption on Shc binding to SHPS-1.** *A,* IGF-I-induced Src association with SHPS-1. SMCs were stimulated with 100 ng/ml IGF-I. Cell lysates were immunoprecipitated (*IP*) with an anti-c-Src antibody, and coprecipitation of SHPS-1 was determined by immunoblotting (*IB*). The immunoprecipitated Src is shown in the *lower panel. B,* Src association with SHPS-1 in cells expressing Src-WT and Src-FF mutant. Cultures were treated with 100 ng/ml IGF-I. Cell lysates were immunoprecipitated with an anti-c-Src antibody, and coprecipitation of SHPS-1 was determined by immunoblotting for SHPS-1. The amount of immunoprecipitated Src is shown in the *lower panel. C,* Shc association with SHPS-1 in cells expressing Src-WT and Src-FF mutant. Cultures were treated with 100 ng/ml IGF-I. Cell lysates were immunoprecipitated with an anti-Shc antibody, and coprecipitation of SHPS-1 was determined by immunoblotting. The immunoprecipitated Shc is shown in the *lower panel*. The experiments in *A–C* were repeated three times.

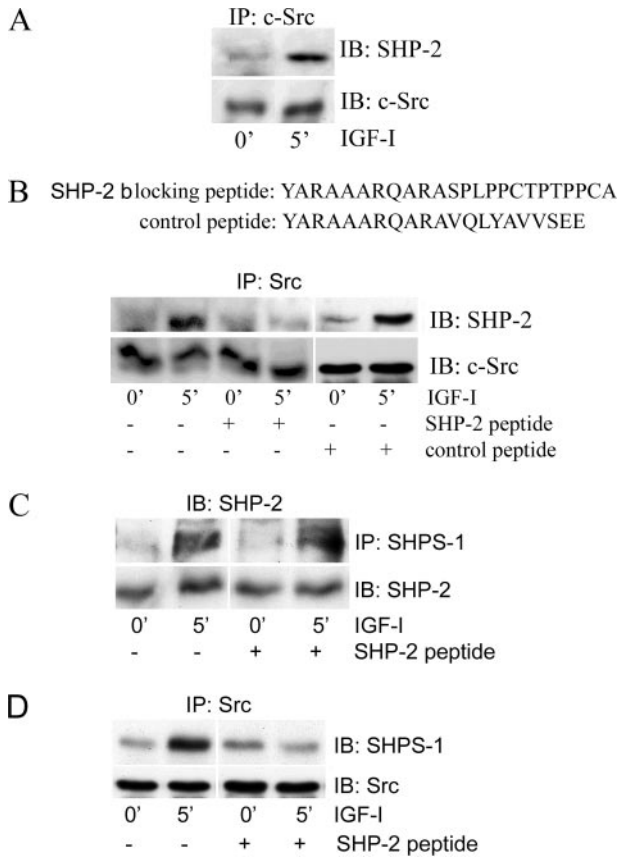

FIGURE 7. **The effect of SHP-2/Src disruption on Src association with SHPS-1.** *A,* IGF-I-induced SHP-2 association with c-Src. Cell lysates were immunoprecipitated (*IP*) with an anti-c-Src antibody, and coprecipitation of SHP-2 was determined by immunoblotting (*IB*). The immunoprecipitated Src is shown in the *lower panel. B,* disruption of SHP-2 association with c-Src. The sequences of the synthetic peptide that contained the polyproline region of SHP-2 and a control peptide that contains Tyr^{328} of Src are shown. SMCs were incubated in the presence or absence of 10 μg/ml of the synthetic peptide and then stimulated with IGF-I. Cell lysates were immunoprecipitated with an anti-Src antibody and then immunoblotted for SHP-2. The immunoprecipitated Src is shown in *lower panel.* The experiments in *A* and *B* were repeated three times. *C,* the effect of the SHP-2 peptide on the SHP-2/SHPS-1 interaction. SHP-2 was precipitated from cell lysates, and the amount of associated SHPS-1 was determined by immunoblotting. The immunoprecipitated SHP-2 is shown in the *lower panel. D,* the effect of SHP-2/Src disruption on Src association with SHPS-1. Cell lysates were immunoprecipitated with an anti-c-Src antibody and then immunoblotted for SHPS-1. The immunoprecipitated Src is shown in the *lower panel.* The experiments shown in *C* and *D* were repeated two times.

decreased from 3.87 ± 1.32-fold to 1.64 ± 0.65-fold in the presence of peptide ($n = 3$, $p < 0.05$) (Fig. 7*B*). In contrast, a control peptide that contained the sequence from Src that contains tyrosine 328 within a Y*XX*L motif that had been shown to block Src/Shc association (Fig. 4*A*) had no effect on Src/SHP-2 association. To further test the specificity of the Src/SHP-2 blocking peptide, we examined its ability to inhibit the SHPS-1/SHP-2 interaction. As shown in Fig. 7*C*, the SHP-2 blocking peptide did not alter IGF-I- induced SHPS-1/SHP-2 association suggesting that its effect is specific for disrupting SHP-2/Src association. To determine whether SHP-2 mediates Src association with SHPS-1, we analyzed the association of Src and SHPS-1 in the presence of the same blocking peptide. The IGF-I-induced increase in Src-SHPS-1 association was decreased in cells treated with blocking peptide suggesting SHP-2 mediates Src/SHPS-1 association (Fig. 7*D*) (2.65 ± 0.99-fold increase in cells with no exposure to peptide compared with 0.65 ± 0.1-fold change in cells exposed to the blocking peptide, $p < 0.01$).

Cells Expressing SHP-2 with Impaired Binding to c-Src Have Reduced MAPK Activation and Mitogenesis in Response to IGF-I—To confirm the importance of SHP-2 in Src/SHPS-1 association, we utilized cells expressing an SHP-2 mutant lacking a proline-rich region (amino acids ^{559}PLPPCTPTPP^{568}), SHP-2Δ10. Cells expressing wild type SHP-2 (SHP-2/WT) were used as a control. The expression levels of SHP-2/WT and SHP-2Δ10 were comparable (Fig. 8*A*). To determine whether expression of SHP-2Δ10 led to disruption of c-Src association, we immunoprecipitated SHP-2 from SHP-2/WT and SHP-2Δ10 cells and immunoblotted with an anti-Src antibody (Fig. 8*B*). The IGF-I-induced increase in c-Src/SHP-2 association that was seen in control SHP-2/WT cells (2.57 ± 0.94-fold) was completely abolished in pSMC-expressing SHP-2Δ10 (0.73 ± 0.39-fold, $n = 3$, $p < 0.05$). To determine the consequences of the loss of this IGF-I-induced association between SHP-2 and

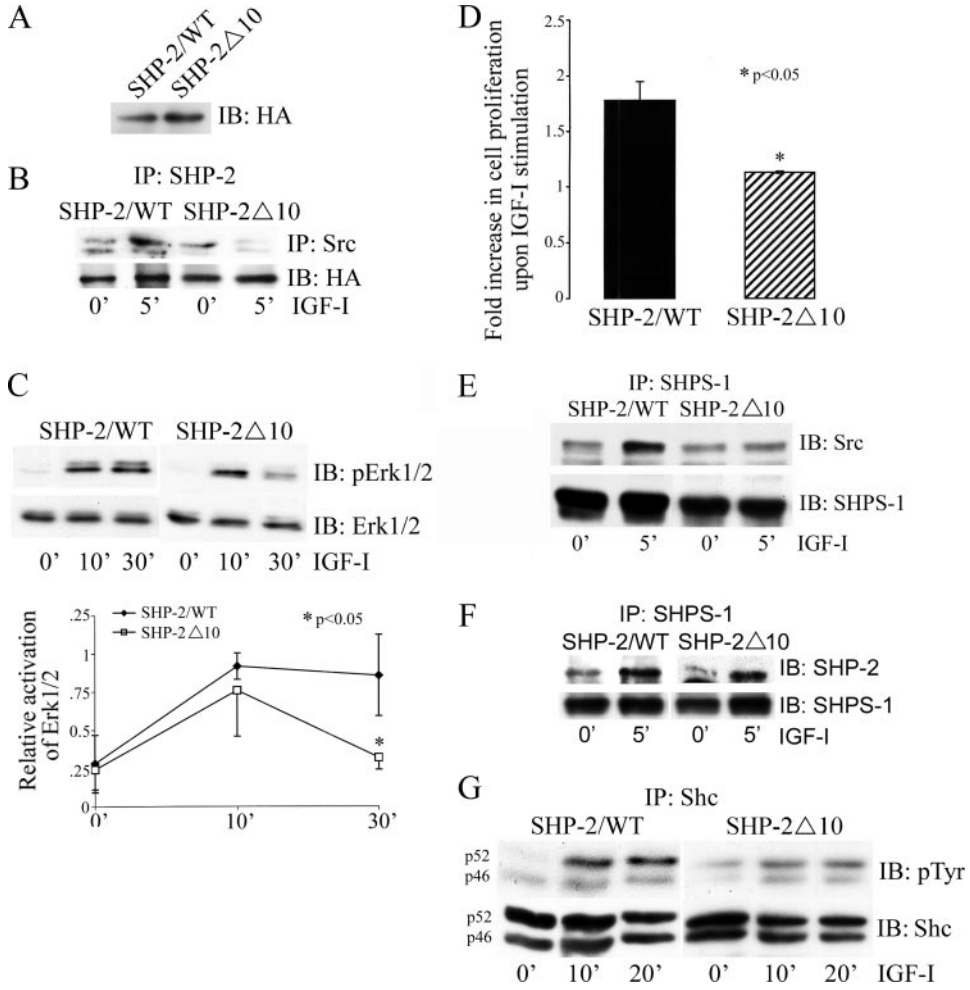

FIGURE 8. **Effect of disrupting SHP-2/Src on IGF-I actions.** *A,* expression of SHP-2/WT and SHP-2Δ10 in pSMC. Cells expressing SHP-2/WT or SHP-2 with deletion of the proline-rich region (SHP-2Δ10) were generated as described under "Materials and Methods." Quiescent cells were lysed in RIPA, and expression of HA-tagged protein was determined by immunoblotting (*IB*). *B,* disruption of SHP-2 association with Src in SHP-2-WT or SHP-2Δ10 cells. Cultures were stimulated with 100 ng/ml. Cell lysates were immunoprecipitated (*IP*) with an anti-SHP-2 antiserum and then immunoblotted for c-Src. The membrane was probed with anti-HA (*lower panel*). The experiments shown in *A* and *B* were repeated three times. *C,* MAPK activation in SHP-2/WT and SHP-2Δ10 cells. Cultures were treated with 100 ng/ml IGF-I. Cell lysates were analyzed for phospho- and total Erk1/2. The phosphorylation of Erk1/2 was quantified using scanning densitometry and normalized by the Erk1/2 protein level. Each point is the mean ± S.E. of three separate experiments. *D,* proliferation of SHP-2/WT and SHP-2Δ10 cells upon IGF-I stimulation. Cells were exposed to serum-free medium or stimulated with 50 ng/ml IGF-I for 48 h, and final cell number in each well was determined. The results represent a mean value of a fold increase (±S.E.) of triplicates from three independent experiments. *E,* Src association with SHPS-1 in SHP-2/WT and SHP-2Δ10 cells. Cultures were stimulated with 100 ng/ml of IGF-I. Cell lysates were immunoprecipitated with an anti-Src antibody and then immunoblotted for SHPS-1. The total immunoprecipitated Src is shown in *lower panel. F,* SHP-2 recruitment to SHPS-1 in SHP-2/WT and SHP-2Δ10 cells. Cultures were stimulated with 100 ng/ml of IGF-I. Cell lysates were immunoprecipitated with an anti-SHPS-1 antibody and then immunoblotted for SHP-2. The total immunoprecipitated SHPS-1 is shown in the *lower panel. G,* Shc phosphorylation in SHP-2/WT and SHP-2Δ10 cells. Cultures were treated with 100 ng/ml of IGF-I. Cell lysates were immunoprecipitated with an anti-Shc antibody and then immunoblotted for phosphotyrosine. The total immunoprecipitated Shc is shown in the *lower panel.* The experiments shown in *E–G* were repeated three times.

Src for IGF-I-dependent actions, we analyzed Erk1/2 activation following IGF-I stimulation in both SHP-2/WT and SHP-2Δ10 cells. As shown in Fig. 8*C*, IGF-I induced MAPK activation in SHP-2/WT-expressing cells that was sustained for 30 min. In contrast, there was a significant impairment in sustained Erk1/2 phosphorylation in SHP-2Δ10 cells (at 30 min decreased by 63 ± 3%, $p < 0.05$). When cell proliferation was assessed, IGF-I induced a 1.78 ± 0.17-fold increase in the SHP-2/WT cells, and this response was significantly reduced in cells expressing SHP-2Δ10 (1.13 ± 0.01-fold, $n = 9$, $p < 0.05$) (Fig. 8*D*).

To confirm that SHP-2 mediates at least in part Src/SHPS-1 association, we then analyzed cells expressing the SHP-2Δ10 mutant. IGF-I did not induce any increase in Src/SHPS-1 association in SHP-2Δ10 cells, whereas in control cells, a significant increase was observed upon IGF-I stimulation (Fig. 8*E*) (1.4 ± 0.06-fold increase in control cells compared with a 1.09 ± 0.14-fold increase in the SHP-2Δ10 cells, $p <$ 0.05). However, as shown in Fig. 7*F*, SHP-2 recruitment to SHPS-1 was not changed in SHPΔ10 cells as compared with control SHP-2/WT-expressing cells (2.39 ± 0.62-fold increase in control compared with 2.23 ± 0.63 increase in SHP-2Δ10, *p* value is not significant). Taken together, these data suggest that Src/SHPS-1 association is dependent on Src binding to SHP-2.

To further confirm that SHP-2 transfer of Src to SHPS-1 was required for an optimal Shc phosphorylation response, we compared Shc phosphorylation in cells expressing either SHP-2/WT or the SHP-2Δ10 mutant. In control cells expressing wild type SHP-2 (SHP-2/WT), IGF-I stimulated a 2.5 ± 1.1-fold increase in Shc phosphorylation at 10 min and a 2.5 ± 0.5-fold increase at 20 min (Fig. 8*G*). In contrast in cells expressing SHP-2Δ10, Shc phosphorylation was not stimulated following IGF-I (1.1 ± 0.3-fold increase at 10 min and 1.3 ± 0.4-fold increase at 20 min, $n = 3$, at 20 min $p < 0.05$). These results further suggest that Src association with SHPS-1 is required for Shc phosphorylation and that its transfer is mediated by SHP-2.

DISCUSSION

Our prior studies have shown that IGF-I induces Shc phosphorylation and that this is necessary for sustained MAPK activation and increased cellular proliferation (4). Shc phosphorylation in response to IGF-I occurs only if Shc is localized to the membrane scaffold protein SHPS-1. Those studies had shown that recruitment of SHP-2 to SHPS-1 was necessary but not sufficient for Shc localization. These studies expand those findings to show in primary, nonimmortalized smooth muscle cell

cultures the following occurs: 1) Src binding to Shc is required for Shc phosphorylation and for its localization on SHPS-1; 2) Src localization on SHPS-1 is mediated by SHP-2; and 3) c-Src activation is induced by IGF-I, and its activation is required for IGF-I-stimulated SMC proliferation.

Our studies used the following three methods to determine the consequences of inhibiting Src activity for IGF-I signaling in SMC. Inhibition of Src activation using the Src family kinase inhibitor, SU-6656, overexpression of the kinase-dead Src mutant, or a reduction in Src protein using RNAi resulted in impaired Shc phosphorylation, decreased MAPK activation, and a decreased mitogenic response. These results demonstrate that IGF-I-stimulated c-Src activation plays an important role in mediating IGF-I-dependent mitogenesis in SMC.

Multiple studies have demonstrated that growth factors can activate Src in vascular cell types. The activation of Src has also been implicated in signaling pathways that are important for atherosclerosis. Sato *et al.* (28) showed that reactive oxygen species generation resulted in increased Src phosphorylation and that this was required for hypoxia-induced reactive oxygen species-mediated damage in SMC. Similarly Cho *et al.* (29) showed that changes in SMC division that were induced by glycated low density lipoprotein required Src activation and that ligand-stimulated, $\alpha V\beta 3$-mediated inhibition of endothelial cell apoptosis required activation of NFκB through an Src-dependent mechanism. Overexpression of a kinase-dead form of Src blocked NFκB activation in this model system (30). Pressure-induced stress in blood vessels has also been shown to be associated with Src activation and cell proliferation, and these changes can be suppressed with Src inhibitors (31). Therefore, Src activation appears to play a role in the vascular cell response to a variety of stress stimuli, and this response may modify the responses of these cells to growth factor stimulation.

Some studies have shown that IGF-I can induce Src kinase activation and that this response may be necessary for activation of downstream signaling. The requirement of c-Src tyrosine kinase activity for IGF-I to stimulate MAP kinase was demonstrated in 3T3 preadipocytes (9). Similarly IGF-I-dependent activation of AKT has been shown to be Src-dependent. Knowlden *et al.* (32) demonstrated that the Src kinase inhibitor, SU6656, inhibited IGF-I-stimulated cell growth and that this effect was epidermal growth factor receptor-dependent. Likewise, Sekimoto *et al.* (33) demonstrated that $\alpha V\beta 3$ ligand occupancy stimulated association of Src with PYK-2 and that this association was required for Src activation in response to IGF-I and downstream MAP kinase activation. Cui *et al.* (34) showed that activation of PI 3-kinase and AKT in oligodendrocytes was dependent upon Src activation in response to IGF-I and that inhibition of an Src family kinase reduced AKT activation and cell survival. Finally, synergy between the actions of angiotensin-II and IGF-I in vascular cells requires transactivation of the IGF-I receptor by Src kinase (35).

To further define the mechanism by which Src kinase participates in IGF-I-dependent Erk1/2 activation, the critical substrates of Src that are phosphorylated need to be identified. Previous studies have shown that IGF-I exposure results in stimulation of Shc phosphorylation and its subsequent binding to Grb-2 (4, 5, 36), and that stimulation of the interaction between these signaling intermediates is necessary for an increase in cell proliferation. Sato *et al.* (37) demonstrated previously that residues Tyr^{239} and Tyr^{240} in Shc are phosphorylated by Src and that this occurs in a phosphatidylinositol 4,5-dependent manner. Because we could detect increased association of Shc and Src in pSMC in response to IGF-I, we tested the possibility that Shc is phosphorylated by Src following IGF-I stimulation. Both cell models wherein WT-Src was decreased (either by expressing the kinase-dead form of Src or using RNAi) and cells expressing a mutant form of Src that does not bind to Shc showed impaired Shc phosphorylation in response to IGF-I. These findings support the conclusion that the Src/Shc interaction is direct and that Shc binding to Src is required for Shc phosphorylation. Furthermore, they suggest but do not prove that c-Src is the kinase that phosphorylates Shc in response to IGF-I in SMC.

The necessity of Src activation for subsequent Shc activation and mitogenic signaling has been demonstrated. Recently IGF-I was shown to induce Src phosphorylation in vascular endothelial cells, and in this model system Shc was also activated (38). Sato *et al.* (39) recently demonstrated that serum stimulation of NIH3T3 cells resulted in Src/Shc association and that this occurred through the IDA domain of Src. Peptides that inhibited this association inhibited mitogenesis. They further demonstrated that Src could activate Shc directly. Sayeski and Ali (40) demonstrated that angiotensin-II stimulated vascular SMC proliferation through increased Src/Shc colocalization, which resulted in Shc phosphorylation and Grb-2 association as well as Erk activation. Pharmacologic inhibition of Src disrupted these molecular signaling events. Other kinases in the Src family have also been studied. Biedi *et al.* (41) demonstrated that IGF-I stimulates the corecruitment of Fyn and Shc to caveolin-1 and that IGF-I signaling requires this colocalization. Similarly, a stretch of SMC results in caveolin-dependent localization of Fyn and Shc, and this localization is required for Erk activation in response to stretch injury. Similar to our findings with SHPS-1, the tyrosines located on the cytoplasmic domain of discoid domain receptor-2 are phosphorylated by Src, and this results in Shc localization (42). These studies support the conclusion that activation of Shc by Src family kinases may be an important component of growth factor-induced signaling and that these events occur in vascular SMC in response to multiple pathophysiologic stimuli.

The mechanism by which Src binds to Shc has not been definitely determined. There are two reports each suggesting a distinct mechanism for Src and Shc interaction. Weng *et al.* (43) reported that their interaction was mediated through the SH3 domain of Src and proline-rich region of Shc. A second type of interaction was described by Sato *et al.* (39, 44) who demonstrated that Src and Shc interact through the activation segment of Src and a specific 19-amino acid sequence in the N-terminal region of Shc. We addressed both possibilities using cells that expressed two different Shc mutants, *e.g.* Shc with prolines substituted to alanines (making proline-rich region nonfunctional) (4) or Shc with deletion of the N-terminal region (amino acids 29–45). Neither of these experimental models showed a significant disruption of Src and Shc associa-

tion (data not shown). In contrast, our results show that in SMC, Src/Shc association can be disrupted using blocking peptides that contain the sequences flanking either Tyr^{328} or Tyr^{358} on Src. In addition, the binding of Shc to Src was disrupted in cells overexpressing the mutant form of Src that contained phenylalanine substitutions for these two tyrosines. Because these two tyrosines are contained in Y*XX*(L/I) motifs, the results support the conclusion that Tyr^{328}/Tyr^{358} of Src binds to the SH2 domains of Shc to mediate the interaction between these two proteins. Cells expressing this mutant had impaired Shc and MAPK phosphorylation and a decreased cell growth response to IGF-I. This further supports the conclusion that Src-mediated Shc activation is required for IGF-I stimulation of these cellular events.

The argument can be raised that the use of the dominant-negative Src kinase may inhibit other signaling pathways that may need to be activated to increase cell proliferation. Although we do not have a model system that we have proven disrupts the Src/Shc interaction exclusively, we present evidence, using cells that express an Src-FF mutant in which the Src/Shc interaction was disrupted, that this change had no effect on Src activation but that it led to impaired Shc phosphorylation and IGF-I-stimulated cell proliferation. Because this would only disrupt Src interaction with SH2 domain-containing proteins that can bind to Tyr^{328} or Tyr^{358} in Src and would have no effect on the ability of Src to phosphorylate other substrates, our findings support the conclusion that this interaction is direct and necessary for Shc phosphorylation.

Previously we have demonstrated that the membrane protein SHPS-1 functions as scaffold protein for the recruitment of SHP-2 and Shc (4). SHP-2 recruitment to SHPS-1 is necessary but not sufficient for Shc to be phosphorylated in response to IGF-I (4). Because our results show that c-Src activation and Src/Shc association are required for IGF-I-stimulated Shc and MAPK phosphorylation, these findings taken together suggest that Src has to be recruited to SHPS-1 for these responses to occur. Indeed we could detect Src/SHPS-1 association in SMC, and this association is enhanced upon IGF-I addition. Moreover, in cells expressing a mutant form of Src that did not bind to Shc, Shc association with SHPS-1 was impaired, although Src association with SHPS-1 was not altered. These data support our conclusion that Src binding to Shc is required for Shc recruitment to SHPS-1.

SHP-2 is an important regulator of IGF-I signaling. SHP-2 recruitment to the plasma membrane via ligand-stimulated activation of the $\alpha V\beta 3$ integrin is required for IGF-I to stimulate SHP-2 transfer to SHPS-1 which is required for optimal MAPK activation (18, 19) and mitogenic signaling (20). Because others have shown that there is an Src/SHP-2 interaction (16) and we could coimmunoprecipitate Src and SHP-2 in SMC, we hypothesized that SHP-2 binding to c-Src plays a role in Src localization to SHPS-1 following IGF-I stimulation. When the association between Src and SHP-2 was disrupted using either cell-permeable peptide homologous to proline-rich regions of SHP-2 or in cells expressing SHP-2Δ10 mutant, this resulted in a decrease in Src/SHPS-1 association, although neither method altered SHP-2 association with SHPS-1. Furthermore, cells expressing a mutant form of SHP-2 that did not associate with Src showed no sustained increase in MAPK activation, and cell proliferation did not increase in response to IGF-I. Taken together with our previous findings, this suggests that neither Src nor Shc can bind directly to SHPS-1 and that their binding to SHPS-1 is mediated through SHP-2. Therefore, we conclude that the SHP-2/Src interaction is necessary for Src and Shc localization to SHPS-1 and for an optimal cell replication in response to IGF-I.

At present there are insufficient data in other cell types to form a definitive conclusion as to the general applicability of this signaling system in mediating IGF-I actions in other cell types. Intestinal smooth muscle cells express $\alpha V\beta 3$, and blocking ligand occupancy of this integrin inhibits IGF-I actions (45). Endothelial cells have abundant $\alpha V\beta 3$, which can function to localize Shc to the cell membrane (41, 46), and our studies have shown that blocking ligand occupancy of endothelial $\alpha V\beta 3$ attenuates IGF-I-stimulated Shc activation and cell growth. Skeletal unloading results in decreased $\alpha V\beta 3$ expression in osteoblasts, and they become refractory to stimulation of the MAP kinase pathway activation and growth by IGF-I (47). Several cell types have been shown to have enhanced $\alpha V\beta 3$ expression, and in some cases this has led to enhanced IGF-I signaling or actions (48). Several studies have shown that a variety of cell types utilize Shc activation in response to IGF-I to signal to MAP kinase (3, 5, 6, 9, 15, 36, 37, 49, 50), and in some cell types IRS-1-mediated signaling has been shown to be down-regulated in response to stress or changes in nutrient availability (51, 52). However, the frequency with which this pathway is activated selectively during normal growth *in vivo* or whether its role is limited to pathophysiologic states such as following vascular injury has not been determined (53).

In summary our results show that Src plays an important role in Shc phosphorylation. Following IGF-I stimulation, a complex assembles on SHPS-1 that contains SHP-2, which binds directly to SHPS-1 and mediates both Src and Shc localization. Formation of this complex is necessary for Shc phosphorylation and MAP kinase activation. SHP-2 recruitment of Shc to SHPS-1 provides a means for linking signaling that is activated by $\alpha V\beta 3$ and the IGF-I receptor. SHP-2 localization to the cell membrane is dependent upon $\alpha V\beta 3$ ligand occupancy that is required for its subsequent transfer to SHPS-1. However, SHP-2 transfer from $\alpha V\beta 3$ to SHPS-1 also requires IGF-I stimulation of SHPS-1 phosphorylation. Because Shc transfer to SHPS-1 is required for Shc phosphorylation and downstream signaling that are linked to the ability of IGF-I to stimulate cell replication, these findings help to further explain how blocking $\alpha V\beta 3$ activation leads to attenuation of IGF-I receptor-linked signaling and biologic actions.

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REFERENCES

1. Imai, Y., and Clemmons, D. R. (1999) *Endocrinology* **140,** 4228 – 4235
2. LeRoith, D., Werner, H., Beitner-Johnson, D., and Roberts, C. T., Jr. (1995)

Endocr. Rev. **16,** 143–163
3. Cobb, M. H., and Goldsmith, E. J. (1995) *J. Biol. Chem.* **270,** 14843–14846
4. Ling, Y., Maile, L. A., Lieskovska, J., Badley-Clarke, J., and Clemmons, D. R. (2005) *Mol. Biol. Cell* **16,** 3353–3364
5. Kim, B., Cheng, H. L., Margolis, B., and Feldman, E. L. (1998) *J. Biol. Chem.* **273,** 34543–34550
6. Sasaoka, T., Ishiki, M., Wada, T., Hori, H., Hirai, H., Haruta, T., Ishihara, H., and Kobayashi, M. (2001) *Endocrinology* **142,** 5226–5235
7. Parsons, J. T., and Parsons, S. J. (1997) *Curr. Opin. Cell Biol.* **9,** 187–192
8. Abram, C. L., and Courtneidge, S. A. (2000) *Exp. Cell Res.* **254,** 1–13
9. Boney, C. M., Sekimoto, H., Gruppuso, P. A., and Frackelton, A. R., Jr. (2001) *Cell Growth & Differ.* **12,** 379–386
10. Peterson, J. E., Kulik, G., Jelinek, T., Reuter, C. W., Shannon, J. A., and Weber, M. J. (1996) *J. Biol. Chem.* **271,** 31562–31571
11. Flossmann-Kast, B. B., Jehle, P. M., Hoeflich, A., Adler, G., and Lutz, M. P. (1998) *Cancer Res.* **58,** 3551–3554
12. Neel, B. G., Gu, H., and Pao, L. (2003) *Trends Biochem. Sci.* **28,** 284–293
13. Shi, Z. Q., Yu, D. H., Park, M., Marshall, M., and Feng, G. S. (2000) *Mol. Cell. Biol.* **20,** 1526–1536
14. Cai, T., Nishida, K., Hirano, T., and Khavari, P. A. (2002) *J. Cell Biol.* **159,** 103–112
15. Noguchi, T., Matozaki, T., Horita, K., Fujioka, Y., and Kasuga, M. (1994) *Mol. Cell. Biol.* **14,** 6674–6682
16. Walter, A. O., Peng, Z. Y., and Cartwright, C. A. (1999) *Oncogene* **18,** 1911–1920
17. Zhang, S. Q., Yang, W., Kontaridis, M. I., Bivona, T. G., Wen, G., Araki, T., Luo, J., Thompson, J. A., Schraven, B. L., Philips, M. R., and Neel, B. G. (2004) *Mol. Cell* **13,** 341–355
18. Maile, L. A., and Clemmons, D. R. (2002) *Endocrinology* **143,** 4259–4264
19. Ling, Y., Maile, L. A., and Clemmons, D. R. (2003) *Mol. Endocrinol.* **17,** 1824–1833
20. Maile, L. A., and Clemmons, D. R. (2002) *J. Biol. Chem.* **277,** 8955–8960
21. Camacho-Hubner, C., Busby, W. H., Jr., McCusker, R. H., Wright, G., and Clemmons, D. R. (1992) *J. Biol. Chem.* **267,** 11949–11956
22. Ho, A., Schwarze, S. R., Mermelstein, S. J., Waksman, G., and Dowdy, S. F. (2001) *Cancer Res.* **61,** 474–477
23. Parker, A., Gockerman, A., Busby, W. H., and Clemmons, D. R. (1995) *Endocrinology* **136,** 2470–2476
24. Mukhopadhyay, D., Tsiokas, L., Zhou, X. M., Foster, D., Brugge, J. S., and Sukhatme, V. P. (1995) *Nature* **375,** 577–581
25. Summy, J. M., Trevino, J. G., Baker, C. H., and Gallick, G. E. (2005) *Pancreas* **31,** 263–274
26. Nam, T. J., Busby, W. H., Jr., Rees, C., and Clemmons, D. R. (2000) *Endocrinology* **141,** 1100–1106
27. Blake, R. A., Broome, M. A., Liu, X., Wu, J., Gishizky, M., Sun, L., and Courtneidge, S. A. (2000) *Mol. Cell. Biol.* **20,** 9018–9027
28. Sato, H., Sato, M., Kanai, H., Uchiyama, T., Iso, T., Ohyama, Y., Sakamoto, H., Tamura, J., Nagai, R., and Kurabayashi, M. (2005) *Cardiovasc. Res.* **67,** 714–722
29. Cho, H. M., Choi, S. H., Hwang, K. C., Oh, S. Y., Kim, H. G., Yoon, D. H., Choi, M. A., Lim, S., Song, H., Jang, Y., and Kim, T. W. (2005) *Mol. Cells* **19,** 60–66
30. Courter, D. L., Lomas, L., Scatena, M., and Giachelli, C. M. (2005) *J. Biol. Chem.* **280,** 12145–12151
31. Morita, N., Iizuka, K., Murakami, T., and Kawaguchi, H. (2004) *Mol. Cell. Biochem.* **262,** 71–78
32. Knowlden, J. M., Hutcheson, I. R., Barrow, D., Gee, J. M., and Nicholson, R. I. (2005) *Endocrinology* **146,** 4609–4618
33. Sekimoto, H., Eipper-Mains, J., Pond-Tor, S., and Boney, C. M. (2005) *Mol. Endocrinol.* **19,** 1859–1867
34. Cui, Q. L., Zheng, W. H., Quirion, R., and Almazan, G. (2005) *J. Biol. Chem.* **280,** 8918–8928
35. Zahradka, P., Litchie, B., Storie, B., and Helwer, G. (2004) *Endocrinology* **145,** 2978–2987
36. Boney, C. M., Gruppuso, P. A., Faris, R. A., and Frackelton, A. R., Jr. (2000) *Mol. Endocrinol.* **14,** 805–813
37. Sato, K., Gotoh, N., Otsuki, T., Kakumoto, M., Aoto, M., Tokmakov, A. A., Shibuya, M., and Fukami, Y. (1997) *Biochem. Biophys. Res. Commun.* **240,** 399–404
38. Bjorndahl, M., Cao, R., Nissen, L. J., Clasper, S., Johnson, L. A., Xue, Y., Zhou, Z., Jackson, D., Hansen, A. J., and Cao, Y. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 15593–15598
39. Sato, K., Iwasaki, T., and Fukami, Y. (2005) *J. Biochem.* (*Tokyo*) **137,** 61–67
40. Sayeski, P. P., and Ali, M. S. (2003) *Exp. Cell Res.* **287,** 339–349
41. Biedi, C., Panetta, D., Segat, D., Cordera, R., and Maggi, D. (2003) *Endocrinology* **144,** 5497–5503
42. Yang, K., Kim, J. H., Kim, H. J., Park, I. S., Kim, I. Y., and Yang, B. S. (2005) *J. Biol. Chem.* **280,** 39058–39066
43. Weng, Z., Thomas, S. M., Rickles, R. J., Taylor, J. A., Brauer, A. W., Seidel-Dugan, C., Michael, W. M., Dreyfuss, G., and Brugge, J. S. (1994) *Mol. Cell. Biol.* **14,** 4509–4521
44. Sato, K., Nagao, T., Kakumoto, M., Kimoto, M., Otsuki, T., Iwasaki, T., Tokmakov, A. A., Owada, K., and Fukami, Y. (2002) *J. Biol. Chem.* **277,** 29568–29576
45. Kuemmerle, J. F. (2006) *Am. J. Physiol.* **290,** G1194–G1202
46. De, S., Razorenova, O., McCabe, N. P., O'Toole, T., Qin, J., and Byzova, T. V. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 7589–7594
47. Sakata, T., Wang, Y., Halloran, B. P., Elalieh, H. Z., Cao, J., and Bikle, D. D. (2004) *J. Bone Miner. Res.* **19,** 436–446
48. Pereira, J. J., Meyer, T., Docherty, S. E., Reid, H. H., Marshall, J., Thompson, E. W., Rossjohn, J., and Price, J. T. (2004) *Cancer Res.* **64,** 977–984
49. Roudabush, F. L., Pierce, K. L., Maudsley, S., Khan, K. D., and Luttrell, L. M. (2000) *J. Biol. Chem.* **275,** 22583–22589
50. Soon, L., Flechner, L., Gutkind, J. S., Wang, L. H., Baserga, R., Pierce, J. H., and Li, W. (1999) *Mol. Cell. Biol.* **19,** 3816–3828
51. Harrington, L. S., Findlay, G. M., and Lamb, R. F. (2005) *Trends Biochem. Sci.* **30,** 35–42
52. Tzatsos, A., and Kandror, K. V. (2005) *Mol. Cell. Biol.* **26,** 63–76
53. Corjay, M. H., Diamond, S. M., Schlingmann, K. L., Gibbs, S. K., Stoltenborg, J. K., and Racanelli, A. L. (1999) *J. Cell. Biochem.* **75,** 492–504