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Role of SHPS-1 in the Regulation of Insulin-like Growth Factor I–stimulated Shc and Mitogen-activated Protein Kinase Activation in Vascular Smooth Muscle Cells

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Insulin-like growth factor I (IGF-I) stimulates smooth muscle cell (SMC) proliferation, and the mitogen-activated protein kinase (MAPK) pathway plays an important role in mediating IGF-I–induced mitogenic signaling. Our prior studies have shown that recruitment of Src homology 2 domain tyrosine phosphatase (SHP-2) to the membrane scaffolding protein Src homology 2 domain–containing protein tyrosine phosphatase substrate-1 (SHPS-1) is required for IGF-I–dependent MAPK activation. The current studies were undertaken to define the upstream signaling components that are required for IGF-I–stimulated MAPK activation and the role of SHPS-1 in regulating this process. The results show that IGF-I–induced Shc phosphorylation and its subsequent binding to Grb2 is required for sustained phosphorylation of MAPK and increased cell proliferation in SMCs. Furthermore, for Shc to be phosphorylated in response to IGF-I requires that Shc must associate with SHPS-1 and this association is mediated in part by SHP-2. Preincubation of cells with a peptide that contains a phospho-tyrosine binding motif sequence derived from SHPS-1 inhibited IGF-I–stimulated SHP-2 transfer to SHPS-1, the association of Shc with SHPS-1, and IGF-I–dependent Shc phosphorylation. Expression of an SHPS-1 mutant that did not bind to Shc or SHP-2 resulted in decreased Shc and MAPK phosphorylation in response to IGF-I. In addition, SMCs expressing a mutant form of the β3 subunit of the αVβ3, which results in impairment of SHP-2 transfer to SHPS-1, also showed attenuated IGF-I–dependent Shc and MAPK phosphorylation. Further analysis showed that Shc and SHP-2 can be coimmunoprecipitated after IGF-I stimulation. A cell-permeable peptide that contained a polyproline sequence from Shc selectively inhibited Shc/SHP-2 association and impaired Shc but not SHP-2 binding to SHPS-1. Exposure to this peptide also inhibited IGF-I–stimulated Shc and MAPK phosphorylation. Cells expressing a mutant form of Shc with the four prolines substituted with alanines showed no Shc/SHPS-1 association in response to IGF-I. We conclude that SHPS-1 functions as an anchor protein that recruits both Shc and SHP-2 and that their recruitment is necessary for IGF-I–dependent Shc phosphorylation, which is required for an optimal mitogenic response in SMCs.

INTRODUCTION

Both vascular smooth muscle cell proliferation and migration in response to growth factor stimulation play important roles in the formation of atherosclerotic plaques, and insulin-like growth factor I (IGF-I) is a potent stimulator of smooth muscle cell proliferation and migration (Jones *et al.*, 1996). In primary cultured smooth muscle cells (pSMCs), IGF-I induces activation of both the phosphatidylinositol (PI)-3 kinase and the mitogen-activated protein (MAP) kinase (MAPK) pathways. Both pathways have been shown to play roles in mediating IGF-I–dependent cell migration and cell proliferation responses (Imai and Clemmons, 1999; Maile *et al.*, 2003).

Previous studies have shown that the protein tyrosine phosphatase SHP-2 plays an important role in mediating growth factor-mediated mitogenic signaling (Noguchi *et al.*, 1994; Bennett *et al.*, 1996; Maroun *et al.*, 2000; Cai *et al.*, 2002; Neel *et al.*, 2003). The catalytic activity of SHP-2 and its recruitment to signaling molecules such as Grb2-associated binder 1 (Gab1) are required for full activation of the MAP kinase pathway in response to epidermal growth factor (EGF) (Holgado-Madruga *et al.*, 1996; Schaeper *et al.*, 2000; Shi *et al.*, 2000; Cai *et al.*, 2002). SHP-2 phosphatase activity also has been shown to be required for insulin to activate MAP kinase in chinese hamster ovary (CHO) cells (Noguchi *et al.*, 1994).

Src homology 2 domain containing protein tyrosine phosphatase substrate-1 (SHPS-1) is a transmembrane protein that plays an important role in mediating various cellular responses, including migration and proliferation. The cytoplasmic domain of SHPS-1 contains four tyrosine residues located within YXXL/I/V motifs that can undergo phosphorylation after growth factor stimulation or cell adhesion and then bind to SH-2 domain containing proteins such as SHP-2 (Fujioka *et al.*, 1996; Takada *et al.*, 1998; Oh *et al.*, 1999). Transfer of SHP-2 to SHPS-1 is required for growth factor-mediated cell proliferation, DNA synthesis, and MAP kinase activation (Takada *et al.*, 1998; Ling *et al.*, 2003; Maile *et al.*, 2003). Disruption of SHP-2 binding to SHPS-1 either by expressing a SHPS-1 mutant that does not bind SHP-2

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Abbreviations used: SHP-2, Src homology 2 domain tyrosine phosphatase; SHPS-1, Src homology 2 domain–containing protein tyrosine phosphatase substrate-1.

(Takada *et al.*, 1998) or by inhibiting SHPS-1 phosphorylation (Maile *et al.*, 2003) leads to impairment of sustained MAP kinase activation. In SMCs, SHP-2 is basally associated with the tyrosine-phosphorylated β3 subunit of the $\alpha V\beta 3$ integrin (Ling *et al.*, 2003). IGF-I stimulation induces the phosphorylation of SHPS-1 and the subsequent binding of SHP-2 to SHPS-1 (Maile and Clemmons, 2002). Inhibition of β3 or SHPS-1 phosphorylation leads to impaired SHP-2 transfer to SHPS-1 in response to IGF-I, and this correlates with a reduction in the cellular DNA synthesis response (Ling *et al.*, 2003; Maile *et al.*, 2003). Although it is clear that SHP-2 transfer to SHPS-1 plays an important role in mediating IGF-I–dependent cellular proliferation, the mechanism by which SHP-2 association with SHPS-1 regulates MAP kinase activation has not been determined.

Tyrosine phosphorylation of adaptor proteins such as IRS-1 and Shc acts to link the activated IGF-I receptor with downstream signaling elements in the PI-3 and/or MAP kinase pathways. In many cell types such as neurons, fibroblasts, and adipocytes, phosphorylation of Shc and its association with the adaptor protein Grb2 are required for growth factor-mediated mitogenic signaling (Kim *et al.*, 1998; Boney *et al.*, 2000; Sasaoka *et al.*, 2001). In pSMCs, IGF-I stimulation induces tyrosine phosphorylation of IRS-1 followed by its association with the regulatory subunit of PI-3 kinase (p85), which leads to activation of the PI-3 kinase pathway (Zheng and Clemmons, 1998). However, it remains unclear whether phosphorylation of Shc mediates IGF-I–stimulated responses in pSMCs; in particular, the role of Shc in mediating IGF-I–dependent mitogenic signaling has not been determined.

The current study was undertaken to determine whether Shc activation was required for sustained MAP kinase activation in response to IGF-I in cultured SMCs, to determine whether Shc binding to SHPS-1 played a role in IGF-I–dependent Shc phosphorylation and whether SHP-2 played a role in their association.

MATERIALS AND METHODS

Human IGF-I was a gift from Genentech (South San Francisco, CA). Immobilon-P membranes were purchased from Millipore (Bedford, MA). DMEM containing 4500 mg glucose/l (DMEM-H) was purchased from Invitrogen (Carlsbad, CA), and streptomycin and penicillin were purchased from Invitrogen. A polyclonal antibody for the β3-subunit of porcine $\alpha V\beta 3$ integrin was generated using two synthetic peptides containing the amino acid sequences encompassing positions 36–63 and 623–648. A polyclonal antibody for SHP-2 was generated using peptides that contained the SHP-2 sequences corresponding to amino acid 367–387 and 486–497. Polyclonal antibodies for IRS-1, SHPS-1, and the hemagglutinin (HA) epitope were obtained from Upstate Biotechnology (Lake Placid, NY). The anti-phospho-tyrosine (p-Tyr) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-extracellular signal-regulated kinase (Erk)1,2, total Erk1,2, Shc, and Grb2 were from BD Transduction Laboratories (Lexington, KY). EZ-link Sulfo NHS SS biotin was purchased from Pierce Chemical (Rockford, IL). Synthetic peptides that contained the TAT cell-permeable sequence (Ho *et al.*, 2001) followed by either an SHPS-1 sequence (underlined) YARAAARQARA^{466}TLTYADLDM^{473} or the proline-rich region of Shc sequence (underlined) YARAAARQARA^{358}LRVPPPPQSM^{367} were prepared. The tyrosine motif control peptide contained the TAT sequence followed by the Tyr-containing motif (YXXL/I) of p85 (underlined) YARAAARQARA^{555}EYREIDKR^{562}. The proline-rich control peptide contained the proline-rich region from downstream of kinase 1 (DOK1) (underlined), YARAAARQARA^{425}PQGPAFPEPG^{434}. The peptides were synthesized by the Protein Chemistry Core Facility at the University of North Carolina at Chapel Hill. Purity and sequence confirmation were determined by mass spectrometry. [^{32}P]orthophosphoric acid (8000–9200 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ).

Cell Culture

pSMCs were prepared from porcine aortas as described previously (Parker *et al.*, 1995). Cells were maintained in DMEM-H with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), streptomycin (100 μg/ml), and penicillin (100 U/ml). pSMCs that were used in these experiments were used between passages 4 and 16.

Generation of pMEP4-Expression Vectors and Establishment of Vector Control and β3-FF Cells

A full-length cDNA encoding a mutant form of human β3 integrin subunit containing two substitutions Y747F and Y759F (β3-FF) was generated in the pMEP4 expression vector as described previously (Ling *et al.*, 2003). The construct contains a FLAG epitope sequence (DYKDDDDK) inserted between the leader sequence and mature protein sequence. Cells that had been transfected with the pMEP4-vector that did not contain β3-FF insert were used as control. pSMCs (passages 4 and 5) were transfected with these constructs by using the poly-L-ornithine method as described previously (Parker *et al.*, 1995; Ling *et al.*, 2003). The cells were maintained in DMEM-H with 15% FBS and 40 μg/ml hygromycin plus streptomycin and penicillin. The cells that were used in these experiments were between passages 6 and 15.

Generation of Plenti-Expression Vectors

pLenti-HA-p52Shc/WT (Wild-Type Shc) and pLenti-HA-p52Shc/3F. (Shc/Y239/240/317F) pcDNA-p52Shc/WT and pcDNA-p52Shc/3F constructs were kindly provided by Dr. Kenneth Yamada (NIH, Bethesda, MD). Mutation of these three tyrosine phosphorylation sites has been shown to generate a dominant negative inhibitor of Shc signaling (Kim *et al.*, 1998). Full-length Shc/WT and Shc/3F were PCR amplified using the following forward and reverse primers: 5′-CACCATGTACCCATACGATGTTCCAGATTACGCTAACAAGCTGAGTGGAGGCGGCGGG-3′ and 5′-TTACAGTTTCCGCTCCACAGGTTGCTG-3′. The PCR products containing a Kozak sequence (CACC) followed by a sequence encoding the HA epitope (underlined) at the 5′ end of the p52 Shc coding sequence were cloned into the plenti6/V5-D-TOPO expression vector (Invitrogen, Carlsbad, CA). The complete sequence was verified by DNA sequencing.

pLenti-Shc4A. Human p52Shc cDNA was cloned in to pENTR/D-TOPO Gateway entry vector according to the manufacturer's instructions (Invitrogen). Wild-type sequences were transferred to plentiCMV Gateway by LR clonase reaction and transformed into Stbl3 competent cells (Invitrogen). Incorporation of the p52 Shc DNA sequence was verified by restriction digest with *Eco*RV. Amino acids 361–364 were changed from prolines to alanines by double-stranded mutagenesis of pENTRp52Shc. One hundred and twenty-five nanograms of complimentary oligonucleotides was annealed to 50 ng of supercoiled plasmid and extended by linear PCR amplification by using Pfx polymerase (Invitrogen). The oligonucleotide sequences were 5′-gaagatgctcttcgggtgGctGcaGctGcccagtcggtgtccatg-3′ forward and 5′-catggacaccgactgggCagCtgCagCcacccgaagagcatcttc-3′ reverse, where the capitalized bases indicate the substitutions. The resulting plasmids were digested by *Dpn*I (New England Biolabs, Beverly, MA) and transformed into TOP10 competent cells (Invitrogen). The cells were plated and colonies isolated. Correct incorporation of the changes was confirmed by DNA sequencing (UNC Genome Analysis Facility, Chapel Hill, NC)

pLenti-HA-SHPS-1/WT and pLenti-HA-SHPS-1/-CD. Full-length human SHPS-1 cDNA was generated by reverse transcription-PCR from mRNA that had been derived from human fibroblasts (GM10; Human Genetic Cell Repository, Camden, NJ) (Maile and Clemmons, 2002). The full-length SHPS-1 sequence was PCR amplified using previously generated pcDNA-SHPS-1 (Maile and Clemmons, 2002) as a template and cloned into the expression vector plenti6/V5-D-TOPO. The forward and reverse primers used to generate the PCR product were 5′-CACCATGGAGCCCGCCGGCCCGGC-3′ and 5′-TTAAGCGTAATCTGGAACATCGTATGGGTACTTCCTCGGGACCTGGACGCT-3′. The wild-type SHPS-1 construct contains an HA sequence at the 3′ end of the coding sequence (underlined). The SHPS-1 coding sequence was truncated at K398, producing a truncated SHPS-1 mutant (SHPS-1/-CD) that contains only the first five residues (amino acids 394–398) of the 110 residues (394–503) within the cytoplasmic domain. The truncated SHPS-1 was generated by PCR amplification with Stratagene (La Jolla, CA) *Pfu* Turbo polymerase by using forward and reverse primers as follows: 5′-CACCATGGAGCCCGCCGGCCCGGC-3′ and 5′-TTAAGCGTAATCTGGAACATCGTATGGGTACTTCTGTCTGATTCGGACGAGGTAGA-3′. The PCR product containing the underlined HA sequence at the 3′ end of the SHPS-1 mutant coding sequence was TOPO-cloned into the plenti6/V5-D-TOPO expression vector. The SHPS-1/-CD sequence was confirmed by DNA sequencing.

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 plate (Corning Glassworks, Corning, NY) the day before transfection in the growth medium (DMEM-H with 10% FBS with streptomycin at 100 μg/ml and penicillin at 100 U/ml). On the day of transfection, the culture medium was replaced with 5 ml of Opti-MEM-I (Invitrogen) without antibiotics or serum. DNA–Lipofectamine 2000 complexes for each transfection sample were pre-

pared according to the manufacturer's protocol (Invitrogen). The next day, the medium containing the DNA–Lipofectamine 2000 complexes was removed and replaced with the growth medium. The virus-containing supernatants were harvested 48–72 h posttransfection and centrifuged at 3000 rpm for 15 min at 4°C to pellet the cell debris. The supernatants were filtered and stored as 1-ml aliquots at −80°C.

Establishment of SMCs Expressing pLenti-Vector Control and pLenti-Constructs

pSMCs (passages 4 and 5) were seeded at 3×10^5/well in each of two wells of a six-well plate (353046; Falcon; BD Biosciences Discovery Labware, Bedford, MA) the day before transduction. The viral stocks were thawed, and the viral complexes precipitated as follows. For each 1 ml of virus stock, 1 μl of an 80 mg/ml solution of chondroitin sulfate (C4384; Sigma-Aldrich, St. Louis, MO) was added and then mixed gently and incubated at 37°C for 10 min. One microliter of 80 mg/ml polybrene (H9286; Sigma-Aldrich) was added and incubated at 37°C for 10 min. The mixture was centrifuged at 10,000 rpm for 5 min to pellet virus, and the supernatant was removed. For transduction, the pellet was resuspended in 1 ml of growth medium, and 1 μl of 40 mg/ml polybrene was added and then the mixture was 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). The cultures were then grown to confluence.

The expression of HA-tagged p52Shc protein was detected by immunoblotting with a 1:1000 dilution of anti-HA antibody using 30 μl of cell lysate.

Detection of Cell Surface Protein Expression

Cells expressing plenti-SHPS-1 constructs were grown to confluence, and the cell surface proteins were biotinylated as described previously (Maile *et al.*, 2001). The cultures were then lysed and an equal amount of protein from each cell lysate was immunoprecipitated using the anti-HA antibody. The immune complexes were then separated by 7.5% SDS-PAGE under nonreducing conditions. Biotinylated SHPS-1 was detected by immunoblotting with peroxidase-conjugated avidin as described below.

Immunoprecipitation and Immunoblotting

Cells were seeded at 5×10^5 cells per 10-cm plate (BD Biosciences, Franklin Lakes, NJ) and grown for 7 d to reach confluence. The cultures were incubated in serum-free DMEM-H for 12–16 h before the addition of 100 ng/ml IGF-I. For the experiments in which the cell-permeable peptides were added, 10 or 20 μg/ml of each peptide was added directly to serum-free medium for 1 h before adding IGF-I. The cell monolayers were lysed in a modified radioimmunoprecipitation assay (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, and 1 μg/ml pepstatin), and phosphatase inhibitors (25 mM sodium fluoride and 2 mM sodium orthovanadate). The cell lysates were centrifuged at 14,000 $\times$ *g* for 10 min at 4°C. The supernatant containing crude membrane and cytosolic proteins was exposed to a 1:330 dilution of anti-IRS-1, anti-Shc, anti-HA, anti-β3, or anti-SHPS-1 antibodies overnight at 4°C. The immunoprecipitates were immobilized using protein A beads for 2 h at 4°C and washed three times with the same lysis buffer. The precipitated proteins were eluted in 40 μl of 2× Laemmli sample buffer, boiled for 5 min, and separated on a 7.5, 8, or 12.5% SDS-PAG. For detection of β3/Shc association and SHP-2/Shc association, the anti-β3 antibody or anti-Shc antibody was used to immunoprecipitate β3 or Shc. After the first round of immunoprecipitation, the supernatant was used for a second round of immunoprecipitation with the same antibodies. Both immunoprecipitates were combined and loaded on 8% SDS-PAG. Further analysis showed that after two rounds of immunoprecipitation >95% of the total amount of either β3 or Shc protein that could be detected by immunoblotting was recovered. The proteins were then transferred to Immobilon-P membranes that were blocked for 1 h in 1% bovine serum albumin in Tris-saline buffer with 0.2% Tween 20. The blots were incubated overnight at 4°C with indicated antibodies (1:500 for p-Tyr; 1:1000 for antibodies against HA, SHPS-1, SHP-2, IRS-1, and Shc; and 1:2000 for anti-Grb2). The levels of β3 protein, Shc, and SHPS-1 that were expressed by each cell type were evaluated by direct immunoblotting of 30 μl of cell lysate by using a 1:1000 dilution of anti-β3 antibody, polyclonal anti-Shc or anti-SHPS-1 antibody. To detect Erk1,2 activation, 30 μl of cell lysate was removed before immunoprecipitation and mixed with 25 μl of 2× Laemmli sample buffer then separated by 8% SDS-PAGE. Anti-phospho-Erk1,2 (1:1000) was used to detect phosphorylated Erk1,2, and total Erk1,2 protein was detected using a monoclonal anti-Erk1,2 antibody (1:1000). The proteins were detected using enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL), and their relative abundance was analyzed using the GeneGnome charge-coupled device image system (Syngene, Cambridge, United Kingdom). The images obtained were also scanned using an Agfa (Ridgefield Park, NJ) scanner. Densitometric analyses of the images were determined using NIH Image, version 1.61. All experiments were performed at least three times. To correct for gel loading differences and differences in the efficiency of immunoprecipitation, the results are expressed as the scanning units divided by the scanning units obtained when the protein that was immunoprecipitated was quantified by immunoblotting.

Concentration of Cell Lysates

pSMCs were serum starved overnight. The cells were scraped off the plates into 900 μl of phosphate-buffered saline that contained protease and phosphatase inhibitors as described above. The samples were frozen and thawed three times before they were centrifuged at 14,000 $\times$ *g* for 10 min at 4°C. The supernatant was then concentrated using Millipore Ultrafree-0.5 centrifugal filter device (Millipore, Billerica, MA). After the cell lysate was concentrated to 100 μl, 0.1 ml of in vitro kinase reaction buffer (RB; 100 mM Tris-HCl, pH 7.2, 125 mM $MgCl_2$, 25 mM $MnCl_2$, 2 mM EGTA, 250 μM sodium orthovanadate, and 2 mM dithiothreitol [DTT]) was added, and the samples were further concentrated to 100 μl. The buffer exchange and concentration steps were repeated one time to a final volume of 100 μl.

Detection of Phosphorylation of the SHPS-1 Peptide In Vivo

Confluent cultures were serum starved overnight. The cultures were incubated for total 3 h in 5 ml of phosphate-free Eagle's minimal essential medium containing 250 μCi of [^{32}P]orthophosphate. The SHPS-1 cell-permeable peptide was added for 30 or 60 min at 10 μg/ml concentration before cells lysed in RIPA buffer. Control cultures were exposed to no peptide or a peptide that does not contain tyrosine residues. The anti-pTyr antibody (1:330) was incubated with the cell lysates and the immunoprecipitation was performed as described above. After washing the protein A beads three times the precipitated proteins were eluted in 40 μl of 2× Laemmli sample buffer and loaded on to 15% SDS-PAG. A duplicate sample of the SHPS-1 peptide that had been labeled with ^{125}I (Markwell, 1982) was used as a marker and run in a parallel lane on the same gel.

Cell Proliferation Assay

Assessment of SMC proliferation was performed as described previously (Nam *et al.*, 2000). Cells were incubated with or without IGF-I (50 ng/ml) in serum-free DMEM containing 0.2% platelet-poor plasma for 48 h, and cell number in each well was counted. Each treatment was analyzed in triplicate, and the results represent mean values of 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. $p < 0.05$ was considered statistically significant.

RESULTS

Shc Phosphorylation Is Required for IGF-I–dependent Mitogenesis in Cultured pSMCs

To determine the necessity for Shc tyrosine phosphorylation for IGF-I–dependent mitogenic signaling, we generated pSMCs expressing a control vector, wild-type p52-Shc (Shc-WT), or p52-Shc with three tyrosine residues (Y239, Y240, and Y317) that had been mutated to phenylalanine (Shc-3F) (Kim *et al.*, 1998). p52Shc-WT and p52Shc-3F were overexpressed in SMCs, and the levels of exogenous Shc were comparable as shown in Figure 1A. There was no significant difference in basal Shc phosphorylation when the Shc-WT– and Shc-3F–expressing cells were compared (p = NS). IGF-I induced a 4.2 ± 0.7 (mean ± SEM, n = 3, $p < 0.01$)-fold increase in p52Shc phosphorylation after 5 min in cells expressing Shc-WT, and the maximum level of phosphorylation was sustained for 20 min (4.0 ± 0.7-fold increase, $p < 0.01$) (Figure 1B). This correlated with increased Grb2 binding to phosphorylated Shc-WT, which also was sustained for 20 min (4.5 ± 1.7-fold increase at 5 min, $p < 0.01$ and 3.6 ± 1.4-fold increase at 20 min, $p < 0.05$, n = 3). In contrast, when the cells expressing Shc 3F were analyzed, there was a significant reduction in p52-Shc phosphorylation (57.6 ± 7.4 and 68.2 ± 5.6% reductions compared with the control cultures at 5 and 20 min, n = 3, $p < 0.01$) and decreased association of Grb2 (84.2 ± 2.2 and 77.3 ± 5.1% reductions at 5 and 20 min, $p < 0.05$) after IGF-I stimulation. Comparison of the time course of Erk1,2 phosphorylation showed that

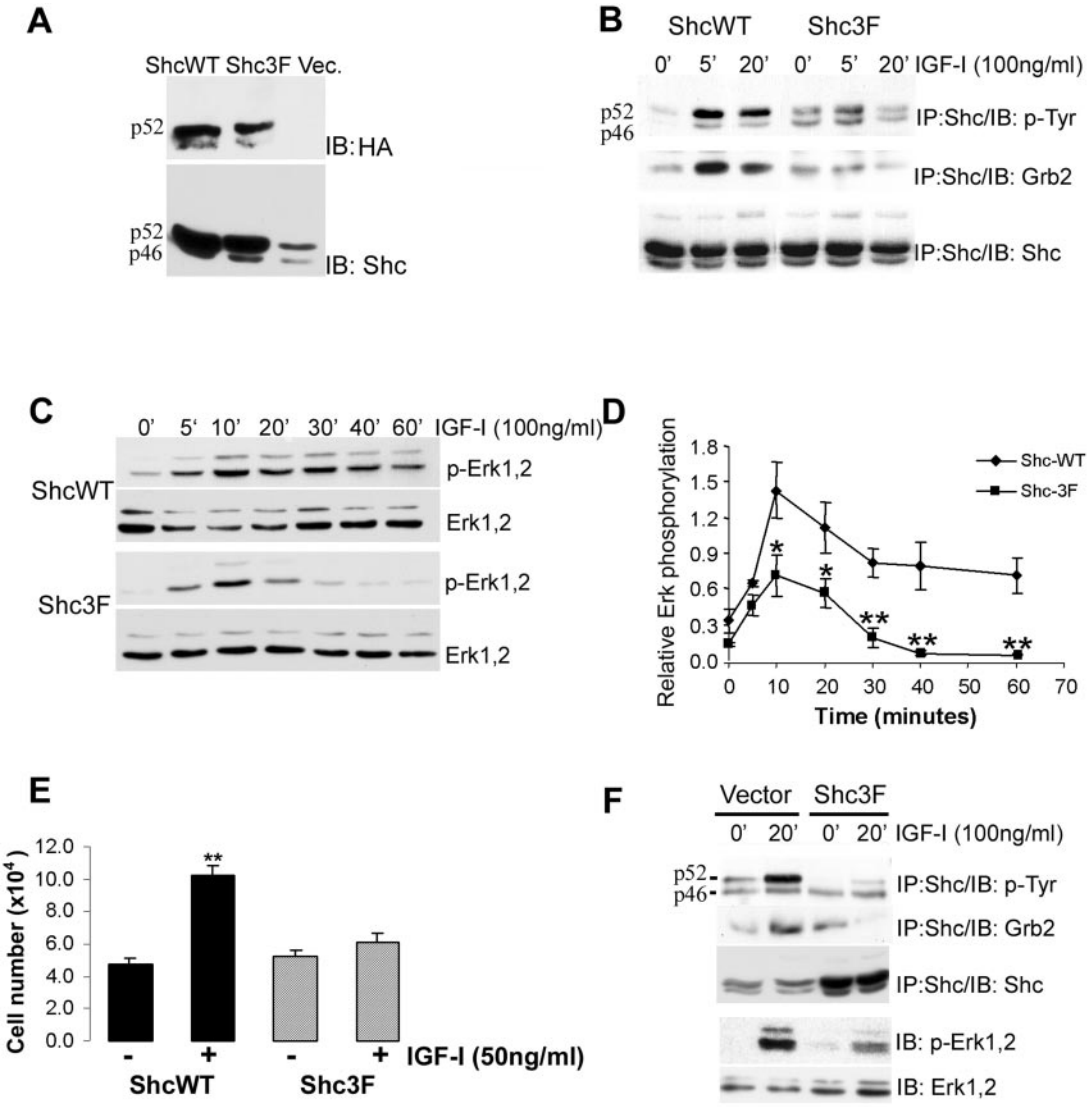

Figure 1. Phosphorylation of Shc mediates IGF-I–dependent mitogenic signaling in SMCs. (A) SMCs expressing wild-type Shc (Shc-WT), tyrosine-mutated Shc (Shc-3F), or vector control were serum starved overnight and analyzed for recombinant protein expression. Thirty microliters of cell lysate was immunoblotted using an anti-HA antibody (top). Separate samples were immunoblotted with a polyclonal anti-Shc antibody to compare the expression levels of total p52Shc in the Shc-transduced cells to vector transduced cells (bottom). (B) SMCs expressing either Shc-WT or Shc-3F were serum deprived for 12–16 h. After IGF-I stimulation for indicated times, cell lysates were immunoprecipitated with an anti-Shc antibody and immunoblotted with p-Tyr (top) followed by reprobing with anti-Shc (bottom). Grb2 association was detected by immunoblotting with an anti-Grb2 antibody after Shc immunoprecipitation (middle). Densitometric scanning units of phospho-Shc and Grb2 were divided by the mean value of total Shc protein that was immunoprecipitated in three experiments and the results are expressed as arbitrary scanning units ± SEM (n = 3). The values for phospho-Shc are 154 ± 35, 618 ± 66, 579 ± 27, 207 ± 60, 256 ± 20, and 185 ± 37. The values for Shc associated Grb2 are 203 ± 63, 784 ± 117, 621 ± 103, 148 ± 39, 124 ± 18, and 147 ± 58. (C) Cells were serum starved then stimulated with IGF-I for indicated times. Thirty microliters of cell lysate was used for detection of phospho-Erk1,2 by immunoblotting with anti-phospho-Erk1,2 antibody. The blots were stripped and reprobed with anti-Erk1,2 antibody. (D) The phosphorylation of Erk1,2 normalized by the protein levels was quantified using scanning densitometry. Each point is the mean ± SEM of three separate experiments. *$p < 0.05$ and **$p < 0.01$ when cells expressing Shc-3F were compared with Shc-WT–expressing cells. (E) The cell proliferation assay was conducted as described in *Materials and Methods*. **$p < 0.01$ when treatment with 50 ng/ml IGF-I is compared with basal state in cells expressing Shc-WT or Shc-3F. (F) IGF-I–induced Shc phosphorylation and Shc binding to Grb2 as well as phosphorylation of Erk1,2 in vector and in Shc-3F–expressing cells are shown. Densitometric units (n = 3) for phospho-Shc divided by total Shc protein immunoprecipitated are 487 ± 47 and 1695 ± 408 in vector control and 199 ± 99 and 269 ± 96 in Shc-3F–expressing cells. The units for Shc associated Grb2 binding are 411 ± 114 and 1100 ± 243 in the control and 209 ± 90 and 218 ± 93 in Shc-3F–expressing cells. The units for phospho-Erk divided by total Erk in control cells are 188 ± 41 and 1329 ± 80 and 216 ± 36 and 253 ± 65 in Shc-3F–expressing cells.

Erk phosphorylation peaked after 10 min and was detectable up to 60 min in cells expressing Shc-WT. However, in cells expressing Shc-3F, this enhanced phosphorylation in response to IGF-I was significantly decreased at 10 min (47.5 ± 18.8% reduction, n = 3, $p < 0.05$) and at the later time points (Figure 1, C and D). There were no significant differences between the two groups basally or at the 5-min time point, indicating the change in Shc phosphorylation correlates with

a change in the sustained phase of Erk1,2 activation. The residual levels of Erk1,2 phosphorylation in Shc-3F cells could be due to incomplete inhibition of endogenous Shc phosphorylation or due to an alternative Erk activation pathway that does not involve Shc. IGF-I stimulated a 2.3 ± 1.2 ($n = 3$, $p < 0.01$)-fold increase in cell proliferation in Shc-WT cells, but this was significantly decreased in cells expressing Shc-3F (1.1 ± 0.1-fold increase, $n = 3$, p = NS) (Figure 1E). To control for excess expression of Shc the results obtained using cells expressing Shc-3F also were compared with cells that had been transfected with vector alone. IGF-I induced a 3.4 ± 0.6-fold increase in Shc phosphorylation at 20 min and a 2.7 ± 0.3-fold increase in Grb2 binding ($n = 3$, $p < 0.05$) in the cells transduced with vector alone. However, cells expressing Shc-3F showed impaired IGF-I induced Shc phosphorylation (1.5 ± 0.2-fold increase compared with control cultures, $n = 3$, P = NS) and decreased Shc binding to Grb2 (1.1 ± 0.4, $n = 3$, p = NS). Correspondingly, IGF-I–induced Erk1,2 phosphorylation was significantly reduced (81.1 ± 4.3% compared with vector expressing cells, $n = 3$, $p < 0.01$) in Shc-3F–expressing cells (Figure 1F). These results demonstrate that IGF-I–induced Shc phosphorylation and its association with Grb2 are required for sustained MAP kinase activation and cell proliferation in cultured pSMCs.

Inhibition of β3 Phosphorylation Impairs IGF-I–dependent Shc and MAPK Activation

We have previously shown that inhibition of β3 phosphorylation led to inhibition of IGF-I–stimulated DNA synthesis (Ling *et al*., 2003), a MAP kinase-dependent event (Imai and Clemmons, 1999). Therefore, we used cells that expressed a mutant form of β3 that could not be phosphorylated to determine whether this change would alter the ability of IGF-I to stimulate both Shc phosphorylation and activation of MAP kinase. Confluent cultures expressing the pMEP4 vector alone had detectible basal tyrosine phosphorylation of the β3 subunit, but cells expressing the β3-FF mutant showed decreased β3 phosphorylation despite the similar levels of expression of total β3 protein (Figure 2A). Similar to SMCs expressing wild-type Shc, IGF-I induced tyrosine phosphorylation of p52-Shc at 5 min (3.5 ± 0.7-fold increase compared with basal, $n = 3$, $p < 0.05$), and this phosphorylation was sustained up to 20 min in pMEP4-vector–expressing cells. This response was associated with enhanced Grb2/Shc association (2.1 ± 0.2-fold increase at 5 min, $n = 3$, $p < 0.05$). In cells expressing the β3-FF mutant, however, IGF-I–induced Shc phosphorylation was reduced by 85.4 ± 6.5% ($n = 3$, $p < 0.05$) at 5 min, and Grb2 association with Shc in response to IGF-I also was reduced (79.3 ± 9.2% decrease compared with control, $n = 3$, $p < 0.05$), despite the fact that comparable amount of Shc was immunoprecipitated (Figure 2B). The significant reduction in both p52Shc phosphorylation and Grb2 binding was sustained to 20 min in these cells. The IRS-1 phosphorylation response also was analyzed. After IGF-I addition, IRS-1 phosphorylation was increased 7.5 ± 1.0-fold ($n = 3$, $p < 0.01$) at 5 min in vector-transfected control cells. In contrast to Shc, IRS-1 phosphorylation was not impaired at 5 min (6.9 ± 1.7-fold increase compared with the basal, $n = 3$, $p < 0.01$), and its phosphorylation was sustained in cells expressing the β3-FF mutant. Correspondingly, there was increased Grb2 association with phosphorylated IRS-1 in response to IGF-I in both control and mutant cells (Figure 2C). The IRS-1 protein level was similar between control and β3-FF–expressing cells. Despite the sustained phosphorylation of IRS-1, there was a lack of sustained phosphorylation of Erk1,2 in response to IGF-I in cells expressing β3-FF mutant (Figure 2, D and E). Together, these results confirm that Shc but not IRS-1 phosphorylation plays a significant role in mediating IGF-I–induced sustained MAP kinase activation in SMCs.

Recruitment of Shc to SHPS-1 but Not to β3 Is Required for IGF-I–dependent Shc Phosphorylation

Recruitment of Shc to specific membrane compartments is required for its subsequent phosphorylation upon growth factor stimulation (Di Guglielmo *et al*., 1994; Chow *et al*., 1998; Sato *et al*., 2000; Biedi *et al*., 2003). Integrin ligand occupancy can facilitate Shc recruitment to the cell membrane, thereby contributing to Shc phosphorylation via direct binding of Shc to phosphorylated β subunits (Cowan *et al*., 2000; Dans *et al*., 2001; Phillips *et al*., 2001b). Because we had shown that Shc phosphorylation was impaired in cells expressing the β3 FF mutant, we determined whether β3 and Shc could be coimmunoprecipitated in response to IGF-I. Sequential immunoprecipitation was performed to ensure that most of the β3 protein was depleted from the cell lysates (Figure 3A). As shown in Figure 3A, Shc was not associated with β3 either basally or after IGF-I stimulation in stably attached, nontransfected SMCs.

Several studies have shown that the transmembrane protein SHPS-1 can recruit signaling molecules to the plasma membrane (Fujioka *et al*., 1996; Oh *et al*., 1999), and our studies have shown that SHPS-1 phosphorylation is required for MAP kinase activation (Maile *et al*., 2003); therefore, we determined whether SHPS-1 could recruit Shc in response to IGF-I. To determine the effect of loss of Shc binding to SHPS-1, we expressed a mutant form of SHPS-1 that lacks most of the cytoplasmic domain (SHPS-1/-CD). SHPS-1/WT was expressed at high level compared with endogenous levels of SHPS-1 (Figure 3B). Cell surface expression of SHPS-1/-CD was comparable with control cells that expressed wild-type SHPS-1 (SHPS-1/WT) (Figure 3B). When the cells expressing SHPS-1/WT were analyzed, IGF-I stimulated a 5.5 ± 0.9 (mean + SEM, $n = 4$)-fold increase in Shc association with SHPS-1 after 5 min. In contrast, there was no detectable SHPS-1/-CD in Shc immunoprecipitates when cells expressing SHPS-1/-CD were analyzed (Figure 3C). Expression of SHPS-1/-CD in these cells did not significantly change levels of basal Shc phosphorylation (p = NS) and its association with Grb2 (P = NS) compared with cells expressing SHPS-1/WT. IGF-I induced significant increases in Shc phosphorylation at 5 min (1.9 ± 0.3-fold increase, $n = 3$, $p < 0.01$) and 10 min (2.2 ± 0.3-fold increase, $p < 0.05$) in SHPS-1/WT cells (Figure 3D). This correlated with increased Grb2 binding to Shc (2.8 ± 0.3-fold increase at 5 min, $p < 0.01$ and 2.9 ± 0.4-fold increase at 10 min, $p < 0.01$). In contrast, IGF-I failed to induce significant increases in Shc phosphorylation and Grb2 binding in cells expressing SHPS-1/-CD (p = NS, $n = 3$ for both Shc phosphorylation and Grb2 binding to Shc) (Figure 3D). IGF-I–induced Erk1,2 phosphorylation was significantly impaired after 10 and 20 min in these cells (Figure 3, E and F). These results suggested that the SHPS-1 cytoplasmic domain is required for IGF-I–mediated mitogenic signaling in SMCs.

SHP-2 Association with SHPS-1 Is Necessary for Shc Phosphorylation and for Shc Association with SHPS-1

Several previous studies have shown that the tyrosine phosphatase SHP-2 is transferred to SHPS-1 in response to mitogens, and in some cases, this has been shown to be required for a mitogenic response (Fujioka *et al*., 1996; Takada *et al*., 1998). In cells expressing the β3-FF mutant, SHPS-1 was phosphorylated in response to IGF-I, but the transfer of

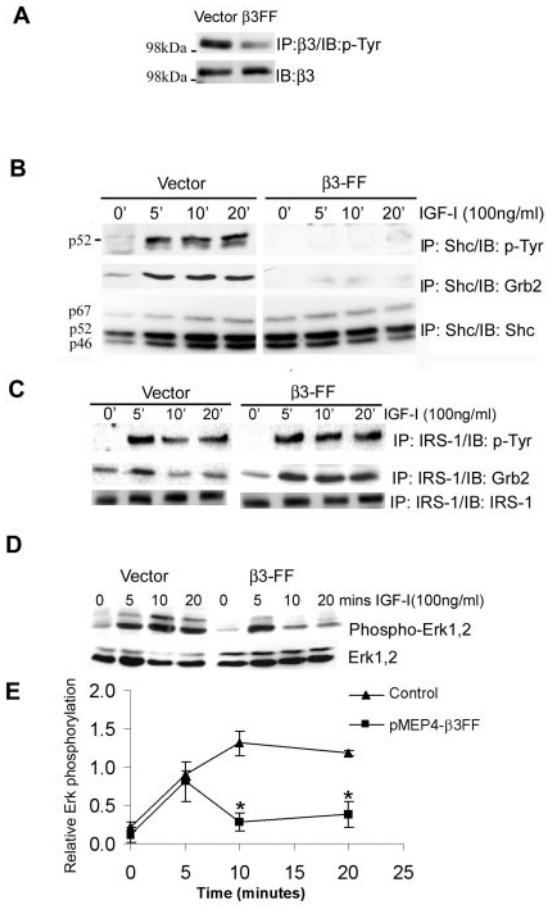

Figure 2. IGF-I–dependent Shc phosphorylation and sustained MAP kinase activation are impaired in cells expressing the β3-FF mutant. (A) Confluent cultures were serum starved overnight and lysed in modified RIPA. β3 phosphorylation was evaluated by immunoprecipitating with an anti-β3 antibody followed by immunoblotting with p-Tyr. Thirty microliters of cell lysate was analyzed by immunoblotting with anti-β3 antiserum for detection of β3 protein. (B) Confluent cultures were serum depleted overnight, and the cells were stimulated with IGF-I for the indicated times. Samples from control and β3-FF cells were immunoprecipitated with anti-Shc and immunoblotted with anti-p-Tyr to detect phosphorylation of Shc (top). Densitometric units of phospho-Shc divided by the total Shc protein levels (n = 3) are 240 ± 29, 835 ± 178, 762 ± 135, and 637 ± 43 in vector cells and 107 ± 24, 126 ± 83, 116 ± 12, and 103 ± 74 in β3-FF cells. Grb2 association was detected by immunoblotting with anti-Grb2 after Shc immunoprecipitation (middle). Densitometric units of Grb2 divided by the total Shc protein levels (n = 3) are 311 ± 74, 636 ± 99, 603 ± 95, 642 ± 112, 119 ± 49, 134 ± 77, 146 ± 55, and 138 ± 37. The levels of Shc protein were assessed by immunoprecipitation followed by immunoblotting with anti-Shc antibody (bottom). (C) Phosphorylation of IRS-1 and its binding to Grb2 were analyzed under the same conditions by using immunoprecipitation with anti-IRS-1 antibody and immunoblotting with p-Tyr (top) or anti-Grb2 antibodies (middle). Densitometric units normalized by the total IRS-1 protein levels (n = 3) are 134 ± 25, 973 ± 58, 577 ± 45, 563 ± 69, 133 ± 22, 889 ± 66, 888 ± 136, and 960 ± 165 for phospho-IRS-1 and 366 ± 34, 726 ± 59, 403 ± 105, 468 ± 66, 327 ± 30, 689 ± 138, 832 ± 200, and 748 ± 240 for Grb2. IRS-1 protein level was analyzed by immunoprecipitation and immunoblotting with an anti-IRS-1 antibody (bottom). (D) Representative immunoblots of phosphorylated Erk1,2 (top) and total Erk1,2 protein (bottom) are shown. Thirty microliters of cell lysate from basal and IGF-I–treated samples was analyzed by immunoblotting as described in *Materials and Methods*. (E) The phosphorylation of Erk1,2 normalized for total Erks was quantified using scanning densitometry. Each point is the mean ± SEM of three separate experiments. *$p < 0.05$ when cells expressing β3-FF are compared with vector-transfected control group.

SHP-2 to SHPS-1 was impaired (Ling *et al*., 2003). This raised the possibility that in cells expressing β3 FF, wherein SHP-2 transfer to SHPS-1 is blocked, SHP-2 transfer is necessary for either Shc binding or for its phosphorylation. Therefore, we used cells expressing the β3 FF mutant to determine whether inhibition of SHP-2 transfer to SHPS-1 was associated with loss of Shc binding. In control cultures expressing the pMEP 4 vector alone, IGF-I induced a 3.2 ± 0.8 (n = 3, $p < 0.05$)-fold increase in Shc and SHPS-1 coprecipitation (Figure 4A) after 5 min. However, in cells expressing the β3-FF mutant Shc binding to SHPS-1 was reduced 86.2 ± 2.1% compared with control cultures (n = 3, $p < 0.01$) (Figure 4A), and this was associated with an inhibition of Shc phosphorylation (Figure 2B). Because in cells expressing both β3 FF and SHPS-1/-CD, SHP-2 transfer to SHPS-1 (Ling *et al*., 2003), Shc recruitment to SHPS-1, and Shc phosphorylation are impaired, this argues strongly for a role for SHP-2 in mediating Shc binding to SHPS-1 and therefore Shc phosphorylation.

To determine directly whether SHP-2 binding to SHPS-1 was necessary for Shc recruitment and phosphorylation, we used a cell-permeable peptide homologous to the site within the SHPS-1 cytoplasmic domain that binds directly to SHP-2 (Takada *et al*., 1998). We predicted that this peptide would bind to SHP-2 and prevent it from binding to SHPS-1, thereby allowing us to examine the role of SHP-2 in mediating Shc binding in nontransfected cells. Because SHP-2 binding to SHPS-1 requires phosphorylation of tyrosine residues within this peptide sequence, we incubated the peptide with SMC cultures as described in *Materials and Methods*. After 30 min, phosphorylation of the peptide was detected as indicated by incorporation of ^{32}P-orthophosphate (our unpublished data). In contrast to control cultures in which IGF-I induced SHP-2 transfer to SHPS-1 after 5 min, exposure to 20 μg/ml peptide completely blocked SHP-2 association with SHPS-1 in response to IGF-I without blocking SHPS-1 phosphorylation (Figure 4B). Furthermore, addition of the peptide impaired both Shc recruitment to SHPS-1 and Shc phosphorylation in response to IGF-I (Figure 4, B and C). After IGF-I stimulation for 5 min, there was a 3.6 ± 0.9 (n = 3, $p < 0.05$)-fold increase in p52Shc phosphorylation and 3.0 ± 0.5 (n = 3, $p < 0.05$)-fold increase in p52Shc binding to SHPS-1 in the control cultures. However, 1.2 ± 0.2- (p = NS) and 1.1 ± 0.1 (p = NS)-fold increases were induced by IGF-I in cultures exposed to the peptide. These effects were not observed when a control peptide that contained a phospho-tyrosine motif within p85 was incubated with the cells (our unpublished data). These results suggest that SHP-2 recruitment is necessary for Shc phosphorylation by regulating the recruitment of Shc to SHPS-1. Further analysis by sequential immunoprecipitation of Shc indicated that SHP-2 can be coimmunoprecipitated with Shc, and this association was enhanced upon IGF-I stimulation (5.6 ± 0.9-fold increase over basal, n = 3, $p < 0.05$) (Figure 4D). Preincubation of the cells with a peptide that contains a proline-rich region of Shc reduced the increased SHP-2 association with Shc that occurred in response to IGF-I (1.4 ± 0.7-fold increase, p = NS). Addition of the peptide did not significantly change the basal association of Shc and SHP-2 (p = NS). Because SHP-2 does not contain a SH3 domain, these results indicate that SHP-2 does not directly bind to Shc and a third protein is involved in mediating SHP-2/Shc association.

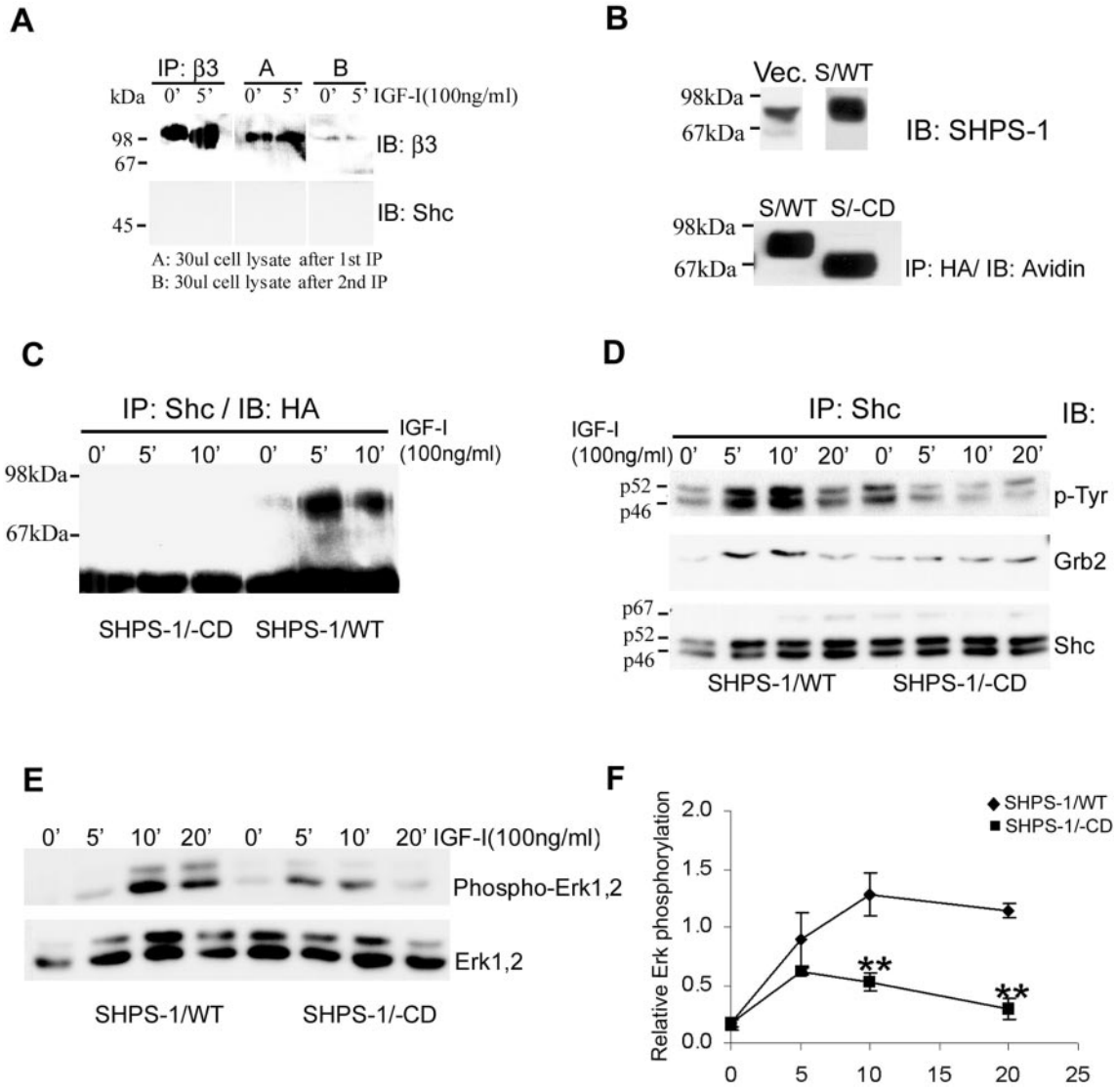

Figure 3. The cytoplasmic domain of SHPS-1 is required for IGF-I–dependent Shc phosphorylation. (A) Nontransfected confluent cultures were serum starved overnight and then stimulated with IGF-I for 5 min. The cells were lysed in the modified RIPA buffer. Sequential immunoprecipitation was performed using an anti-β3 antibody followed by immunoblotting with an anti-Shc antibody or anti-β3 antibody. Thirty microliters of cell lysate was used to analyze the residual β3 after the first "A" and second round "B" of immunoprecipitation by direct immunoblotting with the anti-β3 antibody. (B) Top, expression of endogenous SHPS-1 and HA-labeled SHPS-1/WT (S/WT) was assessed by immunoblotting with anti-SHPS-1 antibody by using 30 μl of cell lysates. Bottom, SMC cultures expressing HA-SHPS-1/WT or SHPS-1/-CD were biotinylated with 0.5 mg/ml biotin as described in *Materials and Methods*. The cells were lysed, and the lysates were immunoprecipitated with anti-HA antibody. The blots were subsequently probed with avidin-horseradish peroxidase to detect surface expression of HA-SHPS-1/WT or HA-SHPS-1/-CD. (C) SMCs expressing HA-SHPS-1/-CD or HA-SHPS-1/WT were serum starved overnight, and IGF-I was added for indicated times before lysing the cells. Prior to immunoprecipitation, the lysates were incubated with protein-A beads for 1 h at 4°C. The samples were centrifuged for 5 min at 14,000 $\times$ *g*, then the supernatants were incubated with 1:330 anti-Shc antibody, and the blots were immunoblotted with anti-HA to detect the association between HA-SHPS-1/WT and Shc. (D) Cells that were expressing SHPS-1/WT or SHPS-1/-CD were serum starved overnight and stimulated with IGF-I for indicated times. Shc phosphorylation and its association with Grb2 were detected by immunoprecipitating with an anti-Shc antibody and immunoblotting with anti p-Tyr (top) or anti-Grb2 antisera (middle). The Shc protein level shown in the lower panel was detected by reprobing the membrane with a monoclonal anti-Shc antibody. The values for phospho-Shc divided by the amount of Shc that was immunoprecipitated are 484 $\pm$ 65, 896 $\pm$ 24, 1068 $\pm$ 200, 374 $\pm$ 38, 425 $\pm$ 130, 300 $\pm$ 51, 252 $\pm$ 56, and 236 $\pm$ 8 (n = 3). The values for Shc-associated Grb2 are 228 $\pm$ 17, 625 $\pm$ 52, 654 $\pm$ 98, 255 $\pm$ 50, 255 $\pm$ 93, 217 $\pm$ 46, 185 $\pm$ 28, and 229 $\pm$ 79. (E) Thirty microliters of cell lysate from the same experiment was used for detection of phospho-Erk1,2 by immunoblotting with anti-phospho-Erk1,2 antibody. The blots were stripped and reprobed with anti-Erk1,2 antibodies. (F) The phosphorylation of Erk1,2 normalized by the protein level was quantified using scanning densitometry. Each point represents the mean + SEM of three separate experiments. **$p < 0.01$ when SHPS-1/-CD cells were compared with cells expressing SHPS-1/WT.

Shc Binding to SHPS-1 Is Required for Shc Phosphorylation

Because in all our previous experiments we had disrupted both SHP-2 and Shc binding to SHPS-1, we wished to determine whether we could inhibit Shc binding to SHPS-1 but retain SHP-2 binding and whether this would alter Shc phosphorylation in response to IGF-I. SMC cultures were exposed to the cell-permeable peptide that contained a pro-

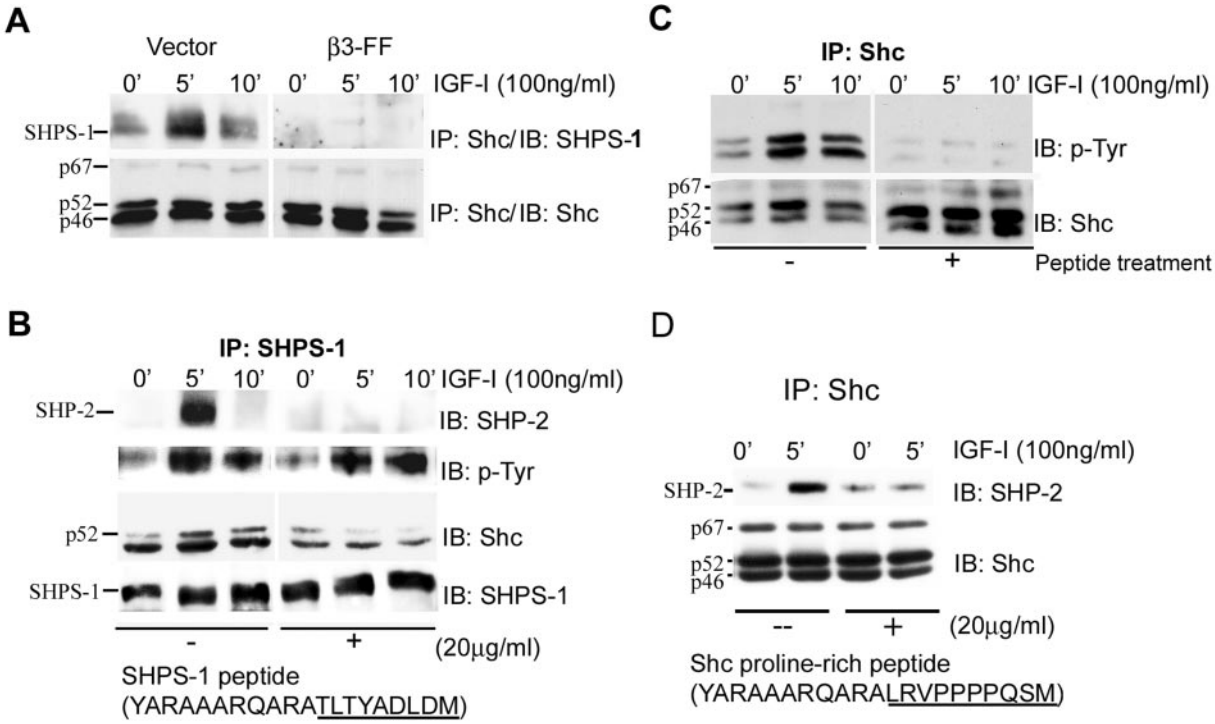

Figure 4. Recruitment of SHP-2 to SHPS-1 is necessary for Shc association with SHPS-1 in response to IGF-I. (A) Cells expressing pMEP4-vector or pMEP4-β3FF were serum depleted overnight before IGF-I was added for the indicated times. The cell lysates were immunoprecipitated with anti-Shc antibody and immunoblotted with anti-SHPS-1 polyclonal antibody. Shc protein level was verified by subsequent immunoblotting with anti-Shc antibody. Densitometric units of SHPS-1 that is associated with Shc normalized by the total Shc protein levels are 409 ± 106, 1193 ± 210, 644 ± 158, 128 ± 32, 172 ± 48, and 160 ± 25. (B) Confluent cultures were serum starved overnight and incubated with or without the SHPS-1 cell-permeable peptide (20 μg/ml) for 1 h. IGF-I was added for indicated times. The cell lysates were immunoprecipitated with anti-SHPS-1 antibody followed by immunoblotting for SHP-2 (first panel) and phosphorylated SHPS-1 (second panel). Densitometric units of SHP-2 normalized by the total SHPS-1 protein levels are 215 ± 45, 978 ± 195, 339 ± 120, 233 ± 67, 207 ± 104, and 308 ± 123. The units for phospho-SHPS-1 divided by total SHPS-1 are 289 ± 153, 1031 ± 227, 764 ± 126, 254 ± 90, 964 ± 149, and 777 ± 145. Association of Shc and SHPS-1 was evaluated by immunoprecipitating with anti-SHPS-1 antibody and immunoblotting with monoclonal anti-Shc antibody (third panel). The densitometric units of Shc that is associated with SHPS-1 are 369 ± 82, 1046 ± 165, 436 ± 133, 323 ± 57, 387 ± 127, and 206 ± 59. SHPS-1 protein level was quantified by reprobing the membrane with anti-SHPS-1 antibody (fourth panel). (C) IGF-I–mediated Shc phosphorylation was analyzed by immunoprecipitating with a polyclonal anti-Shc antibody then immunoblotting with anti-p-Tyr. Normalized densitometric units of Shc phosphorylation are 284 ± 71, 941 ± 213, 788 ± 170, 192 ± 62, 212 ± 81, and 190 ± 84. Shc protein was detected by reprobing the membrane with a monoclonal anti-Shc antibody. (D) The association of SHP-2 and Shc was analyzed by sequential immunoprecipitation of Shc followed by immunoblotting for SHP-2 in the absence or in the presence of the Shc proline peptide (top). Densitometric units of SHP-2 normalized by immunoprecipitated Shc protein are 86 ± 23, 461 ± 112, 130 ± 27, and 175 ± 53. Levels of Shc protein are shown (bottom).

line-rich region of Shc that had been shown to disrupt Shc/SHP-2 association (Figure 4D). In the presence of this peptide, Shc binding to SHPS-1 was impaired. IGF-I induced Shc and SHPS-1 association was reduced by 67.3 ± 7.5% at 5 min ($n = 3$, $p < 0.01$) and by 70.4 ± 11.4% at 10 min ($n = 3$, $p < 0.05$) after exposure to the peptide (Figure 5A). Addition of the peptide did not significantly change basal Shc and SHPS-1 association (p = NS) but was associated with a decrease of both Shc phosphorylation (61.5 ± 8.2% reduction compared with control cultures, $n = 3$, $p < 0.05$) (Figure 5B) and MAP kinase activation (71.3 ± 5.2% reduction, $n = 3$, $p < 0.01$) after IGF-I stimulation for 10 min (Figure 5C). Addition of the peptide also decreased IGF-I–induced Shc phosphorylation at 5 min (62.9 ± 10.7% reduction $n = 3$, $p < 0.05$), but it did not induce a significant reduction in phosphorylation of Erk1,2 after IGF-I stimulation for 5 min. This is consistent with our finding that IGF-I–induced Shc phosphorylation is responsible for sustained MAP kinase activation. To control for a nonspecific effect, a control peptide that contains the proline-rich region of a nonrelated protein, DOK1, was used. This peptide did not inhibit Shc association with SHPS-1, and IGF-I–dependent phosphorylation of Shc and Erk1,2 was stimulated to levels that were similar to cultures not exposed to this peptide (our unpublished data). To determine whether the effect was specific for Shc, SHP-2 binding to SHPS-1 was assessed. In the presence of the blocking peptide SHP-2 binding to SHPS-1 increased after IGF-I to a level that was comparable with control cultures (Figure 5A). Because SHP-2 contains a proline-rich region that has been shown to be required for its association with c-Src (Walter *et al.*, 1999), we also tested the effects of Shc proline peptide on SHP-2/c-Src association. Our results demonstrated that SHP-2/Src association was not disrupted by peptide incubation (our unpublished data). The necessity of the polyproline domain in Shc for SHPS-1 association was confirmed using the cells expressing the Shc mutant with the four proline substitutions (Shc-4A) (Figure 5D). To exclude the possibility of detection of endogenous Shc binding to SHPS-1, we used SMCs expressing either HA-Shc-WT or HA-Shc-4A and immunoprecipitated cell lysates with the anti-HA antibody. Our results indicated that only wild-type Shc but not Shc-4A mutant was associated

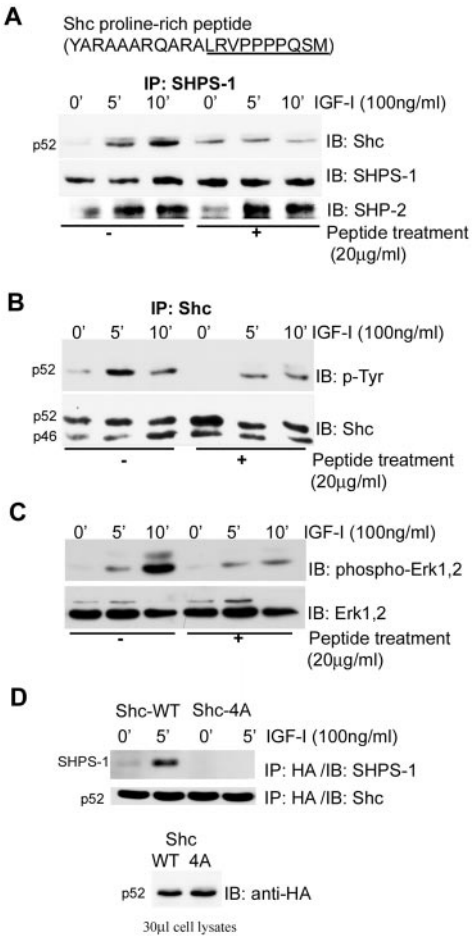

Figure 5. The proline-rich region of Shc mediates its association with SHPS-1. (A) Confluent cultures were serum starved overnight and incubated with or without the cell-permeable Shc proline-peptide (20 μg/ml) for 1 h. IGF-I was added for indicated times. The association of Shc with SHPS-1 was detected by immunoprecipitating with an anti-SHPS-1 antibody followed by immunoblotting with anti-Shc antiserum (top). Densitometric units of Shc normalized by the total SHPS-1 protein levels are 180 $\pm$ 70, 915 $\pm$ 68, 970 $\pm$ 308, 303 $\pm$ 105, 305 $\pm$ 92, and 240 $\pm$ 12. SHP-2 association with SHPS-1 and SHPS-1 protein was detected by reprobing the membrane with anti-SHP-2 (bottom) and anti-SHPS-1 antibodies (middle), respectively. Normalized units of SHP-2 are 307 $\pm$ 70, 1032 $\pm$ 236, 877 $\pm$ 111, 295 $\pm$ 112, 802 $\pm$ 147, and 843 $\pm$ 189. (B) Phosphorylation of Shc in response to IGF-I was analyzed by immunoprecipitation of Shc and immunoblotting with anti-p-Tyr. The scanning units of phospho-Shc divided by total immunoprecipitated Shc are 309 $\pm$ 37, 982 $\pm$ 158, 785 $\pm$ 192, 269 $\pm$ 137, 382 $\pm$ 147, and 296 $\pm$ 67. The amount of immunoprecipitated Shc protein is shown (bottom). (C) IGF-I–dependent phosphorylation of Erk1,2 was evaluated using 30 μl of cell lysate and immunoblotting with anti-phospho-Erk1,2. Equal amounts of total Erk protein were loaded as indicated by immunoblotting the membrane with anti-Erk1,2 antiserum. Normalized units of phospho-Erk are 138 $\pm$ 45, 594 $\pm$ 177, 1296 $\pm$ 21, 112 $\pm$ 24, 389 $\pm$ 107, and 372 $\pm$ 66. (D) Top, SMCs expressing either an Shc-WT or an Shc mutant that had four prolines changed to alanines (Shc-4A) were used to evaluate HA-Shc/SHPS-1 association. Middle, levels of Shc that were immunoprecipitated by an anti-HA antibody. Bottom, levels of HA-Shc-WT or HA-Shc-4A by direct immunoblotting of 30 μl of cell lysate with the anti-HA antibody.

with SHPS-1 upon IGF-I stimulation (Figure 5D), indicating that the presence of the polyproline sequence within Shc was required for SHPS-1 association. The results demonstrate that in SMCs Shc binding to SHPS-1 is absolutely required for Shc phosphorylation and therefore downstream signaling and that this interaction requires the Shc polyproline domain.

DISCUSSION

Ligand occupancy of the αVβ3 integrin and tyrosine phosphorylation of the β3 subunit regulate the recruitment and transfer of SHP-2 to the plasma membrane and to SHPS-1. In SMCs, these transfers are required for IGF-I–stimulated cell migration and proliferation (Zheng and Clemmons, 1998; Maile *et al*., 2001; Ling *et al*., 2003). This study focused on the role of SHPS-1 in providing a site of localization for both SHP-2 and Shc and on how their binding to SHPS-1 regulates IGF-I–dependent activation of the MAP kinase pathway. Our results show that sustained MAP kinase activation in response to IGF-I is mediated by Shc phosphorylation and that this activation step requires recruitment of Shc to SHPS-1. SHP-2 binding to SHPS-1 is also necessary, although not sufficient for IGF-I–dependent Shc phosphorylation. These results support a model in which SHPS-1 functions as a scaffolding protein to recruit key signaling molecules that are required for an optimal mitogenic response to IGF-I.

Integrin activation is required for sustained MAP kinase activation in response to serum (Lin *et al*., 1997; Eliceiri *et al*., 1998; Kim *et al*., 2003). Previous studies have shown that cells must be attached to extracellular matrix proteins to exhibit sustained Erk activation after growth factor stimulation (Lin *et al*., 1997; Renshaw *et al*., 1997; Eliceiri *et al*., 1998; Kim *et al*., 2003). Consistent with those observations, this study shows that in SMCs, β3 phosphorylation is required for sustained Erk phosphorylation in response to IGF-I, supporting the conclusion that in pSMC, integrin activation may be considered as a basal requirement for adequate MAP kinase induction after IGF-I stimulation. This study also shows that Shc phosphorylation is required for sustained MAP kinase activation and that SHP-2 association with Shc is necessary for Shc phosphorylation. Together with our previous findings (Ling *et al*., 2003), these results support a model in which αVβ3 ligand occupancy and subsequent β3 phosphorylation regulate SHP-2 recruitment to SHPS-1 and thus its availability for recruitment of Shc to SHPS-1, which is required for Shc phosphorylation.

Both IRS-1 and Shc are adaptor proteins that undergo tyrosine phosphorylation upon growth factor stimulation. Phosphorylation of IRS-1 is dispensable for IGF-I–induced Erk activation in fibroblasts (Bruning *et al*., 1997), but it has been linked to activation of the PI-3 kinase pathway, which has been shown to contribute to differentiated cellular functions such as cell migration or membrane ruffling (Kim *et al*., 1998; Zheng and Clemmons, 1998; Bartucci *et al*., 2001). In contrast, Shc-mediated signaling is thought to be a major component of growth factor-dependent MAP kinase activation (Ravichandran, 2001; Sasaoka *et al*., 2001). In a variety of cell lines, including 3T3-L1 preadipocytes (Boney *et al*., 2000), CHO cells (Chow *et al*., 1998; Sasaoka *et al*., 2001), and neuroblastoma cells (Kim *et al*., 1998), IGF-I–dependent mitogenesis requires Shc phosphorylation and its subsequent binding to Grb2. Expression of a dominant negative Shc mutant that cannot be phosphorylated in neuroblastoma cells led to decreased mitogenic signaling and defective neurite outgrowth (Kim *et al*., 1998). Our results show that overexpression of the Shc-3F mutant in SMCs leads to impaired phosphorylation of Erks and decreased cell proliferation. Therefore, Shc phosphorylation and its association

with Grb2 are essential for IGF-I–dependent MAP kinase activation and mitogenesis in SMCs. In addition, cells expressing the β3-FF mutant that exhibited impaired IGF-I–dependent DNA synthesis (Ling *et al.*, 2003) also showed impaired Shc but not IRS-1 phosphorylation in response to IGF-I. Together with our previous studies, we conclude that in SMCs, Shc but not IRS-1 phosphorylation is required for sustained MAP kinase activation, whereas IGF-I–dependent phosphorylation of IRS-1 is mainly responsible for activation of the PI-3 kinase pathway that mediates IGF-I stimulation of SMC migration (Zheng and Clemmons, 1998; Inagaki *et al.*, 2000).

Several studies have analyzed Shc recruitment and localization in response to growth factor stimulation. After EGF stimulation, Shc distribution is changed from a diffuse cytosolic pattern to discrete localization along the plasma membrane (Sato *et al.*, 2000), suggesting that Shc has to be recruited to a specific cellular compartment to be phosphorylated (Di Guglielmo *et al.*, 1994; Sato *et al.*, 2000). The requirement of compartmentalization of Shc also has been documented in studies using IGF-I. When Shc was localized in caveolae, IGF-I induced Shc phosphorylation, Grb2 association, and Ras activation, which led to induction of MAP kinase activation (Biedi *et al.*, 2003). These studies suggest that recruitment of Shc to a membrane protein complex may facilitate its subsequent phosphorylation. The αIIbβ3 integrin can facilitate formation of such a complex by directly recruiting Shc to the phosphorylated β3 cytoplasmic domain, thereby leading to Shc phosphorylation (Cowan *et al.*, 2000; Phillips *et al.*, 2001a). However, we were not able to coimmunoprecipitate Shc with β3 integrin, suggesting that in stably attached, primary SMC cultures a membrane protein other than β3 functions to localize Shc in the membrane fraction. Although phosphorylation of β3 does not directly recruit Shc, our results show that by regulating SHP-2 transfer to SHPS-1, β3 phosphorylation contributes to Shc recruitment and that this provides a link between ligand occupancy of the αVβ3 integrin and IGF-I signaling.

Prior observations prompted us to focus on the membrane protein SHPS-1 as a candidate for Shc localization. SHPS-1 functions as a scaffold protein that is capable of binding to various proteins and recruiting them to the plasma membrane (Oshima *et al.*, 2002). These proteins include protein tyrosine phosphatases (Kharitonenkov *et al.*, 1997; Takada *et al.*, 1998), Src family kinases (Gresham *et al.*, 2000), focal adhesion kinase–related cytosolic kinase, PYK2 (Timms *et al.*, 1999), and Janus kinase 2 as well as adapter proteins, e.g., Grb2 (Kharitonenkov *et al.*, 1997). In addition, both the extracellular and cytoplasmic domains of SHPS-1 are important determinants of intracellular signaling responses that regulate both mitogenesis and cell migration (Kharitonenkov *et al.*, 1997; Inagaki *et al.*, 2000; Motegi *et al.*, 2003; Sato *et al.*, 2003). Our results support a role for SHPS-1 in providing a scaffold for Shc localization and clearly indicate that in SMCs Shc binding to SHPS-1 is required for IGF-I–dependent Shc phosphorylation and subsequent downstream signaling. These results are in contrast to the results from Kasuga's group showing that in immortalized fibroblasts derived from mice expressing a mutant SHPS-1 that lacks the cytoplasmic domain, IGF-I–induced phosphorylation of Erk1,2 was not impaired (Inagaki *et al.*, 2000). It is likely that in fibroblasts the role of SHP-2 in growth factor-dependent Erk pathways might be mediated by a docking protein other than SHPS-1, as suggested by the authors.

SHPS-1 also serves as a substrate for phosphatase SHP-2. The binding of SHP-2 to tyrosine-phosphorylated SHPS-1 activates its catalytic activity, and this activation has been shown to be required for sustained MAP kinase phosphorylation in response to cell attachment (Oh *et al.*, 1999) as well as insulin- or serum-stimulated mitogenic responses (Noguchi *et al.*, 1994; Fujioka *et al.*, 1996; Zhang *et al.*, 2002; Ling *et al.*, 2003). In pSMCs, failure to transfer SHP-2 to SHPS-1 correlates with impairment of sustained Erk phosphorylation, decreased DNA synthesis, and cell proliferation in response to IGF-I (Ling *et al.*, 2003; Maile *et al.*, 2003). This study extends those findings to show that IGF-I induces Shc association with SHP-2 and that this association is required for Shc association with SHPS-1 and for Shc phosphorylation. Even though SHP-2 also associates with Shc, their increased association in response to IGF-I seems to be indirect because it requires an intact polyproline region of Shc and SHP-2 has no SH3 domain. Therefore, these results indicate that a third protein that contains an SH3 domain is probably involved in mediating the IGF-I stimulation increase in Shc binding to SHP-2. In this paradigm, IGF-I stimulation may act to enhance binding of SHP-2 to an SH-3 domain containing linker protein and thereby facilitate Shc recruitment to SHPS-1 and its subsequent phosphorylation.

In our studies, we used a blocking peptide approach to evaluate the role of SHP-2/SHPS-1 association in mediating IGF-I–induced mitogenic signaling. Although we did not use phosphorylated SHPS-1 peptides, because this may decrease cell permeability, our results indicated that after a 30-min incubation with cells, the peptides undergo tyrosine phosphorylation. Previous studies indicated that microinjection of protected phosphopeptides can inhibit growth factor-mediated DNA synthesis, whereas nonprotected peptides did not due to its vulnerability to intracellular phosphatase (Xiao *et al.*, 1994). Because several factors such as expression of receptor tyrosine kinases, levels of phosphatases (Lammers *et al.*, 1993) and oxidative status (Meng *et al.*, 2002) can influence intracellular phosphatase activities, it is possible that dephosphorylation of peptides occurs at a much slower rate in our system compared with theirs, thereby prolonging the half-life of the phosphorylated peptide that functions to block SHP-2 binding to SHPS-1.

In summary, we conclude that IGF-I–dependent Shc phosphorylation is required for sustained MAP kinase activation and cell proliferation. Recruitment of SHP-2 and Shc to SHPS-1 in response to IGF-I are both required for Shc phosphorylation and therefore necessary for mediating IGF-I–dependent mitogenic signaling in SMCs.

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