THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 24, pp. 16320–16331, June 13, 2008

Insulin-like Growth Factor-I Stimulates Shc-dependent Phosphatidylinositol 3-Kinase Activation via Grb2-associated p85 in Vascular Smooth Muscle Cells*

Received for publication, March 3, 2008, and in revised form, April 15, 2008 Published, JBC Papers in Press, April 16, 2008, DOI 10.1074/jbc.M801687200

Yashwanth Radhakrishnan[‡], Laura A. Maile[‡], Yan Ling[‡], Lee M. Graves[§], and David R. Clemmons[‡1]

From the Departments of [‡]Medicine and [§]Pharmacology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

Insulin-like growth factor-I (IGF-I) stimulates vascular smooth muscle cell proliferation and migration by activating both MAPK and phosphatidylinositol 3-kinase (PI3K). Vascular smooth muscle cells (VSMCs) maintained in 25 mM glucose sustain MAPK activation via increased Shc phosphorylation and Grb2 association resulting in an enhanced mitogenic response compared with cells grown in 5 mM glucose. PI3K plays a major role in IGF-I-stimulated VSMC migration, and hyperglycemia augments this response. In contrast to MAPK activation the role of Shc in modulating PI3K in response to IGF-I has not been determined. In this study we show that impaired Shc association with Grb2 results in decreased Grb2-p85 association, SHPS-1-p85 recruitment, and PI3K activation in response to IGF-I. Exposure of VSMCs to cell-permeable peptides, which contained polyproline sequences from p85 proposed to mediate Grb2 association, resulted in inhibition of Grb2-p85 binding and AKT phosphorylation. Transfected cells that expressed p85 mutant that had specific prolines mutated to alanines resulted in less Grb2-p85 association, and a Grb2 mutant (W36A/W193A) that attenuated p85 binding showed decreased association of p85 with SHPS-1, PI3K activation, AKT phosphorylation, cell proliferation, and migration in response to IGF-I. Cellular exposure to 25 mM glucose, which is required for Shc phosphorylation in response to IGF-I, resulted in enhanced Grb2 binding to p85, activation of PI3K activity, and increased AKT phosphorylation as compared with cells exposed to 5 mM glucose. We conclude that in VSMCs exposed to hyperglycemia, IGF-I stimulation of Shc facilitates the transfer of Grb2 to p85 resulting in enhanced PI3K activation and AKT phosphorylation leading to enhanced cell proliferation and migration.

Insulin-like growth factor-I (IGF-I)[2] stimulates proliferation and migration in vascular smooth muscle cells (VSMCs) (1). IGF-I-stimulated VSMC migration and/or proliferation requires ligand occupancy of the $\alpha V\beta 3$ integrin (2). Activation of the IGF-I receptor is coupled to downstream signaling events via recruitment and phosphorylation of two major adaptor proteins, Shc or members of the insulin receptor substrate (IRS) family. In VSMCs IGF-I stimulation leads to recruitment of the signaling protein Shc to SHP substrate-1 (SHPS-1) allowing sustained MAPK activation (3). IGF-I stimulation of phosphatidylinositol 3-kinase (PI3K) is also required to induce VSMC migration (4). Hyperglycemia has been shown to increase the responsiveness of smooth muscle cells as well as other cell types to growth factor stimulation (5–9). Following IGF-I stimulation of porcine VSMCs maintained in hyperglycemic conditions (*e.g.* >12 mM glucose) there is an increase in Shc phosphorylation and subsequent Grb2 association resulting in sustained MAPK activation and enhanced cell proliferation. In contrast cells grown in 5 mM glucose do not show these changes (5). Mutation of three Shc tyrosine phosphorylation sites, Tyr-239/240/317, that are required for Grb2 association to phenylalanine (Shc-3F) generates a dominant negative inhibitor of Shc signaling leading to attenuated MAPK activation (10–13). Inhibition of Shc signaling by expressing this mutant form decreases the mitogenic response to IGF-I (3).

PI3Ks are a family of enzymes that catalyze the phosphorylation of the 3-position of the inositol ring in the phosphoinositide 4,5-bisphosphate to yield phosphatidylinositol 3,4,5-trisphosphate that functions to recruit downstream signaling molecules such as AKT to the cell membrane (14). The class IA PI3Ks contain two subunits: the p110 catalytic subunit that contains the enzymatic activity and p85α, the regulatory adaptor subunit. The p85α subunit plays an important role in mediating the activation of p110 in response to tyrosine kinases. These tyrosine kinases may be an integral part of a receptor such as growth factor receptors (*e.g.* platelet-derived growth factor and epidermal growth factor), or they can be nonreceptor tyrosine kinases that either associate with or are activated by receptors (*e.g.* Syk and ZAP70) or the Src family of protein-tyrosine kinases that associate with B cell or T cell receptors. These tyrosine kinases phosphorylate the critical tyrosine residues within activation motifs that can be present on the recep-

* This work was supported, in whole or in part, by National Institutes of Health Grant HL56850. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: Division of Endocrinology, University of North Carolina, CB 7170, 5030 Burnett-Womack, Chapel Hill, NC 27599-7170. Tel.: 919-966-4735; Fax: 919-966-6025; E-mail: endo@med.unc.edu.

[2] The abbreviations used are: IGF-I, insulin-like growth factor-I; VSMC, vascular smooth muscle cell; Shc, Src homology 2 domain-containing protein; Grb2, growth factor receptor-bound protein 2; MAPK, mitogen-activated protein kinase; PI, phosphatidylinositol; PI3K, phosphatidylinositol 3-kinase; SHPS-1, SHP (Src homology 2 domain-containing protein-tyrosine phosphatase) substrate-1; DMEM, Dulbecco's modified Eagle's medium; HA, hemagglutinin; IRS, insulin receptor substrate; Gab, Grb2-associated binding protein; FBS, fetal bovine serum; SH, Src homology; WT, wild type; HG, high glucose; NG, normal glucose.

tors themselves (*e.g.* platelet-derived growth factor or Fc receptors) or present on the receptor-associated proteins, termed adaptors (*e.g.* IRS-1, Gab, and Cbl). These phosphotyrosine residues then act as binding sites for specific SH2 domain-containing effector molecules (15). The SH2 domain of p85α binds to phosphotyrosine residues contained in the sequence pY*XX*M (where pY is phosphotyrosine) on receptor tyrosine kinases or adaptor molecules such as IRS-1 (16). This binding relieves both the basal inhibition of p110 by p85 and recruits the p85-p110 heterodimer to the plasma membrane (17) where it phosphorylates phosphoinositide 4,5-bisphosphate generating phosphatidylinositol 3,4,5-trisphosphate (18). In addition p85 can interact with other adaptors such as Gab2 that function to recruit the p85·p110 complex to the plasma membrane (18), resulting in an increase in the PI3K enzymatic activity. Various proteins that contain SH3 domains (*e.g.* Lyn, Fyn, Abl, and Lck) have been shown to bind to the proline-rich region of p85 (19) and activate PI3K (20). Activation of BaF3 cells by interleukin 3 recruits p85 to the plasma membrane via a complex scaffold formed by three adaptor proteins, Shc, Grb2, and Gab2, and formation of this complex results in PI 3-kinase activation (21). Thus, the recruitment and activation of PI3K can be accomplished by multiple distinct pathways.

In most cell types IGF-I activation of PI3K is dependent on phosphorylation of the adaptor protein IRS-1 that subsequently associates with p85α and functions to recruit the p85·p110 complex to the cell membrane. However, hyperglycemia reduces tyrosine phosphorylation of IRS-1 (5), and IRS-1-mediated PI 3-kinase/AKT activation has been shown to be downregulated in several cell types in hyperglycemic conditions (8, 22–25). Because hyperglycemia has been shown to enhance the ability of IGF-I to stimulate Shc activation and cell migration, this study was undertaken to determine whether Shc activation and subsequent Shc-Grb2 association played a role in PI3K activation and whether Grb2 association with p85 was required for IGF-I-stimulated cell migration.

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-HG) or DMEM containing 900 mg of glucose/liter (DMEM-NG) was purchased from Invitrogen. Streptomycin and penicillin were purchased from Invitrogen. Antibodies against phospho-AKT, total AKT, and β-actin were from Cell Signaling Technology Inc. (Beverly, MA). Polyclonal antibodies for the p85α subunit and Shc were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-phosphotyrosine (PY 99), the hemagglutinin (HA) epitope, and anti-Grb2 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). SHPS-1 polyclonal antiserum was prepared in our laboratory (26). The horseradish peroxidase-conjugated mouse anti-rabbit, goat anti-mouse, and mouse anti-rabbit light chain-specific antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Phosphatidylinositol substrate and the control lipid L-α-phosphatidylinositol 4-phosphate were purchased from Avanti Polar Lipids (Alabaster, AL). Unlabeled ATP was purchased from Sigma, and [γ-^{32}P]ATP was from GE Healthcare. Two synthetic peptides containing the protein transduction domain 4 (27) and one of two proline-rich regions of the p85α subunit, YARAAARQARA^{82}SPPTPKPRPPRPLPV^{97} (underlined and hereafter referred to as peptide 264) and YARAAARQARA^{300}-ERQPAPALPPKPPKPT^{315} (underlined and hereafter referred to as peptide 265), 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.

Cell Culture—Porcine VSMCs were isolated from the porcine aortic explants and were maintained as described previously (28). Cells were maintained in DMEM-high glucose (25 mM) growth medium (referred to as HG) or DMEM-normal glucose (5 mM) growth medium (referred to as NG) with 10% fetal bovine serum (Hyclone, Logan, UT) and 100 μg/ml streptomycin and 100 units/ml penicillin. Both media were supplemented with 1.0 mM pyruvate. Cultured VSMCs were used between passages 4 and 12.

Generation of pLenti Expression Vectors pLenti-HA-p52Shc/Wild Type (Shc-WT) and pLenti-HA-p52Shc/3F (Shc-3F)—pcDNA-p52Shc/WT and pcDNA-p52Shc/3F (Shc/Y239F/Y240F/Y317F) constructs were kindly provided by Dr. Kenneth Yamada (National Institutes of Health, Bethesda, MD). Mutation of these three tyrosine phosphorylation sites has been shown to generate a dominant negative inhibitor of Shc signaling (29). The full-length Shc-WT and Shc-3F cDNAs were prepared and ligated into the pLenti vector as described previously (3). Similarly VSMCs expressing these cDNAs were prepared as described previously (3).

pLenti-HA-p85α Wild Type (p85-WT), pLenti-HA-p85α Mutant Proline Region 1 (p85-P1), and pLenti-HA-p85α Mutant Proline Region 2 (p85-P2)—Human p85α DNA was obtained by PCR using first strand cDNA transcribed from HepG2 cell total RNA. The full-length p85α wild type was cloned into the expression vector pLenti-6DV5 using TOPO methodology as reported previously (30). Using 100 ng of pLenti-p85 wild type as template, PCR amplification was carried out with forward primer 5′-**cacc**atgagtgctgaggggtaccagtaca-3′ and reverse primer 5′-**tta**agcgtaatctggaacatcgtatgggtatcgcctctgctgtgcatatactgggta-3′. The forward primer includes a Kozak sequence (in bold) followed by the ATG start site (underlined). The reverse primer contains the sequence encoding HA epitope (underlined) followed by the stop codon (in bold). The PCR product was cloned into the pENTR/D-TOPO Gateway vector according to the manufacturer's instructions (Invitrogen). The complete sequence was verified by DNA sequencing (University of North Carolina Genome Analysis Facility, Chapel Hill, NC). Two independent mutants were made. In each mutant four prolines within p85α were mutated to alanines. For generating the first proline region mutant (p85-P1), prolines at positions 84, 87, 91, and 94 were mutated to alanine by double-stranded mutagenesis using the pENTRp85-WT as template. Two hundred nanograms of pENTRp85-WT was amplified by PCR using *Pfx* polymerase and complimentary primers (Invitrogen). The sequence of the forward primer was 5′-attggaaggaaaaaaatctcgGctcccacaGcaaagccccggGcacctcgg-Gctcttcctgttgcaccag-3′, and the reverse primer was 5′-ctggtgc-

aacaggaagagCccgaggtgCccggggctttgCtgtgggagCcgagatttttttccttccaat-3′ where the capitalized bases indicate the substitutions. Similarly for generating the second proline region mutant (p85-P2), the prolines at positions 305, 308, 311, and 314 were mutated to alanine using the pENTRp85-WT as template. Two complimentary primers, forward primer 5′-gaatgaacgacagcctgcaGcagcactgGctcctaaaGcaccaaaaGctactactgtagccaacaacg-3′ and reverse primer 5′-cgttgttggctacagtagtagCttttggtgCtttaggagCcagtgctgCtgcaggctgtcgttcattc-3′ were used to generate the p85-P2 mutant. The capitalized bases indicate the substitutions. The mutant products were also sequenced to confirm the correct incorporation of the changes and transferred from the entry vector into pLentiCMV DEST vector (Invitrogen) using the LR Clonase reaction following the manufacturer's instructions (Invitrogen).

pLenti-Grb2 Wild Type (Grb2-WT) and pLenti-Grb2-WA Mutant—Full-length porcine Grb2 was generated using first strand cDNA transcribed from porcine vascular smooth muscle cell total RNA and cloned into the pENTR/D-TOPO Gateway entry vector according to the manufacturer's instructions (Invitrogen). The forward and reverse primers used to generate the PCR product were: forward primer, 5′-**cacc**atggaagccatcgccaaaatatgac-3′; and reverse primer, 5′-**tta**agcgtaatctggaacatcgtatgggtagacgttccggttcacgggggtgac-3′. The forward primer includes a Kozak sequence (bold) placed 5′ to the ATG (underlined) start site. The reverse primer contained the sequence encoding HA epitope (underlined) followed by the stop codon (bold). Using the pENTRGrb2-WT as template, the tryptophans at positions 36 and 193 were mutated to alanine (Grb2-WA) by double-stranded mutagenesis. PCR amplification was carried out using forward primer 5′-ttgaatgaagaatgtgatcagaacGCgtacaaagcagagctcaatg-3′ and reverse primer 5′-cattgagctctgctttgtacGCgttctgatcacattcttcattcaa-3′ where capitalized bases indicate the substitution W36A. Using the product with mutant W36A as template a second PCR was performed using forward primer 5′-ggataactcggaccccaacGCgtggaagggggcttgc-3′ and reverse primer 5′-gcaagccccttccacGCgttggggtccgagttatcc-3′ where the capitalized bases indicate the substitution W193A. After selection of correct clones based on sequencing, the cDNAs encoding the wild type and mutant protein were transferred from the entry vector into pLentiCMV DEST vector using the LR Clonase reaction following the manufacturer's instructions (Invitrogen).

Generation of Virus Stocks—293FT cells (Invitrogen) were prepared for generation of virus stocks of each individual pLenti construct. Cells were plated at 5×10^6/75-cm^2 plate (Corning Glassworks, Corning, NY) the day before transfection in the growth medium (DMEM-HG with 10% FBS, 100 μg/ml streptomycin, and 100 units/ml penicillin). 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 were prepared 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 post-transfection 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 Porcine VSMCs Expressing pLenti Constructs—VSMCs (passage 4–5) were seeded at 3×10^5/well in each of two wells of a 6-well plate (Falcon catalog number 353046) the day before transduction. The viral stocks were thawed, and the viral complexes were precipitated as described previously (3). For transduction, the pellet was resuspended in 1 ml of growth medium, 1 μl of Polybrene (40 mg/ml) was added, and 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 confluency and expanded for experiments or frozen for subsequent experiments. VSMCs expressing all the pLenti constructs and the nontransduced VSMCs used for peptide experiments were maintained in DMEM-HG. The expression of the HA-tagged Shc-WT, Shc-3F, p85-WT, p85-P1 and p85-P2 mutants, Grb2-WT, and Grb2-WA mutant were detected by immunoblotting using a 1:1000 dilution of anti-HA antibody and 30 μl of cell lysate.

Immunoprecipitation and Immunoblotting—Cells were seeded at 5×10^5 cells/10-cm plates (Discovery Labware) and then grown for 5 days to reach confluency. The cultures were incubated in serum-free DMEM-HG or DMEM-NG for 14–18 h before the addition of IGF-I (as indicated). In some experiments, nontransduced VSMCs were preincubated with or without the synthetic peptides that contained a region of sequence from p85 for 1 h before IGF-I was added. Similarly for experiments comparing the effect of glucose, nontransduced VSMCs that had been grown in HG or NG were placed in serum-free DMEM with the respective glucose concentration prior to initiation of the experiment. The VSMCs grown in NG were supplemented to 25 mM mannitol to control for differences in osmolarity. The cell monolayers were lysed in 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 × *g* for 10 min at 4 °C. The supernatants containing crude membrane and cytosolic proteins were exposed to a 1:300 dilution of anti-Grb2, anti-HA, anti-SHPS-1, or anti-p85 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 lysis buffer. The precipitated proteins were eluted in 40 μl of 2× Laemmli sample buffer, boiled for 5 min, and separated by 8 or 11% SDS-PAGE. The proteins were then transferred to Immobilon-P membranes that had been blocked for 2 h in 5% milk or 3% bovine serum albumin in Tris-saline buffer with 0.2% Tween 20. The blots were incubated overnight at 4 °C with the antibody indicated (1:1000 for Tyr(P) mouse) and 1:1000 for anti-HA (rabbit), -Grb2 (mouse), -p85 (rabbit), -SHPS-1 (rabbit), or -Shc (mouse)). To detect phospho-AKT, 15 μg of protein from the cell lysate was removed before immunoprecipitation, mixed with 2× Laemmli sample buffer, and

then separated by SDS-PAGE. Anti-phospho-AKT (Ser-473, Thr-308) (1:1000) was used to detect phosphorylated AKT, and total AKT protein was detected using an anti-AKT antibody (1:1000). The immune complexes were detected using either an anti-mouse (1:10,000) or anti-rabbit (1:20,000) horseradish peroxidase-conjugated antibody and developed with enhanced chemiluminescence following the manufacturer's instructions (Pierce). The images obtained were also scanned using a DuoScan scanning densitometer (Agfa, Morstel, Belgium). Densitometric analyses of the images were determined using National Institutes of Health ImageJ, 1.37v. To determine differences in AKT phosphorylation, arbitrary scanning units obtained for phospho-AKT band intensities were divided by arbitrary scanning units obtained for total AKT or β-actin protein band intensities for each time point analyzed. The average values obtained from at least three independent experiments for each variable were pooled.

PI 3-Kinase Assay—The confluent VSMC cultures were maintained in 0.2% FBS overnight and stimulated with IGF-I (50 or 100 ng/ml) for 10 min before cells were lysed. The cell lysates were prepared, and the PI 3-kinase complexes were immunoprecipitated as described above for immunoprecipitation of p85 or Grb2. The PI 3-kinase assay was performed as described previously (30). Briefly the p85 or Grb2 immunoprecipitates were washed once with phosphate-buffered saline, twice with lysis buffer, and once with the reaction buffer and resuspended in 50 μl of the reaction buffer (31). The reaction was initiated by addition of 25 μl of ATP mixture containing 440 mM cold ATP, 30 μCi of [γ-^{32}P]ATP (GE Healthcare), and 20 μg of phosphatidylinositol (PI3K) substrate with 100 mM $MgCl_2$ and then incubated for 10 min at 22 °C, and the reaction was stopped with addition of 20 μl of 8 N HCl. Then 160 μl of chloroform/methanol (1:1) was added to extract the lipid product from the reaction mixture, and the organic phase was spotted onto a thin layer chromatography plate (catalog number 05-713-318, Fischer Scientific) impregnated with 1% potassium oxylate and resolved by ascending chromatography ($CHCl_3$:CH_3OH:H_2O:NH_4OH at 60:47:11.3:2). The plates were dried, and the lipid products were identified by phosphorimaging and/or autoradiography. The radiolabeled PI3K spots were scraped, and the amount of radioactivity was quantitated by Cherenkov counting using a Beckman LS6500 scintillation counter.

Cell Proliferation—Assessment of VSMC proliferation was performed as described previously (32). Briefly VSMCs were plated at 3×10^4 cells/well in 24-well plates in serum-free medium containing 2% FBS and incubated overnight. The cultures were washed and then incubated in serum-free DMEM containing 0.2% platelet-poor plasma with or without IGF-I (50 ng/ml) for 48 h. Cell number in each well was counted after trypsinization. Each treatment was analyzed in triplicate. The results represent mean values of three independent experiments.

Cell Wounding and Migration Assay—VSMCs were plated in 6-well dishes and grown to confluence. A monolayer of cells was wounded with a razor blade as described previously (33). After wounding, cells were rinsed twice in serum-free medium and incubated in serum-free medium containing 0.2% FBS with or without IGF-I at 100 ng/ml for 48 h at 37 °C. The cells were then fixed and stained (Diff-Quick, Dade Behring Inc., Newark, DE), and the number of cells that migrated at least 5 μm into the wound area was counted. At least five of the 10 previously selected 1-mm areas at the edge of the wound were counted for each data point. Each experiment was repeated three times.

Statistical Analysis—Student's *t* test or the analysis of variance was used to compare the differences between the treatments as appropriate. The results that are shown in all experiments are representative of at least three separate experiments. $p \leq 0.05$ was considered statistically significant.

RESULTS

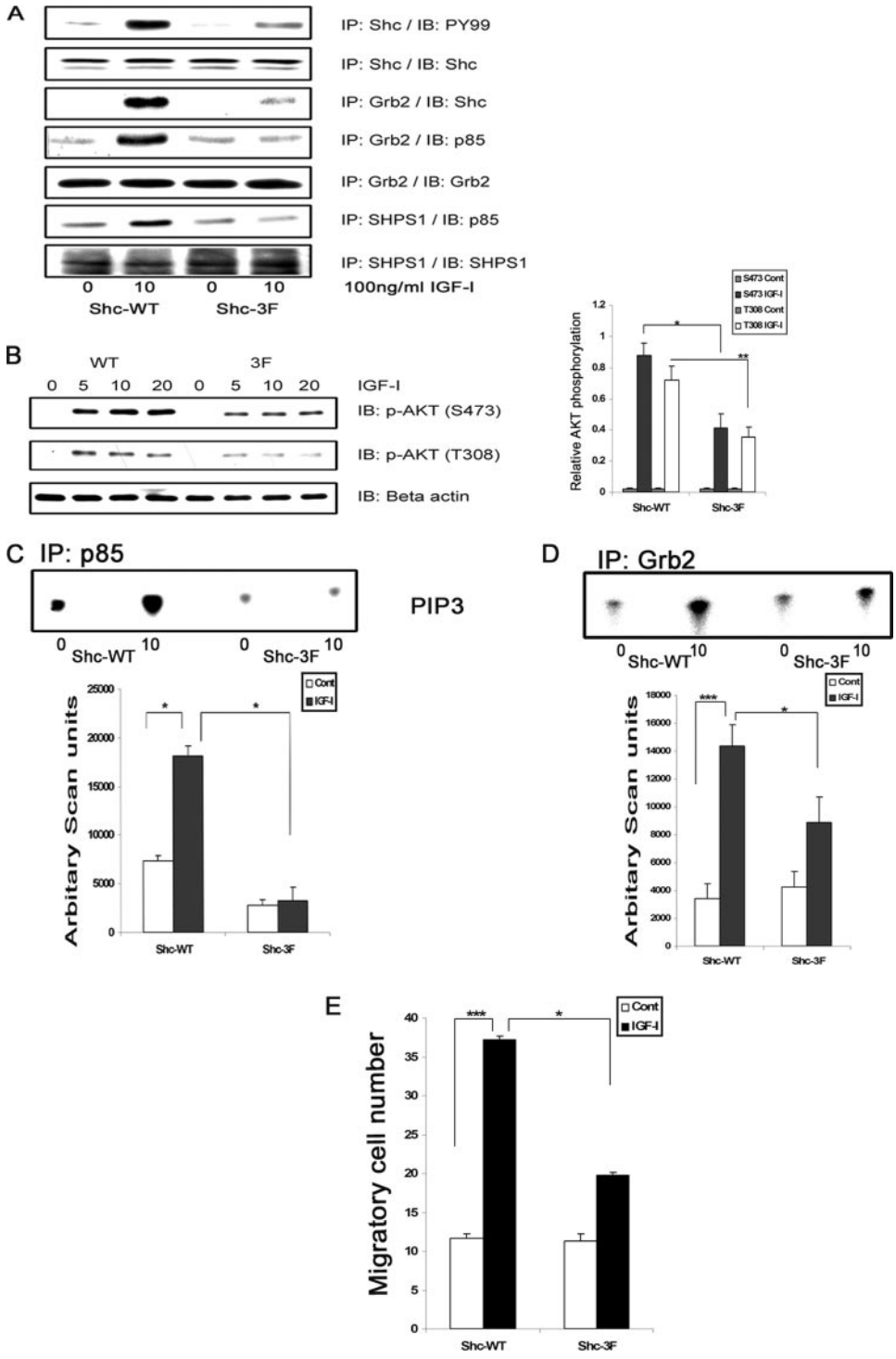
Inhibition of Shc Phosphorylation Impairs PI 3-Kinase Activation and Downstream Signaling—Previously we showed that Shc phosphorylation was required for an optimal mitogenic response to IGF-I in VSMCs maintained in 25 mM glucose and that expression of a p52Shc mutant, Y239F/Y240F/Y317F (Shc-3F), inhibited IGF-I-stimulated mitogen-activated protein kinase activation and cell proliferation (3). Because cell proliferation is decreased in VSMCs expressing the Shc-3F mutant and PI3K activation is required for optimal cell proliferation, we wished to determine whether attenuation of Shc activation would alter the ability of IGF-I to activate PI3K. Therefore we compared VSMCs that expressed wild type Shc (Shc-WT) to cells expressing the Shc-3F mutant that were maintained in 25 mM glucose. Control experiments showed that the cells expressing the Shc-3F mutant had reduced tyrosine phosphorylation and Shc-Grb2 association compared with cells expressing Shc-WT (Fig. 1*A*). Because it had been shown that Grb2 binding to p85 could alter PI3K activation *in vitro* (34) we wished to determine whether IGF-I stimulated Grb2-p85 association. Grb2-p85 association was increased 4.3 ± 0.6-fold ($n = 4$, $p < 0.05$) in the cells expressing wild type Shc in response to IGF-I. Phosphorylated Shc binds to Grb2; therefore, we next determined whether disrupting Shc-Grb2 would result in disruption of Grb2-p85. In contrast to cells expressing Shc-WT, following IGF-I addition there was no change in the association of Grb2 with p85 in the cells expressing Shc-3F (Fig. 1*A*). Because we previously demonstrated that Shc recruitment to plasma membrane protein SHPS-1 is necessary for IGF-I-dependent Shc phosphorylation (3), we wished to investigate the role of Shc in recruitment of Grb2-associated p85 to SHPS-1. Following the IGF-I stimulation, recruitment of p85 to SHPS-1 was significantly increased 3.1 ± 0.4-fold ($n = 4$, $p < 0.05$) in the Shc-WT cells compared with nonstimulated cells, whereas the IGF-I had no effect on the Shc-3F mutant cells (Fig. 1*A*).

To ascertain the consequences of Grb2-p85 disruption we examined PI3K activation and activation of its downstream mediator AKT. In response to IGF-I, cells expressing Shc-WT showed a 2.3 ± 0.1-fold ($n = 3$, $p < 0.05$) greater increase in AKT Ser-473 phosphorylation compared with cells expressing Shc-3F. Similarly stimulation of Thr-308 was increased to a level that was 2.1 ± 0.1-fold ($n = 3$, $p < 0.01$) greater than in the Shc-3F cells (Fig. 1*B*). p85-associated PI 3-kinase activity increased 2.8 ± 0.1-fold in Shc-WT cells in response to IGF-I stimulation, but the Shc-3F-expressing cells showed almost no increase (1.6 ± 0.1-fold) in PI 3-kinase activity in response to

IGF-I (Fig. 1*C*). To determine whether the Grb2·p85 complex showed similar differences in PI 3-kinase activity we immunoprecipitated Grb2 and directly measured PI 3-kinase. Immunoprecipitation of Grb2 followed by quantitation of PI 3-kinase activity showed a 4.9 ± 0.4-fold increase ($n = 3$, $p < 0.001$) in the Shc-WT cells, whereas the cells expressing the Shc-3F mutant showed only a 1.9 ± 0.6-fold rise in activity. This 2.5 ± 0.2-fold difference between the two cell types was significant ($p < 0.05$) (Fig. 1*D*). Because our prior studies had shown that PI 3-kinase activation was required for IGF-I to stimulate cell migration (4) we compared migration in the two cell types. The Shc-3F cells showed no significant change in response to IGF-I, whereas the Shc-WT cells showed a 2.9 ± 0.1-fold increase ($p < 0.001$) (Fig. 1*E*).

Disrupting p85-Grb2 Association Leads to Attenuation of PI 3-Kinase and AKT Activation—Direct association of the SH3 domain of Grb2 with the polyproline region of the p85 subunit of p85 kinase has been demonstrated *in vitro* (Fig. 2*A*) (34). To determine whether disrupting Grb2-p85 would alter AKT activation we used cell-permeable peptides containing each of the two polyproline regions that mediate Grb2 binding. Smooth muscle cells exposed to each of the cell-permeable peptides (peptides 264 and 265) showed significant impairment of Grb2-p85 association in response to IGF-I (Fig. 2*A*).

Exposure to peptide 264 decreased the association of Grb2 and p85 by 1.5 ± 0.3-fold ($p < 0.05$), peptide 265 inhibited it by 2.5 ± 0.2-fold ($p < 0.01$), and the combination resulted in greater inhibition (3.4 ± 0.2-fold; $p < 0.01$) (Fig. 2*B*). Quantitation showed that peptide 265 had a slightly greater effect on disrupting this association as compared with peptide 264 ($p < 0.01$). The combination of both peptides at a low concentration (*i.e.* 5 μg/ml) demonstrated an additive effect. When IGF-I stimulation of phosphorylation of AKT Thr-308 was examined, there was a significant decrease with either peptide 264 (*e.g.* 1.4 ± 0.2-fold; $p < 0.05$) or with peptide 265 (1.8 ± 0.1-fold; $p < 0.05$). The combination of both peptides had a significantly greater inhibitory effect (2.7 ± 0.1-fold; $n = 3$, $p < 0.001$). The ability of the peptides to inhibit Ser-473 phosphorylation was significant ($p < 0.05$) but less than that for Thr-308 (1.4-, 1.6-, and 2.1-fold decreases) (Fig. 2*B*). Because incubation with peptides decreased Grb2-p85 association and AKT phosphorylation that is required for IGF-I-induced cellular functions, we compared proliferation and migration of VSMCs maintained in DMEM-HG with or without peptides 264 and 265 independently or when added together. Cellular proliferation was increased after IGF-I stimulation 2.3 ± 0.2-fold ($p < 0.01$, $n = 4$). In contrast in the presence of both the



peptides cell number was unchanged 1.1 ± 0.1-fold (p not significant, $n = 4$) (Fig. 2*C*). Similarly each peptide alone significantly inhibited the proliferation response (data not shown). Cellular migration was also disrupted by blocking the Grb2-p85 association with the peptides. Cell migration was increased 2.4 ± 0.3-fold ($p < 0.01$, $n = 3$) after IGF-I stimulation compared with nonstimulated cells. In contrast in the presence of both peptides migration was increased 1.4 ± 0.1-fold ($p < 0.05$, $n = 3$) compared with the response without peptide addition (Fig. 2*D*). These results clearly demonstrate that disruption of Grb2-p85 binding by the peptides attenuated VSMC proliferation and migration.

To more definitively determine the role of Grb2 association with p85 in regulating PI3K activity, we prepared cells that expressed mutant forms of p85 in which each of the polyproline regions was mutated to alanine. Immunoblotting for HA showed that cells expressing the wild type p85 or p85 mutants had equivalent expression (Fig. 3*A*). Immunoprecipitation with an anti-Grb2 antibody followed by immunoblotting for p85 revealed that IGF-I stimulated an increase in Grb2-p85 association (*e.g.* 3.2 ± 0.4-fold; $n = 4$, $p < 0.05$) in cells expressing p85-WT (Fig. 3*B*). When cells expressing the P1 site mutant were analyzed for Grb2-p85 association the degree of increase was attenuated 2.2 ± 0.1-fold compared with p85-WT cells ($p < 0.05$). When the cells expressing the P2 mutant were examined there was also a significant attenuation 2.9 ± 0.2-fold compared with p85-WT cells ($p < 0.01$) (Fig. 3*B*). In the cells expressing an empty vector the amount of Grb2-p85 association was comparable to the p85-WT cells (data not shown). Importantly the ability of IGF-I to stimulate PI 3-kinase activation was significantly impaired in the cells expressing the p85-P1 mutant. The increase in p85-associated PI 3-kinase activity was 3.8 ± 0.3-fold above basal in the cells expressing p85-WT, whereas those expressing the P1 mutant showed a 1.4 ± 0.1-fold increase that was not significant (Fig. 3*C*). Consistent with this result, when we measured AKT Ser-473 and Thr-308 phosphorylation (Fig. 3*B*), both were markedly inhibited in the cells expressing the P1 mutant form of p85 as compared with those expressing the p85-WT. IGF-I stimulated a 36.7 ± 1.1-fold increase in AKT Thr-308 phosphorylation and a 32.4 ± 1.1-fold change in AKT Ser-473 phosphorylation in the p85-WT-expressing cells. In contrast the response of the cells expressing the P1 mutant was 10.3 ± 0.5-fold for Ser-473 and 4.5 ± 0.1-fold for Thr-308. Similarly the cells expressing the P2 mutant showed a 4.1 ± 0.3-fold increase for Ser-473 and a 2.8 ± 0.1-fold increase for Thr-308 (Fig. 3*B*). The degree of change for each mutant was significantly less than that of the cells expressing p85-WT ($p < 0.001$). Because IGF-I induced the Shc-mediated recruitment of Grb2-associated p85 to SHPS-1 (Fig. 1*A*), we compared the p85 recruitment to SHPS-1 in the cells expressing the p85-WT and p85-P1 and -P2 mutants. Following IGF-I stimulation the association of p85 with SHPS-1 was significantly increased in the p85-WT cells (3.6 ± 0.3-fold increase; $p < 0.01$) compared with the nonstimulated cells and p85-P1 and p85-P2 mutants. Because IGF-I-stimulated PI 3-kinase activation is also required for IGF-I to stimulate cell proliferation and migration, we compared the cell proliferation and migration responses in cells expressing wild type and the p85-P1 and -P2 mutants. Cell proliferation was increased 2.4 ± 0.4-fold in the p85-WT-expressing cells but only 1.4 ± 0.3-fold in the cells expressing the P1 mutant and 1.3 ± 0.2-fold in the P2 mutant cells (Fig. 3*D*). Cell migration was also impaired when the response of the P1 and P2 mutant cells was compared with cells expressing P85-WT (*e.g.* 2.5 ± 0.2-fold for WT ($p < 0.001$), 1.4 ± 0.2-fold for P1 ($p < 0.05$), and 1.3 ± 0.1-fold for P2 ($p < 0.01$)) (Fig. 3*E*).

Effect of Disruption of Grb2 Binding to p85 Impairs AKT Phosphorylation and Cell Migration—To confirm that Grb2 binding to p85 was required for optimum cellular responses to IGF-I we prepared a mutant in which substitutions were introduced into Grb2 to disrupt its association with p85. This mutant, W36A/W193A, had equivalent expression of HA and Grb2 compared with cells expressing wild type Grb2 (Fig. 4, *A* and *B*). p85-Grb2 association was markedly impaired in the cells expressing the W36A/W193A mutant. Following IGF-I stimulation Grb2 binding to p85 increased 3.6 ± 0.1-fold ($n = 3$, $p < 0.001$) in cells expressing WT Grb2. In contrast cells expressing the mutant showed a 1.8 ± 0.1-fold increase that

FIGURE 1. **Shc mediates IGF-I-stimulated PI 3-kinase/AKT activation and cell migration in VSMCs in 25 mM glucose.** *A*, confluent Shc-WT and Shc-3F cells were serum-starved for 14 h in DMEM-HG and then exposed to IGF-I (100 ng/ml) for 10 min. The extent of Shc phosphorylation was determined by immunoprecipitating p52Shc and then immunoblotting with an anti-phosphotyrosine antiserum. After stripping membranes were then reprobed with anti-Shc antibody to detect total Shc (*upper panels*). Similarly the lysates were immunoprecipitated with anti-Grb2 antibody and immunoblotted for p52Shc and p85. Membranes were then stripped and reprobed with anti-Grb2 antibody to detect total Grb2 (*middle panels*). Further lysates were immunoprecipitated with anti-SHPS-1 antibody and immunoblotted for p85. Subsequently the membranes were stripped and then reprobed with anti-SHPS-1 antibody to detect total SHPS-1 (*lower panels*). *B*, VSMCs expressing Shc-WT and Shc-3F were grown to confluency in DMEM-HG and then placed in serum-free medium for 14 h. Cultures were stimulated with or without IGF-I (100 ng/ml) for the time points indicated. Cell lysates were immunoblotted using the anti-phospho-AKT Ser-473 (*top panel*) and Thr-308 antibodies (*middle panel*). The blots were stripped and reprobed using anti-β-actin antibody. The *bar graph* shows the relative increase in AKT phosphorylation for at least three independent experiments and two independent transductions. *Error bars* represent mean ± S.E. **, $p < 0.01$; *, $p < 0.05$. *C*, confluent Shc-WT and Shc-3F cell cultures were grown to confluency in DMEM-HG, and maintained in this medium containing 0.2% serum for 14 h, and then treated with or without IGF-I (100 ng/ml) for 10 min. The anti-p85 immunoprecipitates were washed, and the pellets were incubated with PI3K and [^{32}P]ATP as described under "Materials and Methods." Radiolabeled PI3K was separated by thin layer chromatography. Representative autoradiograms are shown. The results are expressed as arbitrary scanning units, from three independent kinase assays and the *error bars* represent the mean ± S.E. *D*, confluent Shc-WT and Shc-3F cell cultures were serum-starved overnight and stimulated with IGF-I (50 ng/ml) for 10 min. The PI 3-kinase complex was immunoprecipitated using anti-Grb2 antibody, and the immunoprecipitates were analyzed for kinase activity as described under "Materials and Methods." Radiolabeled PI3K is shown as *PIP3*. The *bar graph* shows the data expressed as arbitrary scan units (mean ± S.E.) ($n = 3$ independent experiments). ***, $p < 0.001$ when kinase activity in the presence of IGF-I is compared with that in nonstimulated Shc-WT cells; *, $p < 0.05$ when the kinase activity in the presence of IGF-I is compared between the Shc-WT and Shc-3F cells. *E*, migration of VSMCs expressing Shc-WT or the Shc-3F mutant. Shc-WT and Shc-3F cells were grown in 6-well dishes in DMEM-HG containing 10% FBS. After wounding they were allowed to migrate with or without IGF-I (100 ng/ml) in medium containing 0.2% FBS for 48 h. The total number of cells migrating past the wound line in the predetermined areas was counted, and the results shown are the mean ± S.E. of three independent experiments. *** indicates $p < 0.001$ and * indicates $p < 0.05$ when the number of cells migrating in response to IGF-I is compared with that in nonstimulated cells for the Shc-WT and Shc-3F cells, respectively. *Cont*, control; *IB*, immunoblot; *IP*, immunoprecipitation; *3F*, Shc-3F; *p-AKT*, phospho-AKT.

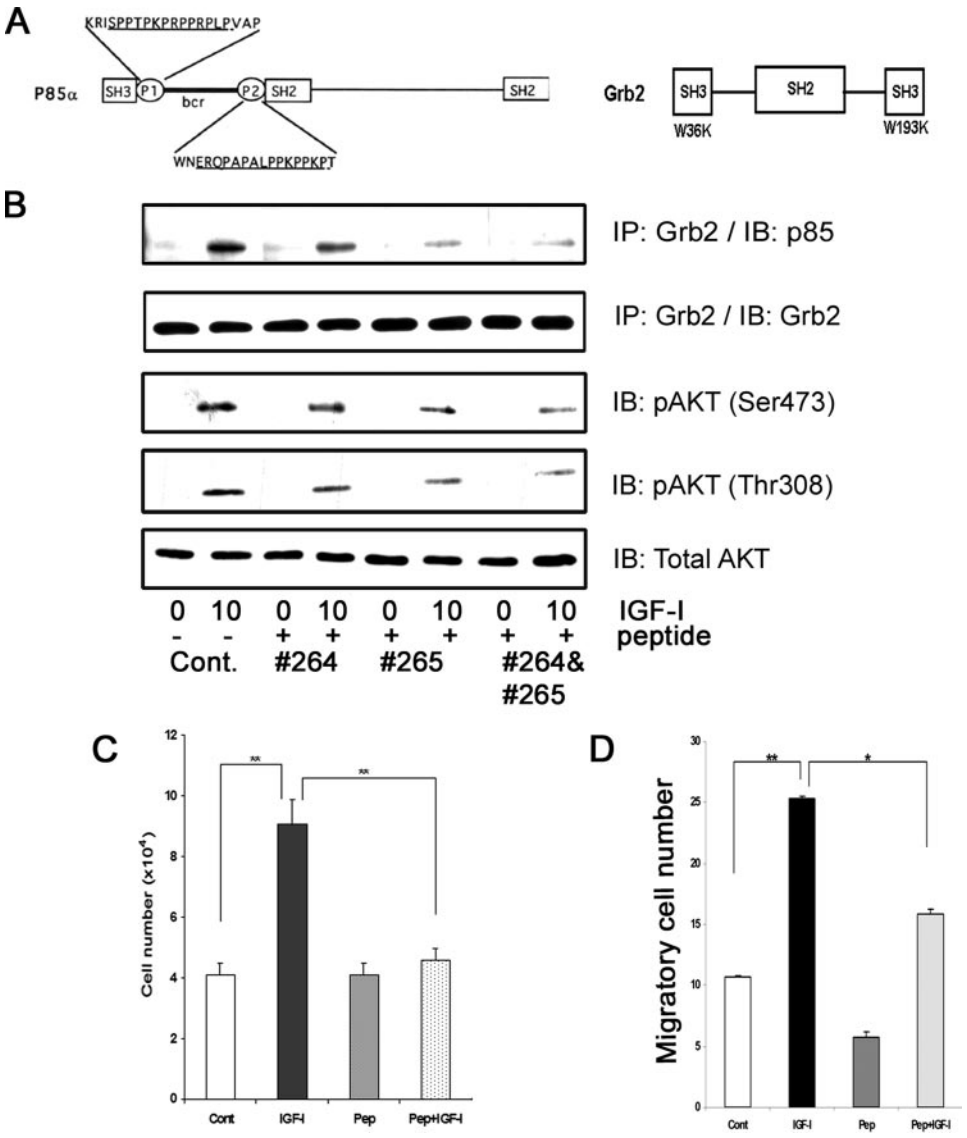


FIGURE 2. **p85 polyproline region disruption attenuates Grb2-p85 association and AKT phosphorylation.** *A*, schematic presentation of p85α subunit. The SH3 domain is located near the N terminus. The breakpoint cluster region is flanked by two proline-rich regions, P1 and P2, on either side. Following P2 is an SH2 domain, and another SH2 domain is located in the C-terminal region. Grb2 contains an SH2 domain and is flanked by two SH3 domains. The proline-rich region of p85 directly associates with the SH3 domain of the Grb2. Mutation of amino acid tryptophan at sites 36 and 193 to lysine in the SH3 domains of Grb2 impairs its association with p85 (modified from Kapeller *et al.* (19)). *B*, confluent VSMCs were serum-starved overnight in DMEM-HG and then incubated with or without cell-permeable Grb2-p85 peptides 264 and 265 (20 μg/ml) each for 2 h either individually or in combination. IGF-I (50 ng/ml) was added for 10 min. Cell lysates were immunoprecipitated with anti-Grb2 antibody and then immunoblotted with anti-p85 antibody (*first panel*). Blots were stripped and reblotted with anti-Grb2 antibody (*second panel*). Cell lysates were directly immunoblotted using anti-phospho-AKT Ser-473 (*third panel*) or Thr-308 (*fourth panel*) and for total AKT (*bottom panel*). *C*, VSMCs were plated at a density of 3×10^4 cells/well in DMEM-HG + 2% FBS in 24-well plates and allowed to attach overnight. They were incubated with or without cell-permeable peptides 264 and 265 (20 μg/ml) together for 2 h before exposure to IGF-I (50 ng/ml) in DMEM + 0.2% platelet-poor plasma. Forty-eight hours after the addition of IGF-I, cell number was determined by trypan blue staining and counting. **, $p < 0.01$ when the cell number in response to IGF-I is compared with that in nonstimulated VSMCs and when the cell number in response to IGF-I is compared with that in cells treated with both peptides. *Error bars* represent the mean ± S.E. *D*, VSMCs were grown to confluency in DMEM-HG, wounded, and allowed to migrate with or without cell-permeable peptides 264 and 265 (20 μg/ml) added together for 2 h before stimulation with IGF-I (100 ng/ml) in medium containing 0.2% FBS for 48 h. The number of cells migrating in the predetermined areas was counted, and the results shown are the mean ± S.E. of three independent experiments. **, $p < 0.01$ when the number of migrating cells in response to IGF-I in cells is compared with that in nonstimulated VSMCs; *, $p < 0.05$ when the cell number in response to IGF-I is compared with that in cells treated with both peptides. *IP*, immunoprecipitation; *IB*, immunoblot; *Cont*, control; *pAKT*, phospho-AKT; *Pep*, peptide.

was significantly less ($p < 0.05$) (Fig. 4*B*).

In parallel, the phosphorylation of AKT at Thr-308 and Ser-473 were markedly attenuated in the cells expressing the Grb2-WA mutant as compared with cells expressing Grb2-WT. In the Grb2-WT cells IGF-I induced a 29.1 ± 1.5-fold increase in AKT (Ser-473) and a 33.9 ± 1.9-fold increase in AKT (Thr-308) phosphorylation, whereas the mutant cells exhibited impaired responses that were increased 10.2 ± 0.9- and 11.9 ± 1.1-fold, respectively (Fig. 4*B*). Cell proliferation increased 2.4 ± 0.1-fold in Grb2-WT-expressing cells compared with 1.2 ± 0.1-fold for Grb2-WA (Fig. 4*C*). The cell migration response was 2.1 ± 0.1-fold ($n = 3, p < 0.005$) after IGF-I in WT compared with 1.5 ± 0.3-fold ($n = 3$, p not significant) in cells expressing Grb2-WA (Fig. 4*D*).

Effect of Hyperglycemia on IGF-I-stimulated Shc-Grb2-p85 Association and PI3K Activation—To determine whether VSMCs maintained in normal (5 mM) glucose or high (25 mM) glucose show changes in signaling elements in the PI 3-kinase pathway, AKT phosphorylation was analyzed. Phosphorylation of AKT in response to IGF-I at both serine 473 and threonine 308 was significantly increased in 25 mM glucose. Phosphorylation of serine 473 increased 20.1 ± 0.1-fold in high glucose compared with 9.2 ± 0.1-fold in normal glucose ($n = 3$, $p < 0.05$), and threonine 308 phosphorylation increased 23.2 ± 0.1-fold in high glucose and 10.3 ± 0.1-fold in normal glucose ($p < 0.001$) following IGF-I stimulation (Fig. 5*A*). Because our previous studies had shown that hyperglycemia led to enhanced Shc phosphorylation and Grb2 association with Shc following exposure to IGF-I (3, 5) and because Wang *et al.* (34) had shown a direct association between Grb2 and p85 we determined whether exposure to 25 mM glucose would alter Grb2 and p85 association. IGF-I stimulated Grb2-p85 association (3.2 ± 0.1-fold), and the degree of increase

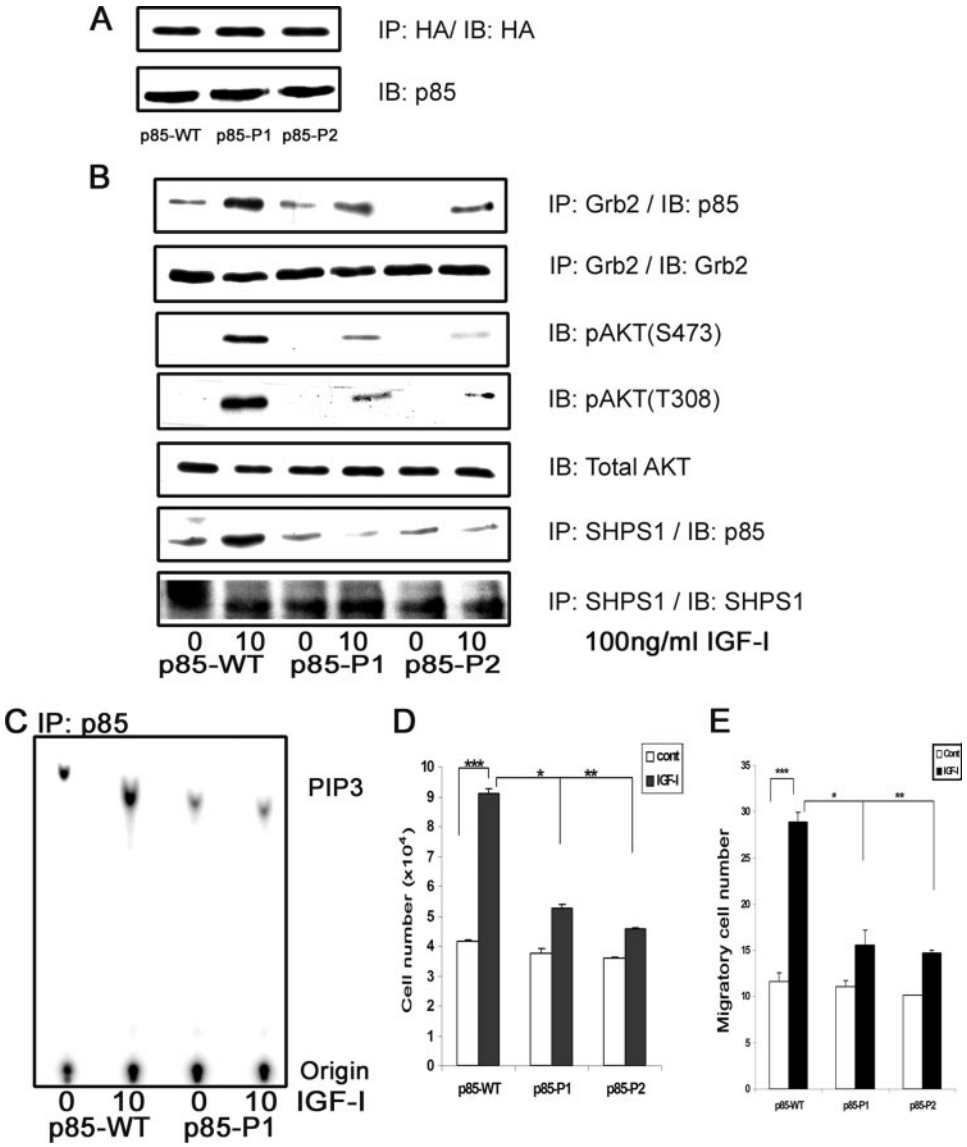


FIGURE 3. **Disruption of p85 binding to Grb2 attenuates IGF-I-stimulated PI 3-kinase activation, AKT phosphorylation, and cellular responses.** *A*, VSMCs expressing p85-WT, mutant p85-P1, and mutant p85-P2 were serum-starved overnight in DMEM-HG and analyzed for recombinant protein expression. Cell lysates were immunoblotted using an anti-HA antibody (*top*) and anti-p85 antibody (*bottom*). *B*, the p85-WT and p85-P1 and p85-P2 mutant cells were serum-starved overnight in DMEM-HG before stimulation with IGF-I (100 ng/ml) for 10 min. The lysates were immunoprecipitated with anti-Grb2 antibody and immunoblotted with anti-p85 antibody (*first panel*). The blots were stripped and reprobed with anti-Grb2 antibody (*second panel*). Similarly cell lysates were immunoblotted with anti-phospho-AKT Ser-473 (*third panel*) and Thr-308 (*fourth panel*) and for total AKT (*fifth panel*). Lysates were also immunoprecipitated with anti-SHPS-1 antibody and immunoblotted for p85 (*sixth panel*). Subsequently the membranes were stripped and then reprobed with anti-SHPS-1 antibody to detect total SHPS-1 (*bottom panel*). *C*, confluent p85-WT and p85-P1 cell cultures were serum-starved (in DMEM-HG, 0.2% FBS) overnight and stimulated with IGF-I (50 ng/ml) for 10 min. The PI 3-kinase complex was immunoprecipitated by using anti-p85 antibody, and the immunoprecipitates were analyzed for kinase activity. PI 3-kinase assay was performed three times, and a representative phosphorimage is shown. *PIP3* indicates the radiolabeled phosphatidylinositol 3,4,5-trisphosphate. The *origin* denotes the location where the reaction products were spotted. *D*, p85-WT, p85-P1, and p85-P2 cells were plated at a density of 3×10^4 cells/well in DMEM-HG, 2% FBS in 24-well plates for 16 h prior to exposure to IGF-I (50 ng/ml) in DMEM + 0.2% platelet-poor plasma. After 48 h, cell number was determined by trypan blue staining and counting. ***, $p < 0.001$ when the cell number after exposure to IGF-I in p85-WT cells is compared with that in nonstimulated p85-WT cells. * indicates $p < 0.05$ and ** indicates $p < 0.01$ when the cell number in response to IGF-I in p85-P1 and p85-P2 mutant cells is compared with that p85-WT cells. *Error bars* represent the mean ± S.E. *E*, cell migration in response to IGF-I in VSMCs expressing p85-WT and p85-P1 mutant cells. p85 wild type and mutant cells were grown to confluency in DMEM-HG, wounded, and allowed to migrate with or without IGF-I (100 ng/ml) in medium containing 0.2% FBS for 48 h. The number of cells migrating in the predetermined areas was counted, and the results shown are the mean ± S.E. of three independent experiments. ***, $p < 0.001$ when the number of p85WT cells migrating in response to IGF-I is compared with that in nonstimulated p85-WT cells. * indicates $p < 0.05$ and ** indicates $p < 0.01$ when the number of cells migrating in response to IGF-I is compared between p85-WT and p85-P1 mutant or p85-P2 mutant cells, respectively. *pAKT*, phospho-AKT; *IB*, immunoblot; *IP*, immunoprecipitation; *Cont*, control.

was significant ($p < 0.05$) in cells exposed to high glucose. In contrast VSMCs exposed to 5 mM glucose showed only a minimal change in Grb2-p85 association (*e.g.* 1.6 ± 0.2-fold). When these two results were compared, there was a significant difference in the maximum response ($p < 0.01$) (Fig. 5*B*). As controls, we measured the Shc phosphorylation response, which was significantly increased in 25 mM glucose (data not shown,) and Grb2-Shc association (Fig. 5*B*). Grb2-Shc association was increased 3.1 ± 0.1-fold in 25 mM glucose but only 1.3 ± 0.3-fold in 5 mM glucose ($p < 0.001$, $n = 4$). The observed change in the ability of Shc and p85 to bind to Grb2 was not due to a change in the total level of Shc or p85 because they remained unchanged with or without IGF-I stimulation and following exposure to either glucose concentration. To confirm that the high glucose-mediated increase in the Grb2-p85 association resulted in amplification of PI3K activity and led to an increase in AKT phosphorylation in response to IGF-I, PI 3-kinase was directly assessed following Grb2 immunoprecipitation. IGF-I-stimulated, Grb2-associated PI 3-kinase was increased 2.9 ± 0.3-fold in VSMCs cultured in 25 mM glucose, whereas cells maintained in normal glucose showed only a 1.4 ± 0.1-fold increase ($n = 2$) (Fig. 5*C*).

DISCUSSION

IGF-I stimulation of the MAPK and PI3K/AKT pathways plays an important role in smooth muscle cell proliferation and migration (1). In this study we focused on the role of Shc and Grb2 in mediating IGF-I-induced activation of PI3K. Our previous studies have shown that there is a significantly greater increase in Shc and MAPK phosphorylation in response to IGF-I when VSMCs are exposed to 25 mM as compared with 5 mM glucose (3, 5). These studies extend those observations to show that this enhancement in Shc phosphorylation and subsequent Grb2 association results in increased recruit-

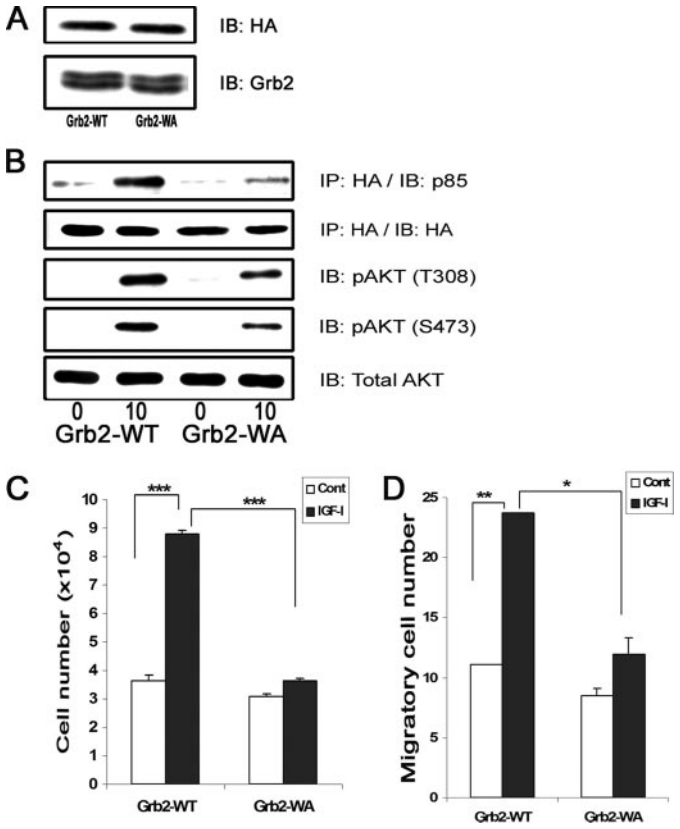


FIGURE 4. **Grb2 mediates IGF-I-stimulated AKT activation, proliferation, and migration responses in VSMCs.** *A*, VSMCs expressing Grb2-WT and VSMCs expressing Grb2-WA were serum-starved in DMEM-HG and analyzed for recombinant protein expression. Cell lysates were immunoblotted using an anti-HA antibody (*top*) and anti-Grb2 antibody (*bottom*). *B*, Grb2-WT- and the mutant (Grb2-WA)-expressing cells were serum-starved in DMEM-HG overnight, and then IGF-I (100 ng/ml) was added for 10 min. Cell lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-p85 antibody (*top panel*). Blots were stripped and reblotted with anti-HA antibody (*second panel*). In addition, cell lysates were immunoblotted with anti-phospho-AKT antibody for Thr-308 (*third panel*) and Ser-473 (*fourth panel*) and for total AKT (*bottom panel*). Each experiment was performed at least three times. *C*, Grb2-WT and Grb2-WA mutant cells (3×10^4) were plated in DMEM-HG + 2% FBS before exposure to IGF-I (50 ng/ml) in DMEM-HG + 0.2% platelet-poor plasma. Forty-eight hours after the addition of IGF-I, cell number was determined by trypan blue staining and counting. ***, $p < 0.001$ when the change in cell number in response to IGF-I in Grb2-WT cells is compared with that in the control or when the response to IGF-I in Grb2-WT cells is compared with that in Grb2-WA cells. *Error bars* represent mean ± S.E. *D*, Grb2 wild type and Grb2-WA mutant cells were grown to confluency in DMEM-HG, wounded, and allowed to migrate with or without IGF-I (100 ng/ml) in DMEM-HG containing 0.2% FBS for 48 h. The number of cells migrating in the predetermined areas was counted, and the results shown are the mean ± S.E. of three independent experiments. **, $p < 0.01$ when the number of Grb2-WT cells after IGF-I is compared with that in nonstimulated control; *, $p < 0.05$ when the number of migrating cells after IGF-I is compared between Grb2-WT and Grb2-WA cells. *IP*, immunoprecipitation; *IB*, immunoblot; *pAKT*, phospho-AKT; *Cont*, control.

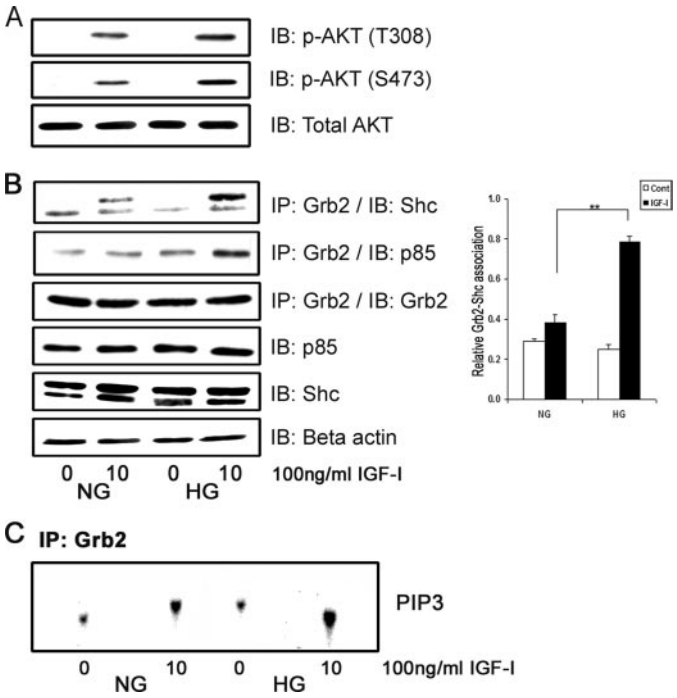


FIGURE 5. **Hyperglycemia increases IGF-I-mediated AKT activation and Shc-Grb2-p85 association in porcine VSMCs.** *A*, quiescent VSMC cultures were incubated in serum-free medium containing 5 (*NG*) or 25 (*HG*) mM glucose overnight. Cells were stimulated with IGF-I (100 ng/ml) for 10 min. After cell lysis aliquots containing equal amounts of protein were separated directly by SDS-PAGE and immunoblotted (*IB*) with anti-phospho-AKT-specific antibodies for phosphorylation of threonine 308 (*upper panel*) and serine 473 (*middle panel*) and also for total AKT (*bottom panel*). *B*, the extent of Shc association with Grb2 was determined by immunoprecipitating (*IP*) cell lysates with an anti-Grb2 antibody and then immunoblotting with an anti-Shc antibody. Similarly the extent of the p85 association with Grb2 was determined by immunoprecipitation using an anti-Grb2 antibody and then immunoblotting with an anti-p85 antibody. Membranes were then stripped and reprobed with anti-Grb2 antibody to detect total Grb2 in each sample. In addition, cell lysates were immunoblotted with anti-p85 (*fourth panel*) and anti-p52Shc antibodies (*fifth panel*). The blots were stripped and reprobed using anti-β-actin antibody to control for differences in loading (*bottom panel*). All experiments were repeated at least three times. *Error bars* represent mean ± S.E. **, $p < 0.01$. *C*, VSMCs cultured in normal glucose or high glucose were serum-starved (0.2% FBS) and stimulated with IGF-I (50 ng/ml) for 10 min. The PI 3-kinase complex was immunoprecipitated by using anti-Grb2 antibody, and the immunoprecipitates were analyzed for kinase activity. The PI 3-kinase assay was performed twice, and a representative phosphorimage is shown. *PIP3* indicates the radiolabeled phosphatidylinositol 3,4,5-trisphosphate. *p-AKT*, phospho-AKT; *Cont*, control.

ment of Grb2-associated p85 to SHPS-1 that leads to increased activation of PI3K in response to IGF-I. AKT activation was also enhanced as were the cellular proliferation and migration responses (Fig. 6). These results present definitive evidence that IGF-I-stimulated Shc activation mediates the enhanced PI3K response through an increase in Grb2-p85 binding.

Growth factor receptor phosphorylation of Shc and its subsequent binding to Grb2 play important roles in various biological responses in variety of cell types (35–38). Several studies have shown that activation of the Shc-Grb2-Sos-MAPK pathway is essential for an optimal mitogenic response (21, 29, 39). Investigators have utilized expression of Shc mutants that do not bind to Grb2 to demonstrate the importance of Shc-Grb2 association in the activation of this pathway (11–13, 29, 37, 40). Previously we used VSMCs expressing this mutant to show that attenuation of Shc-Grb2 binding impairs MAPK activation and hence reduces IGF-I-stimulated cellular proliferation (3). However, activation of PI3K is also an important signal transduction pathway that is used by growth factor receptors (14, 15), and previous studies from our laboratory have demonstrated that IGF-I stimulation of PI3K in VSMCs is required for an optimal mitogenic response and is essential for a significant increase in cell migration (4). Therefore we sought to determine whether Shc activation played a role in this increase in PI3K activity.

Cells expressing the Shc-3F mutant had reduced Grb2-p85 association and a significant reduction in both p85- and Grb2-

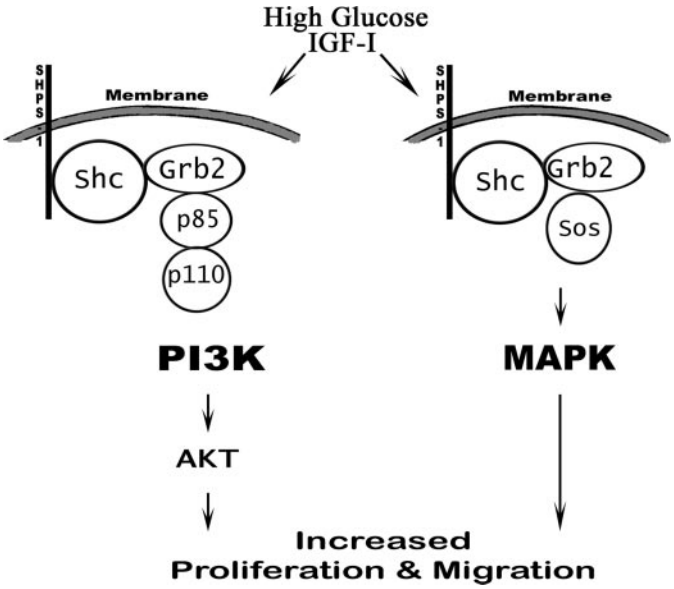


FIGURE 6. **IGF-I-induced activation of PI 3-kinase/AKT and MAPK pathways in VSMCs.** VSMCs maintained in HG conditions upon IGF-I stimulation show enhanced activation of the MAPK and PI3K/AKT pathways leading to increased proliferation and migration compared with cells grown in NG. IGF-I-stimulated Shc mediates activation of MAPK via Grb2-Sos binding. In HG conditions Shc-mediated, Grb2-associated p85 recruitment to SHPS-1 activates the PI3K/AKT pathway increasing VSMC proliferation and migration in response to IGF-I stimulation.

associated PI3K activation following IGF-I stimulation. This finding suggested that Shc mediated Grb2 availability for binding to p85 and that Grb2 binding regulated the ability of p85 to activate PI3K. Because IGF-I addition resulted in major increases in Grb2-p85 association we blocked the Grb2-p85 interaction using two approaches (*e.g.* cell-permeable blocking peptides and mutagenesis). Both approaches resulted in Grb2-p85 disruption and more importantly a reduction in the ability of IGF-I to activate PI3K. Therefore we conclude that this Shc-facilitated increase in Grb2-p85 association is required for enhanced PI3K activation in response to IGF-I.

Our studies show that the Grb2-p85 interaction is mediated through interaction of the SH3 domains of Grb2 and proline-rich regions in p85. Mutagenesis of the proline-rich regions resulted in decreased PI-3K and AKT activation in response to IGF-I. Further our studies confirmed the importance of this interaction by expressing a Grb2 mutant (W36A/W193A) that could not bind to p85 and showing that this resulted in a similar attenuation of activity. Prior studies have reported that proline-rich sequences in p85 interacted with SH3 domains in Abl, Lck, Lyn, and Fyn (19, 20). Pleiman *et al.* (20) showed that addition of synthetic peptides with sequences derived from the SH3 domains of Fyn and Lyn that have sequences that are similar to the SH3 domain of Grb2 resulted in a 5–7-fold enhancement of PI3K activity *in vitro*. They also showed that a cell-permeable synthetic peptide containing the region of p85 (amino acids 80–104) that interacts with Grb2 inhibited PI3K activation in B cells. The importance of Grb2-p85 association for PI3K enzymatic activation was reported by Wang *et al.* (34) who demonstrated that p85 could bind directly to Grb2 both *in vitro* using purified proteins and in NIH-3T3 cells. In addition, they demonstrated that following Grb2-p85 association the p110 subunit of PI3K remained associated with p85 and that the complex retained full catalytic activity.

In terms of the relative activity of the two sites, our findings show that the P2 site appears to be more important for Grb2 binding to p85 and for PI3K activation in response to IGF-I. Other studies have shown a relatively greater effect when the P1 site is altered (20, 34). This difference could be explained by differences in the specificity of various SH3 domains for distinct polyproline sequences or by the fact that different test systems were utilized to compare activity. Because the proline-rich regions of p85 bind to both the internal SH3 domain in p85 and to other proteins with SH3 domains, the normal regulation of PI3K activation has been suggested to involve competition between the exogenous SH3 domains and the internal SH3 domain of p85 thus leading to an alteration in the interaction between p85 and p110 (41). Because the interaction of SH3 domain of Grb2 with p85 increases PI3K activation it is possible that Grb2 binding may function to alter the p85-p110 interaction thus relieving the p85 constraint of p110 activity.

Previously C-terminal SH3 domain of Grb2 binding to Gab1 (42) has been shown to result in tyrosine phosphorylation of Gab1 that generates binding sites for p85 thereby activating PI3K (43). Similarly a complicated signaling scaffold formed by three adaptors, Shc, Grb2, and Gab2, was shown to mediate recruitment of PI3K to the plasma membrane in cells activated by cytokines such as interleukin 3 and interleukin 2 (21, 44). However, our prior studies have shown that VSMCs do not utilize Gab2 for p85 or p110 recruitment.[3] In contrast our prior studies suggest that the transmembrane protein SHPS-1 serves as a scaffold for p85·p110 complex assembly (30). Following its tyrosine phosphorylation in response to IGF-I, SHPS-1 functions as a scaffold for assembly of a complex containing SHP-2·c-Src·Shc and Grb2 (3, 30, 45). Our results show that p85 recruitment to SHPS-1 is impaired in VSMCs expressing the Shc mutant and in p85 mutant cells. This suggests that in VSMCs Grb2 recruitment to SHPS-1 via Shc functions to localize p85 to this SHPS-1 complex. This is supported by a recent report that phosphotyrosyl peptides derived from B and T lymphocyte attenuator, which has strong sequence homology with SHPS-1, associated directly with Grb2 and recruited p85 through Grb2 association (46).

Although only a small fraction of Grb2 is associated with p85 and vice versa, the interaction presents intriguing possibilities for linking PI3K activation to growth factor receptors, such as the IGF-I receptor, that lack the ability to bind PI3K directly. This dependence upon assembly of a Shc·SHPS-1·Grb2 complex to achieve full PI3K activation suggests that recruitment of p85·p110 to the membrane is also important for activation by IGF-I.

Exposure of VSMCs to hyperglycemia modifies the mitogenic response to several growth factors including IGF-I (5, 47, 48). In VSMCs hyperglycemia results in increased proliferation and migration, and these responses are associated with enhanced MAPK activation (5). Similar observations have been reported in streptozotocin-induced diabetic rats that show an

[3] Y. Ling and D. R. Clemmons, unpublished data.

increase in Shc phosphorylation and Grb2 association with insulin stimulation (49). Our studies have shown that this enhanced MAPK activity is dependent upon increased Shc tyrosine phosphorylation and Grb2 association (3, 5). The current results suggest that high glucose is also inducing enhanced p85-Grb2 association in response to IGF-I thus leading to enhanced PI3K activation. In VSMCs maintained in normoglycemic conditions it is well established that IRS-1 mediates the PI3K activation in response to insulin or IGF-I (50). IRS-1 contains several tyrosine phosphorylation sites and six YM*X*M motifs. Upon stimulation tyrosine phosphorylated Y*XX*M motifs associate with SH2 domains of p85 (51), and the engagement of p85 SH2 domain relieves the p85-mediated inhibition of p110 thus increasing the enzymatic activity of p110 (17). However, with hyperglycemia there is a major decrease in IRS-1 tyrosine phosphorylation (5, 52, 53), and hyperglycemia mediates IRS-1 degradation (23, 54), which leads to a decrease in IRS-1-mediated PI3K activation (8, 23, 25). IRS-1 repression has been shown to induce a substantial increase in Shc expression and its tyrosine phosphorylation in response to insulin, and it has been proposed that in the absence of IRS-1 enhanced Shc phosphorylation could represent an alternative pathway to transduce insulin signaling (55, 56). Additionally IGF-I-induced tyrosine phosphorylation of IRS-1 has been shown to be decreased by overexpression of Shc-WT in L6 myoblasts, and in that system IRS-1-associated p85 and PI3K activation were also reduced (10). Because Shc competes with IRS-1 for the same limited pool of Grb2 for binding (57), the preferential increase in Shc-mediated signaling in VSMCs in high glucose in response to IGF-I would be predicted to further reduce the amount of Grb2 that is bound to IRS-1. Under these conditions the increase in PI3K activation that occurs in response to IGF-I in VSMCs appears to be directly dependent upon Shc-mediated Grb2-p85 association.

In summary, we conclude that IGF-I-induced Shc enhances the activation of the PI3K pathway by increasing Grb2-p85 association leading to membrane recruitment. This in turn augments AKT signaling as well as proliferation and migration. In hyperglycemic conditions, preferential Shc activation enhances the PI3K/AKT signaling pathway that leads to vascular dysfunction suggesting a possible role in atherosclerosis.

Acknowledgments—We thank Jane Badley-Clarke for help in preparation of the p85 mutants and Walker H. Busby, Jr. for help in the peptides. We thank Laura Lindsey for assistance in preparing the manuscript.

REFERENCES

1. Clemmons, D. R. (2007) *Nat. Rev. Drug Discov.* **6,** 821–833
2. Ling, Y., Maile, L. A., and Clemmons, D. R. (2003) *Mol. Endocrinol.* **17,** 1824–1833
3. Ling, Y., Maile, L. A., Lieskovska, J., Badley-Clarke, J., and Clemmons, D. R. (2005) *Mol. Biol. Cell* **16,** 3353–3364
4. Imai, Y., and Clemmons, D. R. (1999) *Endocrinology* **140,** 4228–4235
5. Maile, L. A., Capps, B. E., Ling, Y., Xi, G., and Clemmons, D. R. (2007) *Endocrinology* **148,** 2435–2443
6. Anitha, M., Gondha, C., Sutliff, R., Parsadanian, A., Mwangi, S., Sitaraman, S. V., and Srinivasan, S. (2006) *J. Clin. Investig.* **116,** 344–356
7. Campbell, M., and Trimble, E. R. (2005) *Circ. Res.* **96,** 197–206
8. Varma, S., Lal, B. K., Zheng, R., Breslin, J. W., Saito, S., Pappas, P. J., Hobson, R. W., 2nd, and Duran, W. N. (2005) *Am. J. Physiol.* **289,** H1744–H1751
9. Zheng, X. L., Yuan, S. G., and Peng, D. Q. (2007) *Diabetologia* **50,** 881–890
10. Sasaoka, T., Ishiki, M., Wada, T., Hori, H., Hirai, H., Haruta, T., Ishihara, H., and Kobayashi, M. (2001) *Endocrinology* **142,** 5226–5235
11. Salcini, A. E., McGlade, J., Pelicci, G., Nicoletti, I., Pawson, T., and Pelicci, P. G. (1994) *Oncogene* **9,** 2827–2836
12. Pratt, J. C., van den Brink, M. R., Igras, V. E., Walk, S. F., Ravichandran, K. S., and Burakoff, S. J. (1999) *J. Immunol.* **163,** 2586–2591
13. Gotoh, N., Tojo, A., and Shibuya, M. (1996) *EMBO J.* **15,** 6197–6204
14. Cantley, L. C. (2002) *Science* **296,** 1655–1657
15. Hawkins, P. T., Anderson, K. E., Davidson, K., and Stephens, L. R. (2006) *Biochem. Soc. Trans.* **34,** 647–662
16. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., and Cantley, L. C. (1993) *Cell* **72,** 767–778
17. Yu, J., Wjasow, C., and Backer, J. M. (1998) *J. Biol. Chem.* **273,** 30199–30203
18. Cantrell, D. A. (2001) *J. Cell Sci.* **114,** 1439–1445
19. Kapeller, R., Prasad, K. V., Janssen, O., Hou, W., Schaffhausen, B. S., Rudd, C. E., and Cantley, L. C. (1994) *J. Biol. Chem.* **269,** 1927–1933
20. Pleiman, C. M., Hertz, W. M., and Cambier, J. C. (1994) *Science* **263,** 1609–1612
21. Gu, H., Maeda, H., Moon, J. J., Lord, J. D., Yoakim, M., Nelson, B. H., and Neel, B. G. (2000) *Mol. Cell. Biol.* **20,** 7109–7120
22. Nakajima, K., Yamauchi, K., Shigematsu, S., Ikeo, S., Komatsu, M., Aizawa, T., and Hashizume, K. (2000) *J. Biol. Chem.* **275,** 20880–20886
23. Tzatsos, A., and Tsichlis, P. N. (2007) *J. Biol. Chem.* **282,** 18069–18082
24. Tzatsos, A., and Kandror, K. V. (2006) *Mol. Cell. Biol.* **26,** 63–76
25. Salt, I. P., Morrow, V. A., Brandie, F. M., Connell, J. M., and Petrie, J. R. (2003) *J. Biol. Chem.* **278,** 18791–18797
26. Maile, L. A., and Clemmons, D. R. (2002) *J. Biol. Chem.* **277,** 8955–8960
27. Ho, A., Schwarze, S. R., Mermelstein, S. J., Waksman, G., and Dowdy, S. F. (2001) *Cancer Res.* **61,** 474–477
28. Parker, A., Gockerman, A., Busby, W. H., and Clemmons, D. R. (1995) *Endocrinology* **136,** 2470–2476
29. Kim, B., Cheng, H. L., Margolis, B., and Feldman, E. L. (1998) *J. Biol. Chem.* **273,** 34543–34550
30. Kwon, M., Ling, Y., Maile, L. A., Badley-Clark, J., and Clemmons, D. R. (2006) *Endocrinology* **147,** 1458–1465
31. Myers, M. G., Jr., Sun, X. J., Cheatham, B., Jachna, B. R., Glasheen, E. M., Backer, J. M., and White, M. F. (1993) *Endocrinology* **132,** 1421–1430
32. Nam, T. J., Busby, W. H., Jr., Rees, C., and Clemmons, D. R. (2000) *Endocrinology* **141,** 1100–1106
33. Jones, J. I., Prevette, T., Gockerman, A., and Clemmons, D. R. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 2482–2487
34. Wang, J., Auger, K. R., Jarvis, L., Shi, Y., and Roberts, T. M. (1995) *J. Biol. Chem.* **270,** 12774–12780
35. Heinrich, J. N., Kwak, S. P., Howland, D. S., Chen, J., Sturner, S., Sullivan, K., Lipinski, K., Cheng, K. Y., She, Y., Lo, F., and Ghavami, A. (2006) *Cell. Signal.* **18,** 795–806
36. Lord, J. D., McIntosh, B. C., Greenberg, P. D., and Nelson, B. H. (1998) *J. Immunol.* **161,** 4627–4633
37. Ravichandran, K. S. (2001) *Oncogene* **20,** 6322–6330
38. Sayeski, P. P., and Ali, M. S. (2003) *Exp. Cell Res.* **287,** 339–349
39. Baughn, L. B., and Rosenberg, N. (2005) *J. Virol.* **79,** 2325–2334
40. Lai, K. M., and Pawson, T. (2000) *Genes Dev.* **14,** 1132–1145
41. Rodriguez-Viciana, P., Marte, B. M., Warne, P. H., and Downward, J. (1996) *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **351,** 225–232
42. Lock, L. S., Royal, I., Naujokas, M. A., and Park, M. (2000) *J. Biol. Chem.* **275,** 31536–31545
43. Holgado-Madruga, M., Emlet, D. R., Moscatello, D. K., Godwin, A. K., and Wong, A. J. (1996) *Nature* **379,** 560–564
44. Gadina, M., Sudarshan, C., Visconti, R., Zhou, Y. J., Gu, H., Neel, B. G., and O'Shea, J. J. (2000) *J. Biol. Chem.* **275,** 26959–26966
45. Lieskovska, J., Ling, Y., Badley-Clarke, J., and Clemmons, D. R. (2006)

J. Biol. Chem. **281,** 25041–25053
46. Gavrieli, M., and Murphy, K. M. (2006) *Biochem. Biophys. Res. Commun.* **345,** 1440–1445
47. Lee, M. J., Feliers, D., Mariappan, M. M., Sataranatarajan, K., Mahimainathan, L., Musi, N., Foretz, M., Viollet, B., Weinberg, J. M., Choudhury, G. G., and Kasinath, B. S. (2007) *Am. J. Physiol.* **292,** F617–F627
48. Campbell, M., Allen, W. E., Silversides, J. A., and Trimble, E. R. (2003) *Diabetes* **52,** 519–526
49. Paez-Espinosa, V., Rocha, E. M., Velloso, L. A., and Saad, M. J. (2001) *Endocrine* **14,** 295–302
50. Zheng, B., and Clemmons, D. R. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 11217–11222
51. Backer, J. M., Myers, M. G., Jr., Shoelson, S. E., Chin, D. J., Sun, X. J., Miralpeix, M., Hu, P., Margolis, B., Skolnik, E. Y., Schlessinger, J., and White, M. F. (1992) *EMBO J.* **11,** 3469–3479
52. Huang, C., Somwar, R., Patel, N., Niu, W., Torok, D., and Klip, A. (2002) *Diabetes* **51,** 2090–2098
53. Pillay, T. S., Xiao, S., and Olefsky, J. M. (1996) *J. Clin. Investig.* **97,** 613–620
54. Buren, J., Liu, H. X., Lauritz, J., and Eriksson, J. W. (2003) *Eur. J. Endocrinol.* **148,** 157–167
55. Tesseraud, S., Metayer, S., Duchene, S., Bigot, K., Grizard, J., and Dupont, J. (2007) *Domest. Anim. Endocrinol.* **33,** 123–142
56. Taouis, M., Dupont, J., Gillet, A., Derouet, M., and Simon, J. (1998) *Mol. Cell. Endocrinol.* **137,** 177–186
57. Yamauchi, K., and Pessin, J. E. (1994) *J. Biol. Chem.* **269,** 31107–31114