

The Coordinate Cellular Response to Insulin-like Growth Factor-I (IGF-I) and Insulin-like Growth Factor-binding Protein-2 (IGFBP-2) Is Regulated through Vimentin Binding to Receptor Tyrosine Phosphatase β (RPTP β)*

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Background: IGFBP-2 binding to RPTP β is required for IGF-I-stimulated AKT activation.

Results: IGF-I stimulates PKC ζ recruitment and serine phosphorylation of vimentin leading to vimentin/RPTP β association, RPTP β polymerization, and enhanced AKT activation.

Conclusion: Vimentin phosphorylation stimulates vimentin/RPTP β association, which mediates RPTP β polymerization in response to IGF-I and IGFBP-2.

Significance: This study demonstrates how these two receptor systems collaborate to obtain optimal IGF-I signal transduction.

Insulin-like growth factor-binding protein-2 (IGFBP-2) functions coordinately with IGF-I to stimulate cellular proliferation and differentiation. IGFBP-2 binds to receptor tyrosine phosphatase β (RPTP β), and this binding in conjunction with IGF-I receptor stimulation induces RPTP β polymerization leading to phosphatase and tensin homolog inactivation, AKT stimulation, and enhanced cell proliferation. To determine the mechanism by which RPTP β polymerization is regulated, we analyzed the protein(s) that associated with RPTP β in response to IGF-I and IGFBP-2 in vascular smooth muscle cells. Proteomic experiments revealed that IGF-I stimulated the intermediate filament protein vimentin to bind to RPTP β , and knockdown of vimentin resulted in failure of IGFBP-2 and IGF-I to stimulate RPTP β polymerization. Knockdown of IGFBP-2 or inhibition of IGF-IR tyrosine kinase disrupted vimentin/RPTP β association. Vimentin binding to RPTP β was mediated through vimentin serine phosphorylation. The serine threonine kinase PKC ζ was recruited to vimentin in response to IGF-I and inhibition of PKC ζ activation blocked these signaling events. A cell-permeable peptide that contained the vimentin phosphorylation site disrupted vimentin/RPTP β association, and IGF-I stimulated RPTP β polymerization and AKT activation. Integrin-linked kinase recruited PKC ζ to SHPS-1-associated vimentin in response to IGF-I and inhibition of integrin-linked kinase/PKC ζ association reduced vimentin serine phosphorylation. PKC ζ stimulation of vimentin phosphorylation required high glucose and vimentin/RPTP β -association occurred only during hyperglycemia. Disruption of vimentin/RPTP β in diabetic mice inhibited

RPTP β polymerization, vimentin serine phosphorylation, and AKT activation in response to IGF-I, whereas nondiabetic mice showed no difference. The induction of vimentin phosphorylation is important for IGFBP-2-mediated enhancement of IGF-I-stimulated proliferation during hyperglycemia, and it coordinates signaling between these two receptor-linked signaling systems.

The insulin-like growth factor-binding proteins were initially proposed as inert carriers of IGF-I whose primary function was to limit IGF-I access to receptors (1). Subsequent studies demonstrated that IGFBP³ modulated IGF-I cellular actions and that they had effects that were believed to be IGF-I-independent (2–4). Although several empiric observations have been published, the cell surface receptors that mediate these events and the signaling components that are required have rarely been characterized (5–7). Our recent studies demonstrated that IGFBP-2 significantly enhanced the ability of IGF-I to stimulate vascular smooth muscle cell (VSMC) proliferation and preosteoblast differentiation (8, 9). We further determined that IGFBP-2 binding to the specific cell surface receptor RPTP β was required to mediate these effects (9). Knockdown of RPTP β resulted in an inability of IGFBP-2 to stimulate these responses. Following RPTP β polymerization, its phosphatase activity is inhibited. Utilization of a substrate-trapping mutant showed that the primary target of RPTP β was PTEN and that RPTP β polymerization was associated with enhanced PTEN tyrosine phosphorylation (9). PTEN tyrosine phosphorylation inhibited its activity leading to enhanced AKT activation. Therefore stimulation of RPTP β polymerization following

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³ The abbreviations used are: IGFBP-2, insulin-like growth factor-binding protein-2; RPTP β , receptor tyrosine phosphatase β ; ILK, integrin-linked kinase; VSMC, vascular smooth muscle cell; PTEN, phosphatase and tensin homolog.

exposure to IGFBP-2 and IGF-I resulted in enhanced VSMC migration. IGFBP-2 binding to RPTP β is also required for IGF-I to stimulate preosteoblast differentiation (10), and IGFBP-2 knockdown results in attenuation of osteoclast differentiation (11). Those studies showed that in addition to IGFBP-2 binding to RPTP β , IGF-I receptor activation was required, and inhibition of the IGF-I receptor tyrosine kinase inhibited RPTP β polymerization. Because activation of the IGF-I receptor as well as IGFBP-2 binding to RPTP β was required to induce RPTP β polymerization, we wished to determine the mechanism by which IGF-I functioned to stimulate this interaction. These studies were undertaken to determine post-receptor signaling events that led RPTP β polymerization.

EXPERIMENTAL PROCEDURES

Human IGF-I was a gift from Genentech (San Francisco, CA). Immobilon-P membranes, an ILK inhibitor (Cpd 22), a myristoylated form of cell-permeable PKC ζ pseudosubstrate inhibitor (catalog no. 539624) (12), and protein A- and G-agarose were purchased from EMD-Millipore (Billerica, MA). Dulbecco's modified medium (DMEM) containing 25 mM glucose, streptomycin, and penicillin were purchased from Life Technologies, Inc. PQ401 was purchased from Tocris Bioscience (Ellisville, MO). Antibodies against PTEN and phospho-AKT(S473) were from Cell Signaling Technology Inc. (Beverly, MA). The anti-phosphotyrosine (pY99), ILK, and vimentin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). An anti-phosphoserine (Ser(P)) antibody was purchased from Abcam (Cambridge, MA). An anti-RPTP β monoclonal antibody was purchased from BD Biosciences. SHPS-1 polyclonal antiserum was prepared as described previously (13). The IGFBP-2 antibody was prepared as described previously (8). The horseradish peroxidase-conjugated mouse anti-rabbit, goat anti-mouse, and mouse anti-rabbit light chain-specific antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). All other reagents were purchased from Sigma unless otherwise stated. A synthetic peptide containing the cell permeability sequence of the protein transduction domain and the head domain sequence (underlined) from vimentin (YARAAARQARAR-SVSSSYRRMF), hereafter referred as a disrupting peptide, and a peptide containing YARAAARQARAR-SVASAYRRMF, wherein three serines were substituted with alanine (underlined), serving as a control peptide, were synthesized by the Protein Chemistry Core Facility at the University of North Carolina at Chapel Hill. Purity and the sequence were confirmed by mass spectrometry.

Cell Culture—The VSMC were isolated from the aortic explants obtained from 3-week-old pigs and were maintained as described previously (14). Cells were maintained in DMEM high glucose (25 mM) or DMEM with normal glucose (5 mM) with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 100 μ g/ml streptomycin, and 100 units/ml penicillin. In some experiments, 20 mM glucose was added to cells that had been exposed to normal glucose for different times prior to IGF addition. The cultures were used between passages 5 and 14.

Identification of RPTP β Interaction Proteins by Tandem Mass Spectrometry (MALDI-TOF/MS)—VSMCs with or without IGF-I (50 ng/ml) treatment were lysed in the modified

radioimmunoprecipitation assay (RIPA) buffer. Cell lysates (3 mg) were precleared with protein A/G-agarose for 30 min to eliminate nonspecific protein interactions and then incubated with an anti-RPTP β antibody (6 μ g) and protein A/G-agarose at 4 °C with gentle agitation for 3 h. The immunocomplexes were separated on a 4–20% gradient SDS-PAGE. The gel was stained with colloidal Blue G-250 (Thermo Fisher Scientific, Rockford, IL). The protein band that was increased in response to IGF-I was excised from the stained gel, destained with 200 mM ammonium bicarbonate (pH 8.0), 40% acetonitrile, twice at 37 °C for 30 min. The gel was exposed for 10 min to 100 mM ammonium bicarbonate, cut into small pieces, then dehydrated with 100% acetonitrile, and vacuum-dried. In-gel digestion was performed by adding 30 μ l of modified porcine trypsin solution (Promega, Madison, WI) at 20 ng/ μ l in 50 mM ammonium bicarbonate following by a 14-h incubation at room temperature. Peptides were extracted with 50% (v/v) acetonitrile and 0.1% trifluoroacetic acid twice at 37 °C for 30 min, and the solution was completely dried in speed vacuum prior to MALDI-MS analysis conducted by the Proteomics Core Facility at the University of North Carolina at Chapel Hill.

Generation and Purification of Wild Type IGFBP-2 and an IGFBP-2 Mutant—Wild type mouse IGFBP-2 and heparin binding domain mutated IGFBP-2 (MT1) were generated and purified following the procedure described previously (9).

Establishment of VSMCs Expressing Wild Type SHPS-1 and Cytoplasmic Domain Truncated SHPS-1—VSMCs expressing wild type SHPS-1 (SHPS-1/WT) and cytoplasmic domain truncated SHPS-1 (SHPS-1/-CD) were prepared as described previously (15).

Establishment of VSMCs Expressing IGFBP-2 shRNA and Control shRNA—Based on Invitrogen website design tools, sequences containing 21 oligonucleotides (GG AGT TCT GAC ATG CGT ATT T) were used to construct a short hairpin RNA (shRNA) template plasmid in order to knock down IGFBP-2. Two-nucleotide substitutions (underlined GGAGTTCTGT-GATGCGTATT) were inserted as a control shRNA. VSMCs expressing shRNA targeting IGFBP-2 and control shRNA were established following the procedure described previously (9).

Transient Transfection with siRNA Targeting Vimentin—siRNA targeting vimentin (sc-29523) and a control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology, Inc. VSMCs were transfected using a concentration of 40 nM and the PepMute Plus reagent (SignaGen Laboratories) following the manufacturer's instructions. The experiments were initiated 72 h after transfection.

Immunoprecipitation and Immunoblotting—The immunoprecipitation and immunoblotting procedures were performed as described previously (9). Immunoprecipitation was performed by incubating 0.5 mg of cell lysate protein with 1 μ g of each of the following antibodies: anti-vimentin, ILK, pY99, and Ser(P) at 4 °C overnight. Immunoblotting was performed using a dilution of 1:1000 for anti-pAKT(S473), PTEN, vimentin, and β -actin antibodies, a dilution of 1:500 for anti-RPTP β antibody, and a dilution of 1:2000 for anti-IGFBP-2 antibody. The proteins were visualized using enhanced chemiluminescence (Thermo Fisher Scientific, Rockford, IL).

Vimentin/RPTP β Interaction Mediates IGF-I Signaling

Chemical Cross-linking—The chemical cross-linking procedures were performed as described previously (9). Briefly, cells were washed three times with PBS and then incubated with 2 mg/ml bis[sulfosuccinimidyl]suberate, BS3 (Thermo Fisher Scientific, Rockford, IL) in PBS for 1 h on ice. Cross-linking was terminated by adding 50 mM Tris for 15 min. The cells were lysed, and the lysate was separated onto a 6% SDS-polyacrylamide gel.

Induction of Hyperglycemia in Mice and Preparation of Aortas for Analysis—All mouse experiments were approved by the Institutional Animal Care and Use Committees of the University of North Carolina at Chapel Hill. Hyperglycemia was induced in C57/B6 mice (Taconic, Hudson, NY) using the low dose streptozotocin (16). The mean weight and serum glucose concentration was 27.1 ± 0.6 g and 120.6 ± 22.6 mg/dl before streptozotocin injection. One week after injection, mean serum glucose concentration was 311.6 ± 21.4 mg/dl. The mice were maintained for another 2 weeks before any treatment. The mean weight and serum glucose concentration was 26.0 ± 0.9 g and 430.3 ± 59.2 mg/dl before sacrifice. There were 12 mice per treatment group. The disrupting peptide (4 mg/kg) or control peptide (4 mg/kg) was administered intraperitoneally twice (24 and 1 h before sacrifice) and IGF-I (1 mg/kg) or PBS was administered intraperitoneally 15 min before sacrifice. The aortic extracts were prepared following the procedure described previously (17). The protein concentration of aortic extracts was measured using a BCA assay (Thermo Scientific). Equal amounts of protein were used for each analysis.

Statistical Analysis—The results that are shown in all experiments are representative of at least three independent experiments and expressed as the mean \pm S.D. The Student's *t* test was used to compare differences between two treatments for *in vitro* experiments. The Bonferroni correction was used when multiple variables were compared. One-way analysis of variance was applied for all data obtained from *in vivo* studies. In addition, repeated measures-analysis of variance was used where appropriate. $p < 0.05$ was considered statistically significant.

RESULTS

To determine whether a specific protein(s) associated with RPTP β in response to IGF-I stimulation, we exposed VSMCs to IGF-I for 10 min in the presence of IGFBP-2 and then immunoprecipitated RPTP β . The proteins that coimmunoprecipitated were separated by SDS-PAGE, and Colloidal Blue staining showed a major increase in a 58,000-kDa band in response to IGF-I stimulation (Fig. 1A). Amino acid sequence analysis revealed that the band was the intermediate filament protein vimentin. That vimentin bound to RPTP β in response to IGF-I was confirmed using immunoprecipitation (3.2 ± 0.6 -fold increase) (Fig. 1B). To determine whether vimentin binding to RPTP β mediated RPTP β polymerization, siRNA was utilized to knock down vimentin, which resulted in $86 \pm 5\%$ inhibition of vimentin synthesis ($p < 0.001$) (Fig. 2A). Transient knockdown of vimentin did not result in a change in cell morphology, cytoskeletal structure, or increased cell death (data not shown). Exposure of cells expressing a control siRNA to IGF-I plus IGFBP-2 resulted in a 7.0 ± 0.4 -fold greater stimulation of

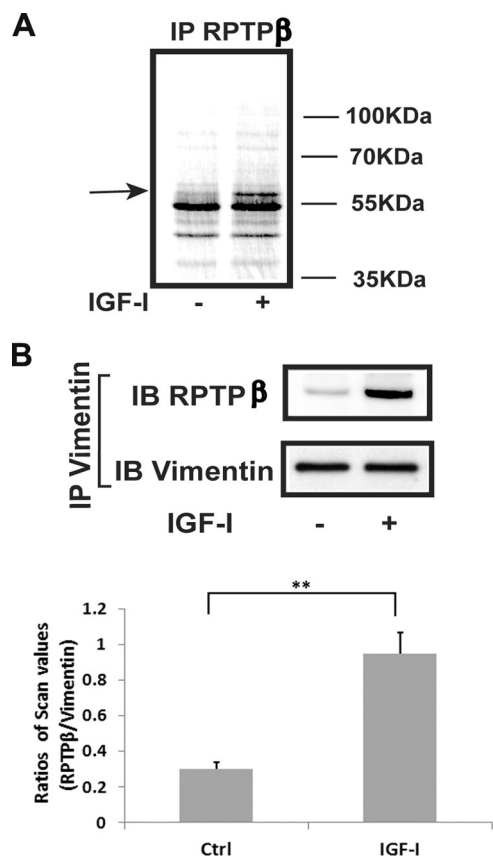


FIGURE 1. IGF-I stimulates vimentin/RPTP β interaction in VSMCs during hyperglycemia. VSMCs were cultured in DMEM containing high glucose (25 mM) plus 10% FBS and then serum-deprived for 16 h before stimulation without (–) or with (+) IGF-I (50 ng/ml) for 10 min. *A*, protein band (arrow) that was increased in response to IGF-I was analyzed by MALDI-TOF/MS as described under “Experimental Procedures.” *B*, cell lysates were immunoprecipitated (*IP*) with an anti-vimentin antibody and immunoblotted with an anti-RPTP β antibody. To control for loading, the blot was stripped and reprobed with an anti-vimentin antibody. Each bar is the ratio of the mean value of the scanning units for vimentin-associated RPTP β divided by total vimentin. *Ctrl*, control. **, $p < 0.01$ indicates significant differences between two treatments.

vimentin binding to RPTP β compared with cells expressing the vimentin siRNA ($p < 0.001$) (Fig. 2B). To assess the functional significance of loss of RPTP β /vimentin association, we determined the effect of IGF-I plus IGFBP-2 on RPTP β polymerization. As shown in Fig. 2C, cells expressing vimentin siRNA had complete attenuation of RPTP β polymerization indicating that vimentin binding to RPTP β was absolutely required. To assess the functional significance of loss of RPTP β polymerization, we analyzed PTEN tyrosine phosphorylation because RPTP β specifically dephosphorylates this substrate (9). As shown in Fig. 2D, IGF-I stimulated a 4.3 ± 0.3 -fold increase in PTEN tyrosine phosphorylation, whereas vimentin knockdown resulted in near complete loss of the IGF-I/IGFBP-2-stimulated response (e.g. 1.4 ± 0.2 -fold increase) ($p < 0.01$ compared with control). IGF-I-stimulated a 7.2 ± 1.4 -fold increase ($p < 0.001$) in AKT phosphorylation in control cells, and this response was significantly attenuated in cells treated with vimentin siRNA ($p < 0.01$) (Fig. 2E). Therefore, vimentin association with RPTP β is required for IGF-I-stimulated RPTP β polymerization and optimum enhancement of AKT activation.

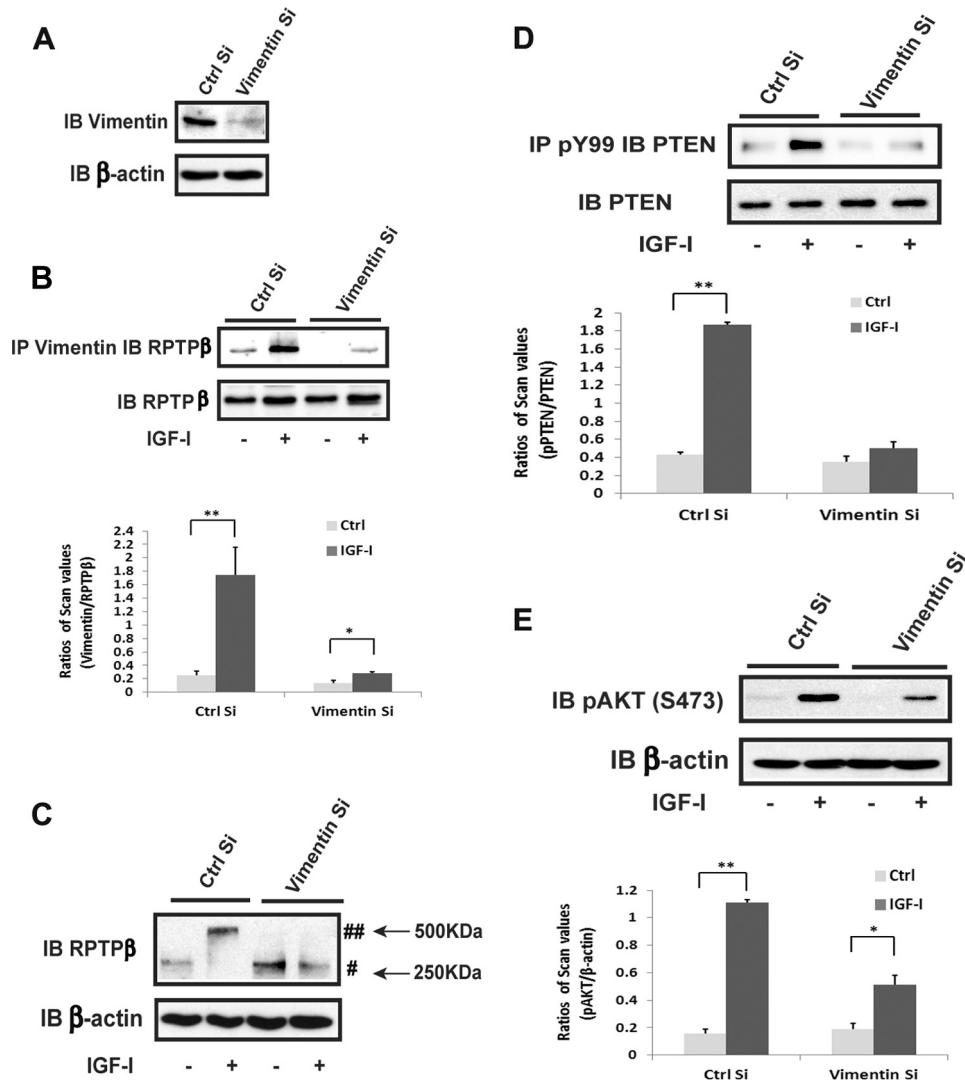


FIGURE 2. Vimentin is required for IGF-I-stimulated RPTP β polymerization, PTEN tyrosine phosphorylation, and AKT activation. VSMCs treated with the siRNA targeting vimentin (*vimentin Si*) and a control siRNA (*Ctrl Si*) were serum-deprived for 16 h and then incubated without (–) or with (+) IGF-I (50 ng/ml) for 10 min. *A*, cell lysates were immunoblotted (IB) with an anti-vimentin antibody. To control for loading, the blot was stripped and reprobed with an anti- β -actin antibody. *B*, cell lysates were immunoprecipitated (IP) with an anti-vimentin antibody and immunoblotted with an anti-RPTP β antibody. To control for loading, the same amount of lysate was immunoblotted with an anti-RPTP β antibody. Each bar is the ratio of the mean value of the scanning units for vimentin-associated RPTP β divided by total RPTP β . *C*, before lysis, cells were exposed to the noncell-permeable cross-linker bis[sulfosuccinimidyl]suberate as described under “Experimental Procedures.” The cell lysates were immunoblotted with an anti-RPTP β antibody. To control for loading, the same amount of lysate was immunoblotted with an anti- β -actin antibody. #, monomer; ##, dimer. *D*, cell lysates were immunoprecipitated with an anti-pY99 antibody and immunoblotted with an anti-PTEN antibody. The control lysate was immunoblotted with an anti-PTEN antibody. Each bar is the ratio of the mean value of the scanning units for pPTEN divided by total PTEN. *E*, cell lysates were immunoblotted with an anti-pAKT(S473) antibody. The blot was stripped and reprobed with an anti- β -actin antibody. Each bar is the ratio of the mean value of the scanning units for pAKT divided by β -actin. *, $p < 0.05$, and **, $p < 0.01$, indicate significant differences between two treatments.

To determine the relative importance of IGFBP-2 binding to RPTP β and whether IGF-I activation of IGF-IR is required for stimulation of vimentin binding to RPTP β , we used cells expressing an IGFBP-2 shRNA that had been shown to have impaired RPTP β polymerization (9). Cells expressing the IGFBP-2 shRNA had a $93 \pm 8\%$ decrease in IGFBP-2 secretion and showed no vimentin/RPTP β binding (Fig. 3A). Exposure of the knockdown cells to IGFBP-2 and IGF-I rescued vimentin/RPTP β association (5.4 ± 0.7 -fold increase compared with 6.8 ± 0.2 -fold in control cells). IGF-I receptor activation was also required for vimentin to bind to RPTP β , because in the presence of an IGF-IR tyrosine kinase inhibitor, PQ401, there was a $72 \pm 10\%$ ($p < 0.01$) reduction in stimulation of vimentin/

RPTP β association (Fig. 3B). To determine the region of IGFBP-2 that was interacting with RPTP β to facilitate vimentin binding, we utilized cells expressing an IGFBP-2 mutant (MT-1), which had been shown previously to have attenuated RPTP β binding. This mutant has no reduction in its affinity for IGF-I. Stimulation of the cultures with IGF-I and the mutant form of IGFBP-2 resulted in significant attenuation of stimulation RPTP β /vimentin association ($79 \pm 6\%$ reduction, $p < 0.001$) (Fig. 3C).

To determine the mechanism by which IGF-I receptor activation stimulated vimentin/RPTP β association, we analyzed the role of vimentin serine phosphorylation because serine phosphorylation of the vimentin head domain has been shown to

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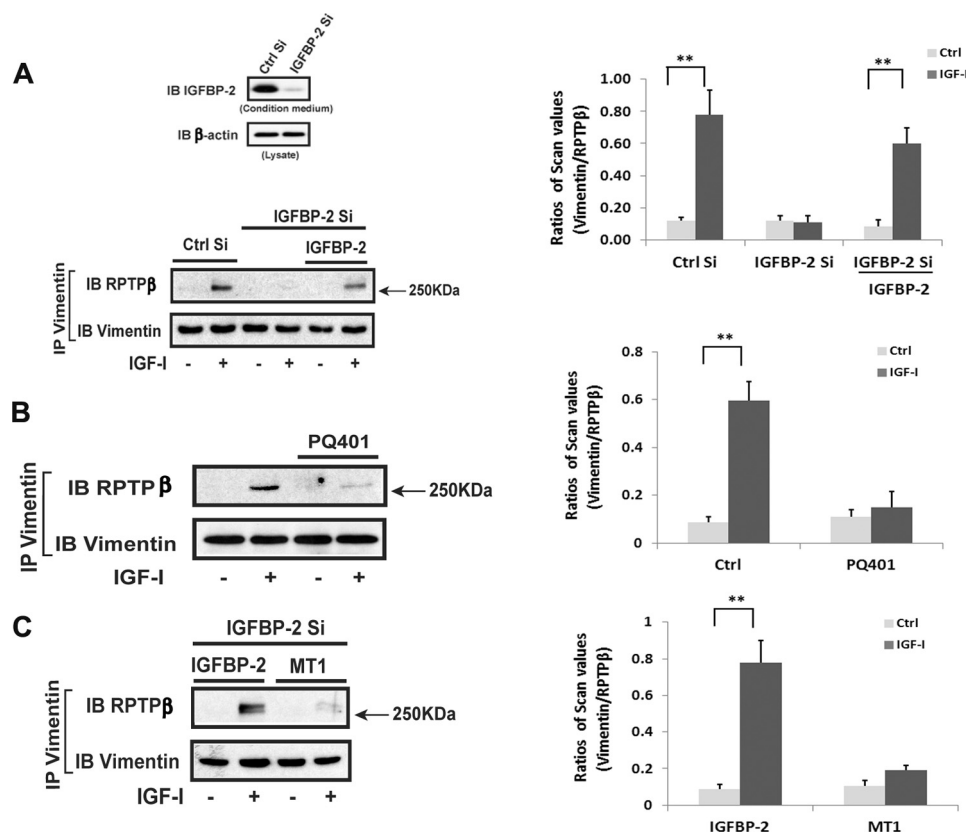


FIGURE 3. IGF-I-stimulated vimentin/RPTP β association requires IGF-I receptor activation and the presence of IGFBP-2. A and C, VSMCs were transfected with control (*Ctrl si*) or IGFBP-2 shRNA (*IGFBP-2 Si*) template plasmids, and conditioned medium was analyzed for IGFBP-2. Cell lysates were immunoblotted (IB) with an anti- β -actin antibody as a loading control (A). Cells were serum-deprived for 16 h before incubation without (–) or with (+) IGF-I (50 ng/ml) for 10 min. VSMC expressing IGFBP-2 shRNA were incubated with or without wild type IGFBP-2 (200 ng/ml) (A) or an IGFBP-2 mutant (MT1) (200 ng/ml) (C) for 2 h prior to IGF-I stimulation. B, VSMCs were serum-deprived for 16 h and then incubated with the IGF-I receptor tyrosine kinase inhibitor, PQ401, or vehicle for 1 h prior to IGF-I (50 ng/ml) stimulation (+) or no stimulation (–) for 10 min. A–C, cell lysates were immunoprecipitated (IP) with an anti-vimentin antibody and immunoblotted with an anti-RPTP β antibody. The blot was stripped and reprobed with an anti-vimentin antibody. Each bar is the ratio of the scanning units for vimentin-associated RPTP β divided by vimentin. **, $p < 0.01$ indicates significant differences.

mediate protein/protein interactions (18). Following IGF-I stimulation, there was a 5.1 ± 0.4 -fold increase in serine phosphorylation of vimentin (Fig. 4A). The IGF-IR tyrosine kinase inhibitor significantly inhibited IGF-I-stimulated vimentin serine phosphorylation (1.7 ± 0.3 -fold increase) ($82 \pm 12\%$ reduction compared with control, $p < 0.001$) (Fig. 4A). In contrast, when cells expressing IGFBP-2 shRNA were analyzed, there was no inhibition of IGF-I-stimulated vimentin phosphorylation (e.g. an 3.6 ± 0.6 -fold increase in control cells and an 3.3 ± 0.9 -fold increase in IGFBP-2 knockdown cells) (Fig. 4B). The vimentin head domain sequence contains 16 known serine phosphorylation sites (18). To identify the kinase that phosphorylated vimentin, we prepared cell-permeable peptides that contained sequence motifs that had been shown to be phosphorylated by specific kinases that were known to phosphorylate vimentin, and we then screened them for their ability to disrupt RPTP β /vimentin association. The response to a cell-permeable peptide that contained the consensus sequence of a known PKC ζ phosphorylation site located between residues 2 and 11 in the vimentin N terminus was compared to a control peptide in which three of the serines in this sequence had been substituted with alanine. The native peptide completely inhibited RPTP β /vimentin association in response to IGF-I, whereas the control peptide had no effect (Fig. 5A). To assess the functional

consequence of inhibiting vimentin/RPTP β association, we repeated the experiment and then assessed the effect on RPTP β polymerization. As shown in Fig. 5B, this was completely inhibited, and the ability of IGF-I/IGFBP-2 to stimulate PTEN tyrosine phosphorylation was also significantly decreased (Fig. 5C). Subsequent analysis of IGF-I-stimulated AKT activation revealed the predicted increase and a significant reduction (e.g. $76 \pm 8\%$ decrease, $p < 0.01$) in the degree of stimulation following exposure to the vimentin/RPTP β -disrupting peptide (Fig. 5D). Exposure to the disrupting peptide had no effect on vimentin phosphorylation (Fig. 5E), thereby excluding the possibility that it functioned to inhibit kinase activity.

The results shown in Fig. 5 suggested PKC ζ is the kinase that phosphorylates vimentin in response to IGF-I. Our prior studies have shown that in this cell type exposure to hyperglycemia and IGF-I specifically activates PKC ζ and that PKC ζ is required for optimal IGF-I stimulation of downstream signaling (17). Based on that result, we stimulated cultures with IGF-I and then examined vimentin serine phosphorylation in the presence or absence of a cell-permeable peptide containing the sequence of a PKC ζ pseudosubstrate peptide inhibitor (12). IGF-I-stimulated vimentin serine phosphorylation was significantly attenuated in the cultures exposed to the PKC ζ inhibitor (Fig. 6A), leading to impaired vimentin/RPTP β association

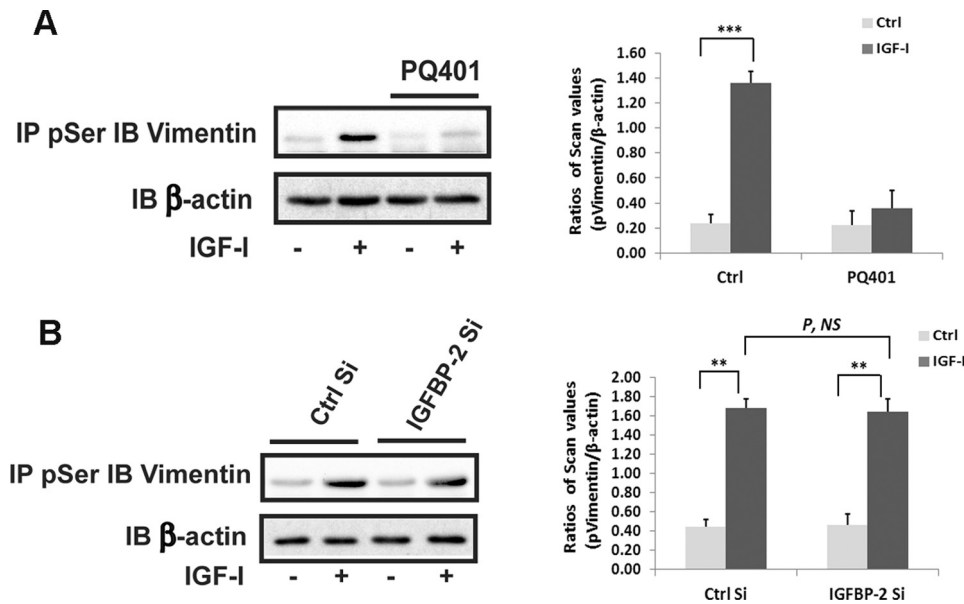


FIGURE 4. IGF-I-stimulated vimentin serine phosphorylation requires IGF-I receptor activation but not IGFBP-2 stimulation. *A*, VSMCs were serum-deprived for 16 h and then incubated with the IGF-I receptor tyrosine kinase inhibitor, PQ401, or vehicle for 1 h prior to IGF-I (50 ng/ml) stimulation (+) or no stimulation (–) for 10 min. *B*, VSMCs expressing the shRNA targeting control (*Ctrl Si*) and IGFBP-2 (*IGFBP-2 Si*) were serum-deprived for 16 h before stimulation without (–) or with (+) IGF-I (50 ng/ml) for 10 min. *A* and *B*, cell lysates were immunoprecipitated (IP) with an anti-Ser(P) (*pSer*) antibody and immunoblotted (IB) with an anti-vimentin antibody. The same amount of lysate was immunoblotted with an anti- β -actin antibody. Each bar is the ratio of the scanning units for serine phosphate vimentin divided by β -actin. ***, $p < 0.001$ indicates significant differences between two treatments. *P*, *NS* indicates no significant difference.

(Fig. 6*B*). In contrast, when cells were maintained in 5 mM glucose, there was no increase of vimentin serine phosphorylation and no vimentin/RPTP β association (Fig. 6, *C* and *D*). To confirm that the peptide was inhibiting vimentin/RPTP β association by inhibiting vimentin phosphorylation and not by inhibiting vimentin binding to PKC ζ , we immunoprecipitated vimentin and immunoblotted for PKC ζ following IGF-I stimulation. IGF-I stimulated PKC ζ /vimentin association, and the pseudosubstrate inhibitor had no effect on this protein/protein interaction (Fig. 6*E*). We subsequently determined that exposure to the pseudosubstrate inhibitor completely inhibited RPTP β polymerization (Fig. 6*F*) indicating that recruitment of this kinase to vimentin and its subsequent phosphorylation is critical for PKC ζ stimulation of vimentin serine phosphorylation and RPTP β polymerization.

In VSMC exposed to hyperglycemia, IRS-1 is down-regulated, and IGF-I receptor-linked signaling occurs through assembly of a signaling complex on the plasma membrane-associated scaffold, SHPS-1. SHPS-1 is tyrosine-phosphorylated in response to IGF-I receptor stimulation, and inhibition of formation of this signaling complex on the SHPS-1 scaffold results in major attenuation of AKT activation (19). Therefore, to identify the proximal signaling components that were required for PKC ζ recruitment to vimentin, we investigated the role of the SHPS-1 signaling complex. Our prior proteomic screening studies had shown that ILK is preferentially recruited to SHPS-1 in response to IGF-I stimulation (15). Based on that result, we determined whether PKC ζ was recruited to ILK following IGF-I stimulation. As shown in Fig. 7*A*, following IGF-I stimulation PKC ζ was recruited to ILK, and this was inhibited by an ILK inhibitor (72 \pm 8% decrease, $p < 0.01$). More importantly, exposure to the inhibitor also disrupted PKC ζ recruitment to vimentin (Fig. 7*B*), and IGF-I stimulated vimentin ser-

ine phosphorylation (77 \pm 7% reduction with 5 μ M, compared with control, $p < 0.01$) (Fig. 7*C*). This was associated with the failure to recruit vimentin to RPTP β (Fig. 7*D*) and loss of IGF-I-stimulated AKT phosphorylation (Fig. 7*E*). To determine whether ILK was facilitating PKC ζ /vimentin association through recruitment of PKC ζ to SHPS-1, we utilized cells expressing an SHPS-1 cytoplasmic domain-truncated mutant that did not bind ILK (15). Expression of this SHPS-1 mutant resulted in failure to recruit PKC ζ to vimentin (Fig. 7*F*), and this was associated with inhibition of IGF-I-stimulated vimentin phosphorylation (Fig. 7*G*). Subsequently, we determined that in the absence of the SHPS-1 cytoplasmic domain, PKC ζ was not recruited to SHPS-1 (Fig. 7*H*). This confirmed that the SHPS-1 scaffold was necessary for PKC ζ /vimentin association. This failure to recruit PKC ζ to SHPS-1 resulted in nearly complete loss of IGF-I-stimulated vimentin binding to RPTP β (Fig. 7*I*).

To determine the significance of these signaling events *in vivo*, mice were made diabetic and then exposed to the peptide that inhibited vimentin/RPTP β association. Analysis of the aortas following the injection of a biotinylated form of the peptide showed that it was taken up by the aortas in 1 h (data not shown). Following injection of IGF-I, there was marked stimulation of RPTP β /vimentin binding, and this was significantly inhibited in the presence of disrupting the peptide (Fig. 8*A*). In contrast, the control peptide had no effect (Fig. 8*A*). IGF-I stimulated a 4.3 \pm 0.7-fold increase in vimentin serine phosphorylation that was attenuated with exposure of the mice to the PKC ζ inhibitor (Fig. 8*B*). In contrast, nondiabetic mice showed no increase in vimentin serine phosphorylation (Fig. 8*C*). The disrupting peptide also inhibited IGF-I-stimulated PTEN tyrosine phosphorylation (Fig. 8*D*) and AKT activation (a 78 \pm 7% reduction compared with control, $p < 0.01$) (Fig. 8*E*). We have reported that these signaling events are attenuated in nondia-

Vimentin/RPTP β Interaction Mediates IGF-I Signaling

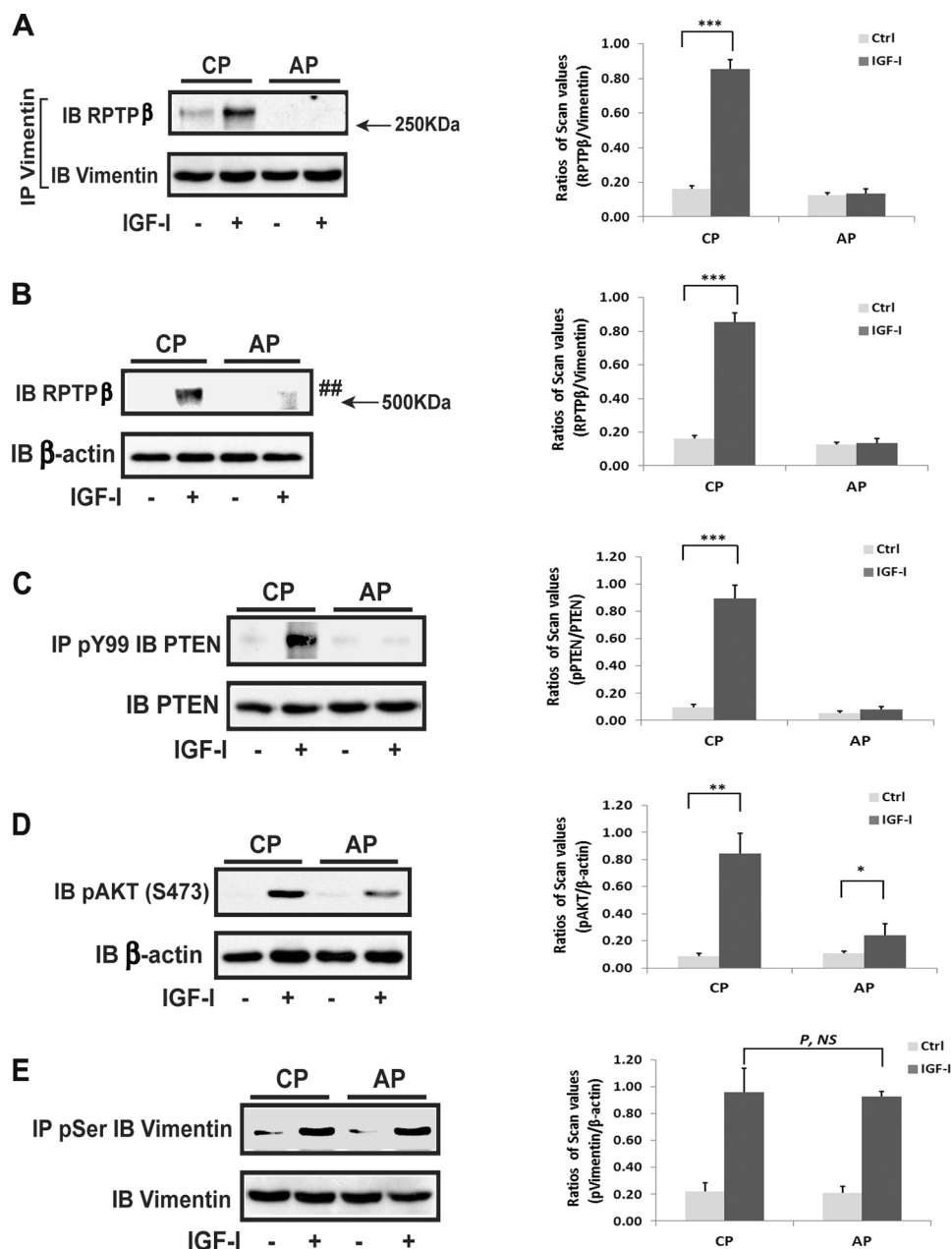


FIGURE 5. Disruption of vimentin/RPTP β association impaired IGF-I-stimulated RPTP β polymerization, PTEN tyrosine phosphorylation, and AKT activation. VSMCs were serum-deprived for 16 h and then incubated with a control (Ctrl) peptide (CP) or a peptide that disrupted vimentin/RPTP β (AP) for 2 h prior to IGF-I (50 ng/ml) stimulation (+) or no stimulation (-) for 10 min. *A*, cell lysates were immunoprecipitated (IP) with an anti-vimentin antibody and immunoblotted (IB) with an anti-RPTP β antibody. To control for loading, the blot was stripped and reprobed with an anti-vimentin antibody. *B*, before lysis, cells were exposed to the noncell-permeable cross-linker bis[sulfosuccinimidyl]suberate as described under "Experimental Procedures." The cell lysates were immunoblotted with an anti-RPTP β antibody. The same amount of lysate was immunoblotted with an anti- β -actin antibody. ##, dimer. *C*, cell lysates were immunoprecipitated with an anti-pY99 antibody and immunoblotted with an anti-PTEN antibody. The same amount of lysate was immunoblotted with an anti-PTEN antibody. *D*, cell lysates were immunoblotted with an anti-pAKT(S473) antibody. The blot was stripped and reprobed with an anti- β -actin antibody. *E*, cell lysates were immunoprecipitated with an anti-Ser(P) antibody and immunoblotted with an anti-vimentin antibody. The value of each bar is the ratio of the scanning units for vimentin-associated RPTP β divided by vimentin (*A*), RPTP β divided by β -actin (*B*), pPTEN divided by PTEN (*C*), pAKT divided by β -actin (*D*), and serine phosphorylated vimentin divided by vimentin (*E*). **, $p < 0.01$, and ***, $p < 0.001$, indicate significant differences between two treatments. *P*, *NS* indicates no significant difference.

betic mice (9, 20). Therefore, this interconnected series of signaling events that had been delineated in smooth muscle cells in culture could be reproduced in intact aorta in diabetic mice.

DISCUSSION

Our prior studies showed that IGFBP-2 stimulates RPTP β polymerization leading to inhibition of its tyrosine phosphatase

activity and enhanced tyrosine phosphorylation of PTEN, its primary target in vascular smooth muscle cells and preosteoblasts (9, 10). This increase in PTEN tyrosine phosphorylation led to reduced PTEN enzymatic activity resulting in enhanced AKT activation and stimulation of vascular smooth muscle migration or osteoblast differentiation responses to IGF-I (9–11). Although binding of IGFBP-2 to RPTP β through its

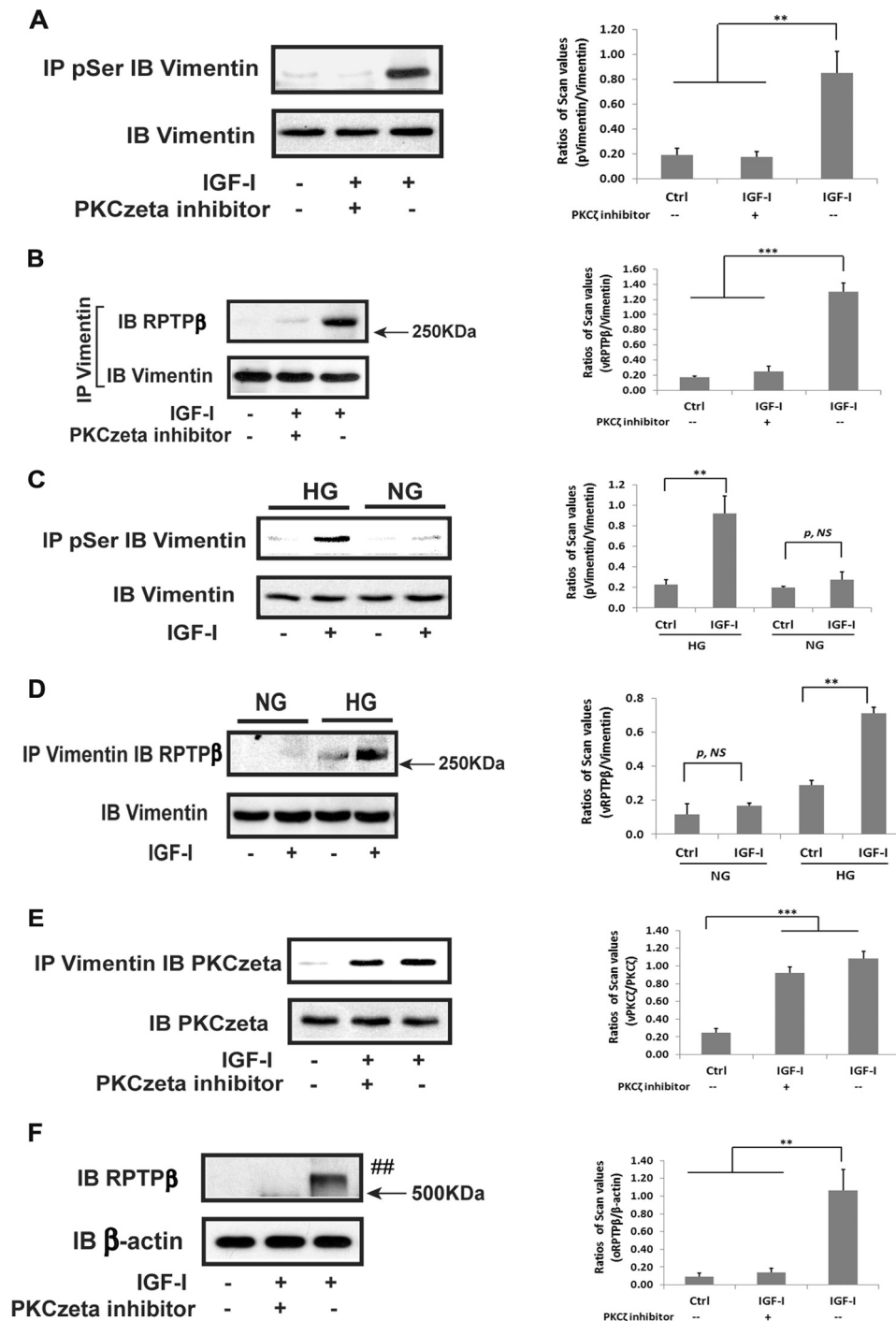


FIGURE 6. PKC ζ mediates IGF-I-stimulated vimentin serine phosphorylation. VSMCs were serum-deprived for 16 h and then incubated without or with a PKC ζ inhibitor (10 μ M) for 1 h prior to IGF-I (50 ng/ml) stimulation (+) or no stimulation (-) for 10 min. *A*, cell lysates were immunoprecipitated (*IP*) with an anti-Ser(P) (*pSer*) antibody and immunoblotted (*IB*) with an anti-vimentin antibody. Loading controls were immunoblotted with an anti-vimentin antibody. *B*, cell lysates were immunoprecipitated with an anti-vimentin antibody and immunoblotted with an anti-RPTP β antibody. The blot was stripped and reprobbed with an anti-vimentin antibody. *C* and *D*, VSMCs cultured in normal glucose (5 mM, NG) or high glucose (25 mM, HG) were serum-deprived for 16 h before IGF-I stimulation for 10 min with (+) or without stimulation (-). Cell lysates were immunoprecipitated with an anti-Ser(P) antibody and immunoblotted with an anti-vimentin (*C*) or with an anti-vimentin antibody and immunoblotted with an anti-RPTP β antibody (*D*). Loading controls were immunoblotted with an anti-vimentin antibody. *E*, cell lysates were immunoprecipitated with an anti-vimentin antibody and immunoblotted with an anti-PKC ζ antibody. The same amount of lysate was immunoblotted with an anti-PKC ζ antibody. *F*, before lysis, cells were exposed to the noncell-permeable cross-linker bis[sulfosuccinimidyl]suberate as described under "Experimental Procedures." The cell lysates were immunoblotted with an anti-RPTP β antibody or an anti- β -actin antibody. ##, dimer. The mean scanning units for serine-phosphorylated vimentin divided by vimentin (*A*), vimentin-associated RPTP β divided by vimentin (*B*), serine-phosphorylated vimentin divided by vimentin (*C*), vimentin-associated RPTP β divided by vimentin (*D*), vimentin-associated PKC ζ divided by PKC ζ (*E*), and RPTP β divided by β -actin (*F*). **, $p < 0.01$, and ***, $p < 0.001$, indicate significant differences between two treatments. *p*, *NS* indicates no significant difference.

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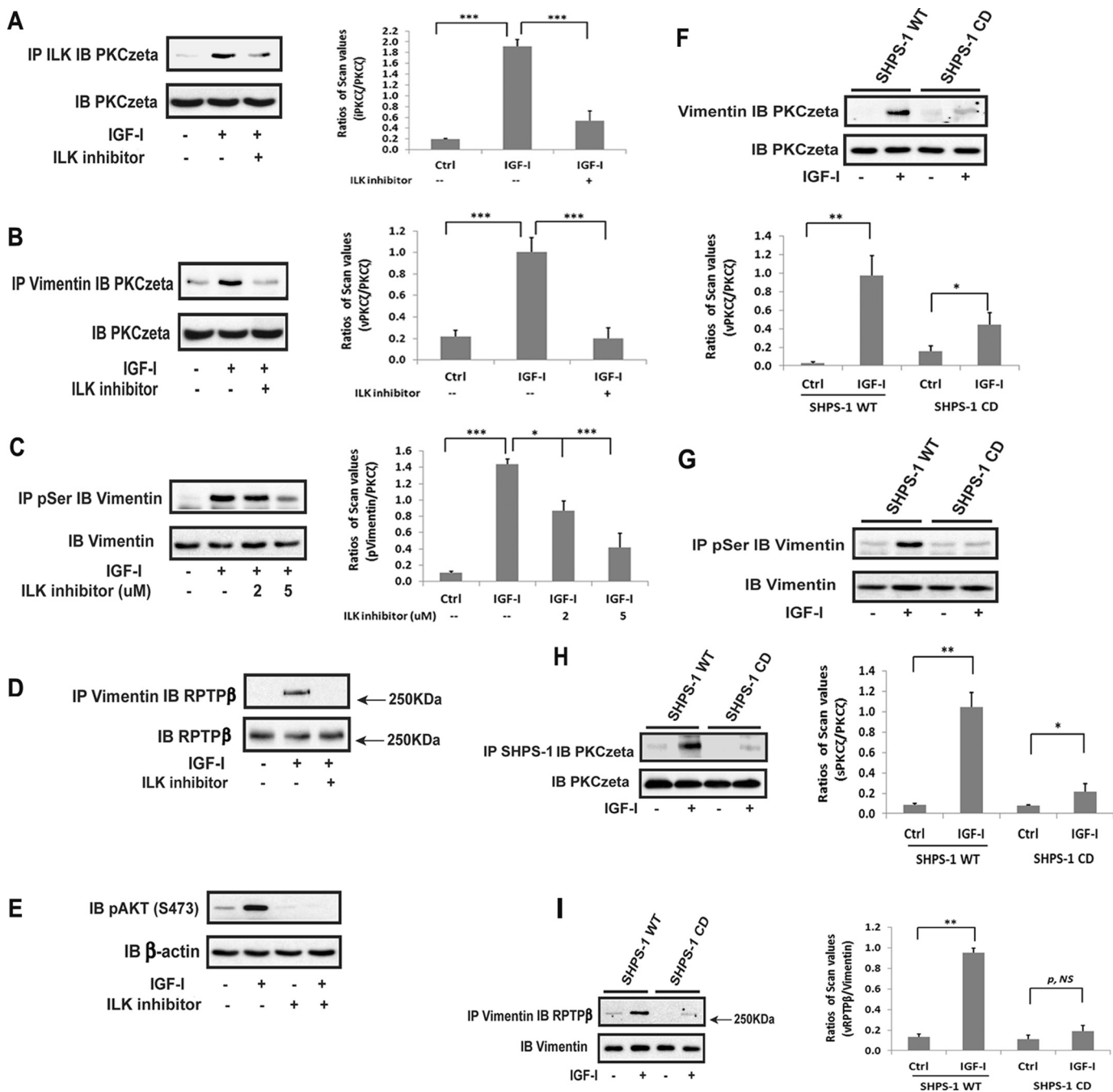


FIGURE 7. ILK is recruited to SHPS-1 and mediates IGF-I-stimulated vimentin/PKC ζ interaction. *A–E*, VSMCs were serum-deprived for 16 h and then incubated without or with an ILK inhibitor (5 μ M, or indicated concentrations) for 1 h prior to IGF-I (50 ng/ml) stimulation (+) or no stimulation (–) for 10 min. Cell lysates were immunoprecipitated (IP) with an anti-ILK (*A*) or anti-vimentin (*B* and *D*) or anti-Ser(P) (*pSer*) (*C*) antibody and immunoblotted with an anti-PKC ζ (*A* and *B*) or anti-vimentin (*C*) or anti-RPTP β (*D*) antibody. To control for loading, the same amount of lysate was immunoblotted with an anti-PKC ζ (*A* and *B*) or anti-vimentin (*C*) or anti-RPTP β (*D*) antibody. *E*, cell lysates were immunoblotted with an anti-pAKT(S473) antibody. The blot was reprobed with an anti- β -actin antibody. *F–I*, VSMCs expressing wild type SHPS-1 (*SHPS-1 WT*) or cytoplasmic domain-truncated SHPS-1 (*SHPS-1 CD*) were serum-deprived for 16 h before stimulation without (–) or with (+) IGF-I (50 ng/ml) for 10 min. Cell lysates were immunoprecipitated with an anti-vimentin (*F* and *I*) or anti-Ser(P) (*G*) or anti-SHPS-1 (*H*) antibody and immunoblotted with an anti-PKC ζ (*F* and *H*) or anti-vimentin (*G*) or anti-RPTP β antibody (*I*). To control for loading, the same amount of lysate was immunoblotted with an anti-PKC ζ (*F* and *H*) or an anti-vimentin antibody (*G* and *I*). The mean value of the scanning units for ILK-associated PKC ζ divided by PKC ζ (*A*), vimentin-associated PKC ζ divided by PKC ζ (*B* and *F*), serine-phosphorylated vimentin divided by vimentin (*C*), SHPS-1-associated PKC ζ divided by PKC ζ (*H*), and vimentin-associated RPTP β divided by vimentin (*I*). *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$, indicate significant differences between two treatments. *P*, *NS* no significant difference.

heparin binding domain was required for RPTP β polymerization, concomitant activation of the IGF-I receptor was also required. Therefore, these studies were undertaken to determine the mechanism by which IGF-I enhanced the ability of IGFBP-2 to stimulate RPTP β polymerization. The results

definitively show that IGF-I receptor activation stimulates vimentin binding to RPTP β and that this is absolutely required for RPTP β polymerization. Disruption of vimentin binding either by vimentin knockdown or the utilization of a cell-permeable peptide that inhibited the interaction of the two pro-

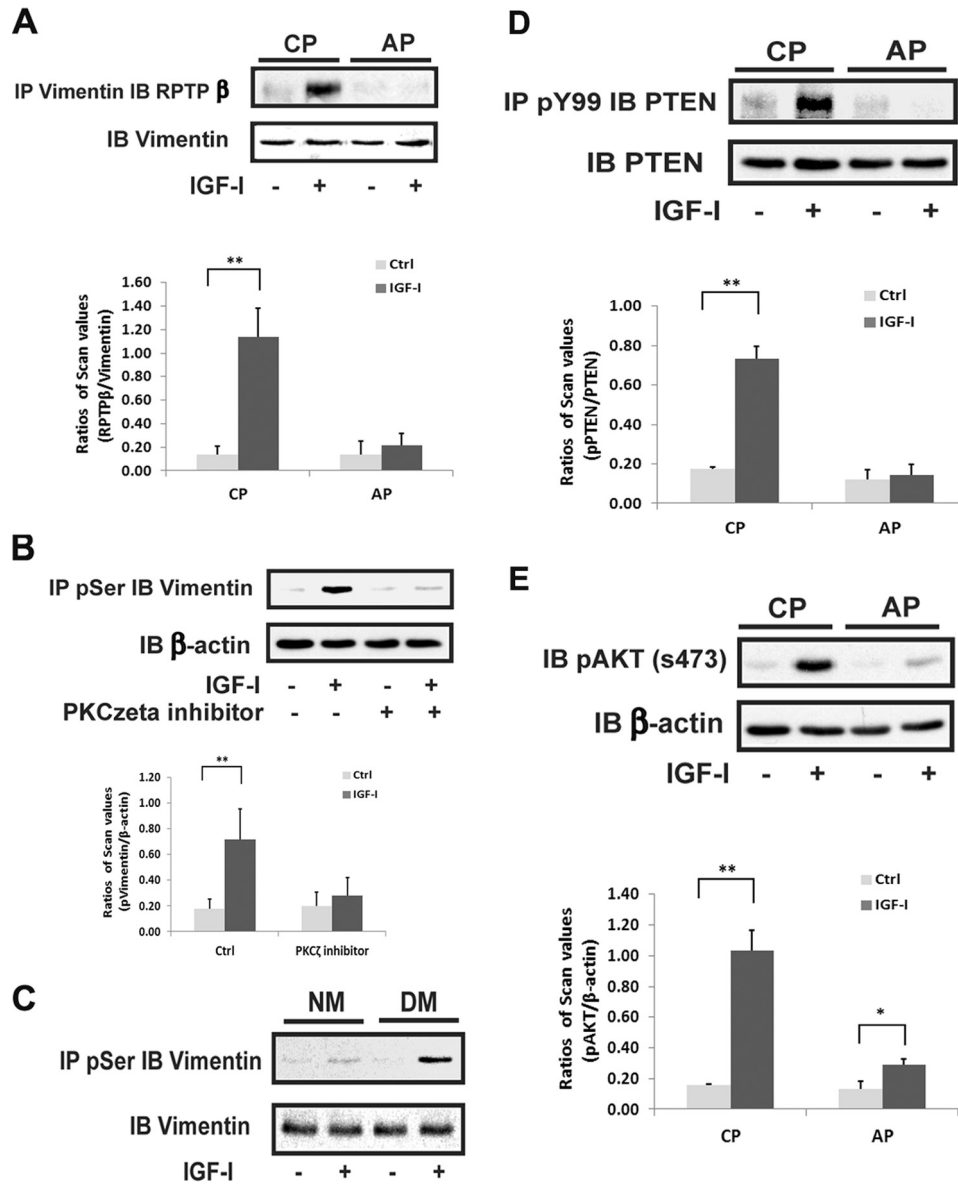


FIGURE 8. Disruption of vimentin/RPTP β association impaired IGF-I-stimulated PTEN tyrosine phosphorylation and pAKT activation *in vivo*. A control peptide (CP) or a disrupting peptide (AP) (A, C, and D) or a PKC ζ inhibitor (B) was injected (intraperitoneally) into the mice ($n = 6$ for each group) 24 and 1 h before sacrifice. IGF-I (+) or PBS (-) was injected (intraperitoneally) 15 min before sacrifice. A, aortic extracts prepared as described under "Experimental Procedures" were immunoprecipitated (IP) using an anti-vimentin and immunoblotted (IB) using an RPTP β antibody. The same amount of extract was immunoblotted using an anti-vimentin antibody. Each bar is the ratio of the mean value of the scanning units for vimentin-associated RPTP β divided by vimentin. B, aortic extracts were immunoprecipitated using an anti-Ser(P) (pSer) antibody and immunoblotted using an anti-vimentin antibody. The extracts were immunoblotted using an anti- β -actin antibody to control for a protein input. Each bar is the ratio of the mean value of the scanning units for serine phosphate vimentin divided by β -actin. C, aortic extracts from diabetic (DM, $n = 4$) or nondiabetic mice (NM, $n = 4$) that had been injected with IGF-I (+) (1 mg/kg, 15 min before sacrifice) or PBS (-) were immunoprecipitated with an anti-Ser(P) antibody and immunoblotted with anti-vimentin antibody. For a loading control, the same amount of aortic extract was immunoblotted with an anti-vimentin antibody. D, aortic extracts were immunoprecipitated using an anti-pY99 antibody and immunoblotted using an anti-PTEN antibody. Each bar is the ratio of the mean value of the scanning units for pPTEN divided by PTEN. E, aortic extracts were immunoblotted using an anti-pAKT(S473) antibody. The blots were stripped and reprobed with an anti- β -actin antibody. Each bar is the ratio of the scanning units for pAKT divided by β -actin. *, $p < 0.05$, and **, $p < 0.01$, indicate significant differences between two treatments.

teins resulted in failure of IGFBP-2/IGF-I to stimulate RPTP β polymerization. That both IGFBP-2 and IGF-I were required was shown by utilizing cells in which IGFBP-2 had been knocked down to demonstrate that although IGF-I stimulated vimentin serine phosphorylation, it could not stimulate vimentin/RPTP β association in the absence of IGFBP-2. Furthermore, addition of an IGF-I receptor tyrosine kinase inhibitor resulted in failure of IGF-I to stimulate vimentin/RPTP β association even in the presence of optimal concentrations of

IGFBP-2. Therefore, coordinate activation of both receptor signaling systems is required for this interaction to occur.

Vimentin binding to RPTP β was mediated by its serine phosphorylation. Exposure of cells to IGF-I resulted in an increase in vimentin serine phosphorylation, and the serine/threonine kinase that phosphorylates vimentin was shown to be PKC ζ . Inhibition of PKC ζ activation resulted in complete attenuation of vimentin-stimulated RPTP β polymerization. More importantly, a synthetic peptide that contained the sequence from the

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vimentin head domain that contained a consensus PKC ζ phosphorylation site disrupted vimentin/RPTP β binding, RPTP β polymerization, PTEN tyrosine phosphorylation, and AKT activation. Therefore, these results definitively show that IGF-I-stimulated serine phosphorylation of the head domain of vimentin leads to RPTP β polymerization. Blocking the vimentin/RPTP β interaction in mice utilizing the disrupting peptide showed that it was required for RPTP β polymerization and AKT activation. Therefore, we conclude this is the mechanism by which IGF-I and IGFBP-2 function to modulate the AKT activation *in vivo*. Our prior studies have shown that optimal activation of AKT in VSMC in response to IGF-I requires the presence of hyperglycemia and that in cells maintained in 5 mM glucose or in nondiabetic mouse aorta, IGF-I-stimulated AKT activation (9, 20) and ki67 labeling are attenuated (16, 17). This is due in part to reduced SHPS-1 phosphorylation (19) which results in a decrease in PKC ζ recruitment and activation (17). The findings in this study are consistent with these results, showing that in the presence of normal glucose, IGF-I-stimulated vimentin serine phosphorylation and vimentin/RPTP β association were decreased. We conclude that hyperglycemia is required for PKC ζ activation and IGF-I-stimulated PKC ζ recruitment to the SHPS-1 signaling complex. PKC ζ phosphorylates vimentin, which then binds to RPTP β . This association resulted in enhanced downstream signaling and biological responses.

The molecular mechanism by which vimentin was shown to interact with RPTP β was through serine phosphorylation of the head domain. This domain, which encompasses the first 74 amino acids of the vimentin N terminus, contains multiple serine phosphorylation sites (21). A consensus sequence motif that is a known PKC ζ phosphorylation site is encompassed by residues 2–11. Proteins containing this sequence motif are preferentially serine-phosphorylated by this kinase (22). Our studies confirmed the importance of this domain by showing that a peptide containing this sequence could completely disrupt vimentin/RPTP β association thereby inhibiting RPTP β polymerization and downstream signaling *in vitro* and *in vivo*. Confirmation that PKC ζ -phosphorylated vimentin was established by using a specific PKC ζ pseudosubstrate inhibitor and showing that it inhibited IGF-I stimulated vimentin serine phosphorylation as well as vimentin binding to RPTP β . Other protein kinases have been shown to phosphorylate the head domain of vimentin including CAM kinase II, an important regulator of smooth muscle cell proliferation (23). In addition, IGF-I has been shown to induce CAM kinase II activation under certain circumstances (24). However, IGF-I also induces protein kinase C activation (25); therefore, taken together with the observation that a specific PKC ζ inhibitor attenuated vimentin phosphorylation, we conclude that PKC ζ is the kinase that phosphorylates vimentin and that it is activated by IGF-I receptor stimulation. Our observation that a peptide containing the PKC ζ phosphorylation site inhibited vimentin binding to RPTP β and RPTP β polymerization suggests that this is the region of vimentin that is phosphorylated and that it interacts with RPTP β to stimulate polymerization.

Serine phosphorylation of the vimentin head domain has been shown to lead to multiple protein/protein interactions

and changes in target protein function (26). Tzivion *et al.* (27) demonstrated that phosphorylation of vimentin sequestered 14-3-3 and that this resulted in differential binding of signaling proteins, such as Raf, to vimentin thereby altering cellular signaling. Similarly phosphorylation of serine 56 by PAK-1 kinase was shown to alter p47 phox association with vimentin thereby regulating smooth muscle cell contraction (28, 29). Vimentin phosphorylation in smooth muscle has also been shown to regulate Crk-associated substrate association as well as translocation of Rho kinase (28). Phosphorylation of serines in the head domain regulates intermediate filament assembly and disassembly in smooth muscle cells, and this results in differential protein/protein interactions (18). This reassembly of intermediary filaments is thought to be an important regulator of cell migration (30). Phosphorylation of vimentin has also been shown to correlate with formation of glomerular lamellipodia, which is essential for migration (26).

Disruption of vimentin/RPTP β association had effects on RPTP β polymerization and downstream signaling events that were similar to those observed following vimentin knockdown. The mechanism by which vimentin and IGFBP-2 binding to RPTP β coordinately regulate RPTP β polymerization has not been determined. The proposed mechanism of RPTP β polymerization has been thought to be due to solely ligand occupancy of the extracellular domain because the binding of ligands such as pleiotropin and midkine facilitates RPTP β polymerization, presumably in the absence of concomitant binding of intracellular proteins (31). It is clear from our studies that IGFBP-2 association with RPTP β alone is not sufficient to stimulate polymerization, and vimentin binding to the RPTP β cytoplasmic domain is required. RPTP β polymerization is thought to occur through formation of a wedge between catalytic domains in the dimer and that wedge formation effectively blocks substrate availability thereby inhibiting tyrosine dephosphorylation (32). The mechanism by which serine phosphorylation of vimentin would facilitate this interaction has not been defined.

Because regulation of PKC ζ subcellular localization has been shown to be an important regulatory variable for determining substrate specificity, we analyzed factors that might result in differential PKC ζ subcellular localization in response to IGF-I stimulation. In smooth muscle cells exposed to hyperglycemia, the primary target of the IGF-I receptor kinase is SHPS-1; therefore, we examined proteins that were differentially recruited to SHPS-1 phosphotyrosines (15). Our proteome-wide screening studies had shown that ILK is recruited to the SHPS-1 signaling complex in response to IGF-I. ILK is a pseudokinase and does not have catalytic activity; therefore, it was not a good candidate for directly phosphorylating vimentin (33). However, ILK has been shown to be important for the recruitment and subcellular localization of signaling molecules (34). ILK forms a complex with two other proteins, PINCH and parvin, that recruit a variety of signaling intermediates to this complex (35). To determine whether ILK mediated PKC ζ recruitment, we utilized an ILK inhibitor that regulates protein recruitment to the PINCH-parvin-ILK complex (35–37). Addition of the ILK inhibitor resulted in inhibition of IGF-I-stimulated ILK/PKC ζ association as well as vimentin phosphoryla-

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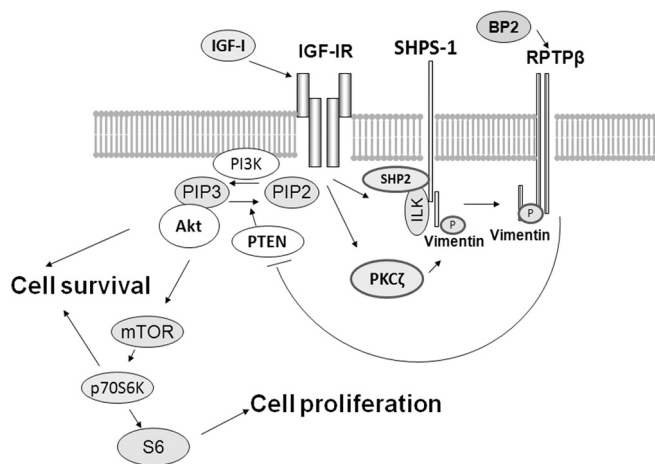


FIGURE 9. Vimentin/RPTP β mediates IGF-I/IGFBP-2 cooperative signaling during hyperglycemia. Hyperglycemia activates PKC ζ and IGF-I stimulates formation of an SHPS-1-associated signaling complex that includes SHP-2/ILK/PKC ζ /vimentin. Activated PKC ζ phosphates vimentin, which creates a binding site for RPTP β . IGFBP-2 binding to RPTP β stimulates phospho-vimentin/RPTP β association, leading to RPTP β polymerization, which attenuates its phosphatase activity. This results in enhanced PTEN tyrosine phosphorylation, impaired PTEN enzymatic activity, and increased AKT activation.

tion leading to loss of vimentin/RPTP β association. The importance of IGF-I stimulated recruitment of ILK to SHPS-1 was validated by showing that cells expressing the SHPS-1 cytoplasmic domain-truncated mutant failed to recruit PKC ζ to vimentin. Therefore, we conclude that IGF-I-stimulated phosphorylation of SHPS-1 is required to recruit ILK-1 and that ILK or the ILK-PINCH-parvin complex recruits PKC ζ , thereby facilitating PKC ζ /vimentin interaction.

Several studies have demonstrated that assembly of the PINCH-parvin-ILK complex is important for subcellular localization of signaling molecules (35, 38). Rho kinase, MAPK, paxillin, NCK-2, PP-1, and β 1 integrins are all recruited to this complex under various conditions (38, 39). During stimulation of cell migration, ILK interacts with multiple integrin receptors, principally the β -1 integrin, and recruitment of ILK to β 1 in focal adhesions is thought to play an important role in this process (40). The ability of ILK to recruit specific signaling components to focal adhesions has been proposed as an important growth regulator mechanism (35). Overexpression of ILK has been shown to regulate smooth muscle cell differentiation (41), and the state of differentiation is an important component of maintenance of signaling through the SHPS-1/PI 3-kinase pathway, which is necessary for IGF-I-stimulated migration (17). Our results are consistent with this model because they show that ILK recruitment of PKC ζ results in an enhancement of IGF-I-stimulated AKT activation in VSMC, which is important for optimal IGF-I-stimulated cell migration.

In summary, these studies have determined the mechanism by which IGF-I receptor stimulation collaborates with IGFBP-2 binding to RPTP β to lead to enhancement of AKT pathway-dependent functions in vascular smooth muscle cells. IGF-I receptor stimulation is required for activation of PKC ζ -stimulated vimentin serine phosphorylation, and this results in direct binding of vimentin to RPTP β , which facilitates IGFBP-2-mediated RPTP β polymerization (Fig. 9). These results emphasize

the importance of collaborative signaling between the two receptor systems to obtain optimal IGF-I responsiveness and how these signaling pathways may be altered in diabetes. The findings clearly emphasize the need for analysis of similar interactions occurring between other IGF-binding proteins and their respective receptors and the role of these interactions mediating specific cellular functional responses following IGF-I stimulation.

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