Insulin-like growth factor-I (IGF-I) plays a role in mutually exclusive processes such as proliferation and differentiation in a variety of cell types. IGF-I is a potent mitogen and mitogen for dedifferentiated vascular smooth muscle cells (VSMCs) in vivo and in vitro. However, in differentiated VSMCs, IGF-I is only required for maintaining the differentiated phenotype. Here we investigated the VSMC phenotype-dependent signaling and biological processes triggered by IGF-I. In differentiated VSMCs, IGF-I activated a protein-tyrosine phosphatase, SHP-2, recruited by insulin receptor substrate-1 (IRS-1). The activated SHP-2 then dephosphorylated IRS-1 Tyr(P)-95, resulting in blockade of the pathways from IRS-1/Grb2/Sos to the ERK and p38 MAPK. Conversely, such negative regulation was silent in dedifferentiated VSMCs, where IGF-I activated both MAPKs via IRS-1/Grb2/Sos interaction-linked Ras activation, leading to proliferation and migration. Thus, our present results demonstrate that the IRS-1/SHP-2 interaction acts as a switch controlling VSMC phenotype-dependent IGF-I-induced signaling pathways and biological processes, and this mechanism is likely to be applicable to other cells.

The insulin-like growth factors (IGFs) are involved in a variety of biological processes regulating cell proliferation, migration, survival, size control, and differentiation (1, 2). IGFs activate the IGF-I receptor (IGF-IR) tyrosine kinase, resulting in the tyrosine phosphorylation of downstream signaling molecules and/or direct interaction with them (1). The major targets of IGF-IR are three adaptor proteins: IRS-1, Shc, and Gab1. IRS-1, an important substrate for both the insulin receptor and IGF-IR, contains multiple tyrosine phosphorylation sites that recognize Src homology 2 domain-containing signaling molecules, such as Grb2, Nck, the p85 subunit of phosphoinositide 3-kinase (p85 PI3K), and SHP-2 (1). Of these, the binding of Grb2 associated with Sos to tyrosine-phosphorylated IRS-1 activates Ras, which switches on the Raf-1/mitogen-activated protein kinase (MAPK) kinase (MEK)/extracellular signal-regulated kinase (ERK) cascade (3). The ligand-engaged IGF-IR triggers another pathway involving PI3K. The interaction between tyrosine-phosphorylated IGF-IRβ or insulin receptor substrate-1 (IRS-1) and p85 PI3K activates PI3K and its downstream molecules, protein kinase B (PKB(Akt)) and p70S6K (4). Shc, an Src homology 2 domain-containing substrate for receptor tyrosine kinases, interacts directly with IGF-IR (5). After tyrosine phosphorylation of Shc, it recruits the Grb2-Sos complex and activates the Ras/Raf-1/MEK/ERK axis (3). Gab1 functions as an adaptor protein downstream of receptor tyrosine kinases including IGF-IR (6). Tyrosine-phosphorylated Gab1 interacts with Grb2, p85 PI3K, and SHP-2 (7). Thus, the cross-talk among IGF-induced signaling pathways is critical for regulating the variety of biological processes described above.

Recent studies (8) have demonstrated the involvement of IGF-induced signals in the mutually exclusive processes of cell proliferation and differentiation. For instance, pharmacological study showed that although the MAPK pathway induced by IGF-I is involved in the mitogenic response of myoblasts, the PI3K/p70S6K pathway is critical for myogenic differentiation. In hematopoietic cells, IGF-I signaling via IRS-1 plays a role in proliferation and via Shc in differentiation (9). In 3T3-L1 preadipocytes, the IGF-I-induced tyrosine phosphorylation of Shc, Shc-Grb2 complex formation, and ERK activation is observed in proliferating, but not differentiating, cells (10). In osteoblasts, IGF-IR-mediated signals via the PI3K, ERK, and p38 MAPK pathways are all involved in differentiation (11). Thus, the downstream pathways of IGF-I signaling seem to depend on the cellular context. IGF-I is also a potent mitogen and motogen for vascular smooth muscle cells (VSMCs) in vivo and in vitro (12, 13); disruption of the IGF-I/IGF-IR interaction by a neutralizing anti-IGF-I/IGF-IR antibody (14) and suppression of IGF-IR expression by antisense oligonucleotides (15) and transcripts (16) inhibit IGF-I-induced VSMC proliferation and migration in culture. Targeted overexpression of IGF-I in arteries in vivo (17) and infusion of IGF-I into the circulatory system (18) have been shown to accelerate neointimal formation after vascular injury. On the other hand, we recently reported a primary culture system of VSMCs that exhibit a differentiated phenotype, in which isolated VSMCs are cultured on laminin under IGF-I-stimulated conditions (19, 20). By using...
this culture system, we demonstrated that IGF-1 is critically involved in maintaining the differentiated phenotype via the PI3K/PKB(Akt) pathway and that the coordinated activation of ERK and p38 MAPK triggered by other growth factors induces VSMC dedifferentiation. Taking these findings together, we concluded that changes in the balance between the strengths of the PI3K/PKB(Akt) and ERK and p38 MAPK pathways determines the VSMC phenotype. However, none of these studies, including those using VSMCs, revealed the cellular phenotype-dependent switching machinery of the IGF-I anti-ERK, anti-p38 MAPK, anti-IGF-IR, anti-ERK, anti-p38 MAPK, anti-IGF-IR, anti-Ras antibody and then quantified.

**PTPase Assay—**VSMCs cultured under quiescent conditions were stimulated with IGF-I (100 ng/ml) or platelet-derived growth factor-BB (PDGF-BB) (20 ng/ml) for 5 min. The SHP-2 protein in the cell extracts was immunoprecipitated using an anti-SHP-2 antibody. The PTPase activities in the immunoprecipitates were assayed using tyrosine-phosphorylated Raytide as a substrate (19).

**Cell Proliferation and Migration Assays—**VSMCs of both phenotypes were cultured under quiescent conditions for 24 h and then stimulated with the indicated amounts of IGF-I. VSMC proliferation was determined by the incorporation of 5-bromo-2-deoxyuridine (BrUrd) (20 μM) for 20 h. Incorporated BrUrd was visualized by staining with an anti-BrUrd antibody, followed by labeling with secondary antibodies conjugated with Alexa 546 (Molecular Probes) and Hoechst. The migration of differentiated VSMCs (four 1.5-mm diameter fields in each experiment) was monitored for 48 h by a cooled CCD camera (Roper Scientific, Tucson, AZ) mounted on an Olympus IX-70 microscope. The migration of dedifferentiated VSMCs was assayed by wound healing.

**VSMC Phenotype-dependent IGF-I Signaling Pathways—**As demonstrated previously (19, 20), primary cultured VSMCs plated on laminin under IGF-I-stimulated conditions showed the differentiated phenotype, as indicated by a spindle-like cell shape, carbachol (CCH)-induced contractility (Fig. 1A), and high expression of SMC markers, such as h-Cad, CN, and MHC SM2, at the mRNA and protein levels (Fig. 1B). By contrast, passage VSMCs, even when cultured under quiescent conditions, displayed a fibroblast-like shape change (Fig. 1C).
1A), loss of CCH-induced contractility (Fig. 1A), and down-regulation of SMC marker expression in addition to isoform conversion of CaD from the h- to l-form (Fig. 1B), indicating the dedifferentiated phenotype. By using both phenotypes of VSMCs, we first examined the dose-dependent response to IGF-I (Fig. 1, C–E). Consistent with our previous study (20), IGF-I (2 ng/ml) markedly activated PKB(Akt) in differentiated and dedifferentiated VSMCs, and its activation reached a maximum (more than 10-fold activation) at 20 ng/ml IGF-I (Fig. 1C). R3IGF-I, an IGF-I analog lacking affinity for IGF-binding proteins, showed a potency equivalent to IGF-I in activating PKB(Akt) in both phenotypes of VSMCs, at concentrations from 2 to 100 ng/ml (data not shown), suggesting that the IGF-I-induced signaling is IGF-binding protein-independent. The PKB(Akt) activation by IGF-I in both VSMC phenotypes was suppressed by LY294002 (10–30 μM) (Fig. 1D). The p38 MAPK activation by IGF-I was only suppressed by SB203580 (10–20 μM, Fig. 1E) or SB220025 (10–20 μM, data not shown). These results indicate that IGF-I activates distinct VSMC phenotype-dependent signaling pathways.

IGF-I-induced Interactions between Signaling Molecules—Signaling molecules, including IGF-IR, IRS-1, P13K, Gab1, PKB(Akt), Sos, Grb2, Ras, ERK, p38 MAPK, SHP-2, SHPS-1, and Shc, have been shown to be involved in IGF-I signaling in a variety of cells, including VSMCs (1, 3, 14, 16, 17). We compared the expression of these signaling molecules (Fig. 2). The IGF-IR, IRS-1, Shc, Gab1, SHPS-1, and Ras proteins were markedly up-regulated in dedifferentiated VSMCs compared with differentiated VSMCs, whereas ERK, p38 MAPK, p85 PI3K, PKB(Akt), SHP-2, and Grb2 proteins were equally expressed in both VSMC phenotypes. Thus, some signaling molecules show different expression profiles in different VSMC phenotypes.

To elucidate the molecular mechanism involved in VSMC phenotype-dependent IGF-I signalings, we examined the ty-
Rosine phosphorylation and interactions of the signaling molecules. After IGF-I (2 and 100 ng/ml) stimulation, an anti-IGF-IR antibody specifically coimmunoprecipitated two tyrosine-phosphorylated proteins with molecular masses of 97 and 185 kDa in both VSMC phenotypes. We identified these proteins as IGF-IR (97 kDa) and IRS-1 (185 kDa) by using their respective antibodies (Fig. 3, A and D). An anti-IRS-1 antibody also coimmunoprecipitated IGF-IR in an IGF-I dose-dependent manner (data not shown). Tyrosine-phosphorylated IGF-IR and/or IRS-1 formed a complex with p85 PI3K but did not associate with Shc in either VSMC phenotype (Fig. 3, A and B, and D–F). We detected a stable complex of Grb2 and Sos in the anti-Grb2 (Fig. 3C) and anti-Sos (data not shown) immunoprecipitates of both VSMC phenotypes with or without IGF-I stimulation. Immunoblotting of the anti-Grb2 immunoprecipitates with anti-phosphotyrosine, anti-IRS-1, and anti-Sos antibodies showed that a complex of tyrosine-phosphorylated IRS-1 and Grb2/Sos formed in the dedifferentiated, but not in differentiated, VSMCs (Fig. 3C), suggesting that the tripartite complex composed of IRS-1, Grb2, and Sos depends on the IGF-I-induced tyrosine phosphorylation of IRS-1. Immunoblotting of the anti-Sos immunoprecipitates with anti-IRS-1 and anti-Grb2 antibodies showed the same interactions in the IGF-I-stimulated dedifferentiated VSMCs (data not shown). The same tripartite complex was also detected in the anti-IRS-1 immunoprecipitates (Fig. 3B). Neither the IGF-I-induced tyrosine-phosphorylated Shc nor an Shc and IGF-IR or Grb2/Sos interaction was detected in either VSMC phenotype. As a control, PDGF-BB markedly induced tyrosine phosphorylation of Shc and an interaction between Shc and Grb2/Sos (Fig. 3F). The protein recognized by anti-phosphotyrosine antibody in the Shc immunoprecipitates from both phenotypes of VSMC was an IgG heavy chain, because anti-phosphotyrosine antibody cross-reacted with IgG heavy chain in rabbit antibodies (Fig. 3F). Because PDGF-BB or 18:1 lysophosphatidic acid potently induced the tyrosine phosphorylation of Shc in both VSMC phenotypes (Fig. 3F and data not shown), IGF-I signaling in VSMCs is Shc-independent. Thus, our results suggest that tyrosine-phosphorylated IRS-1 preferentially recruits the Grb2-Sos complex in dedifferentiated but not in differentiated VSMCs.

**VSMC Phenotype-dependent Interaction of Tyrosine-phosphorylated IRS-1 and SHP-2**—IRS-1 is phosphorylated on multiple tyrosine residues in response to IGFs. Among them, phosphorylated Tyr-895 of IRS-1 has been identified as a binding site for Grb2 (26). We analyzed the phosphorylation of IRS-1 Tyr-895 by using an anti-IRS-1 Tyr(P)-895 antibody. The IRS-1 Tyr-895 was markedly phosphorylated by IGF-I stimulation only in dedifferentiated VSMCs (Fig. 4A). This is consistent with the IGF-I-induced tripartite complex formation between tyrosine-phosphorylated IRS-1 and Grb2/Sos in dedifferentiated, but not in differentiated, VSMCs (Fig. 3). It is well documented that tyrosine-phosphorylated IRS-1 interacts with SHP-2, the ubiquitous PTPase containing the SH2 domain, leading to the positive or negative regulation of receptor tyrosine kinase-linked events (27–31). IRS-1 has two tyrosine residues for SHP-2 binding, Tyr-1172 and Tyr-1222 (32). We characterized the tyrosine phosphorylation of these SHP-2-binding sites using anti-IRS-1 Tyr(P)-1172 and Tyr(P)-1222 antibodies, respectively. In a preliminary experiment, the former antibody specifically recognized IRS-1 Tyr(P)-1172, but the latter did not recognize Tyr(P)-1222 (data not shown). We therefore analyzed the phosphorylated Tyr-1172 of IRS-1 in IGF-I-stimulated
VSMCs. In response to IGF-I stimulation, IRS-1 Tyr-1172 was significantly phosphorylated in both VSMC phenotypes (Fig. 4A). We then examined the possible involvement of SHP-2 in the VSMC phenotype-dependent interaction between IRS-1 and Grb2/Sos. In both VSMC phenotypes under quiescent and IGF-I-stimulated conditions, almost all of the SHP-2 protein was extractable by nonionic detergents. Notably, IGF-I dose-dependently enhanced the PTPase activity of SHP-2 in differentiated VSMCs, whereas its effect was less significant in dedifferentiated ones. As a control, PDGF-BB enhanced the PTPase activity of SHP-2 in both the VSMC phenotypes (Fig. 4B). The PTPase activities might be mainly originated from the SHP-2 PTPase activity itself, because exogenously expressed Myc-tagged PTPase-dead (dominant-negative) SHP-2 (myc-SHP-2DN) (34) in IGF-I-stimulated differentiated VSMCs showed the low PTPase activity itself, because exogenously expressed Myc-tagged PTPase-dead (dominant-negative) SHP-2 (myc-SHP-2DN) (34) in IGF-I-stimulated differentiated VSMCs showed the low PTPase activity itself. The interaction reached a maximum 5 min after IGF-I stimulation, and decreased thereafter (Fig. 4C). This interaction process coincided well with the progressive changes in phosphorylation of Tyr-1172 (Fig. 4D). Thus, the extent of this interaction in differentiated VSMCs may depend on the phosphorylation of Tyr-1172 and possibly Tyr-1222 in IRS-1. No IRS-1-SHP-2 interaction was detected in dedifferentiated VSMCs, even when IRS-1 Tyr-1172 was phosphorylated by IGF-I stimulation (Fig. 4D), suggesting that an additional inhibitory mechanism of this interaction may exist in dedifferentiated VSMCs. No interactions between SHP-2 and other tyrosine-phosphorylated adaptor proteins, such as SHPS-1, Gab1, and Shc, were detected in either VSMC phenotype, even under the reported assay conditions (33, data not shown). Together, our results suggest that SHP-2 recruited to IRS-1 might rapidly dephosphorylate the IRS-1 Tyr-895 in differentiated VSMCs only.

Regulation of the IRS-1 and Grb2/Sos Interaction by SHP-2—We examined the effects of wild-type and dominant-negative SHP-2 on the IGF-I-induced interaction between IRS-1 and Grb2/Sos. The extracts from IGF-I-stimulated, differentiated, and dedifferentiated VSMCs cotransfected with expression plasmids for FLAG-tagged Grb2 (FLAG-Grb2) and myc-SHP-2wt or myc-SHP-2DN were immunoprecipitated with an anti-FLAG antibody, followed by immunoblotting with an anti-IRS-1 antibody (Fig. 5A). Overexpressed myc-SHP-2DN caused a new interaction between IRS-1 and Grb2, but myc-SHP-2wt did not. These results suggest that overexpressed SHP-2DN competes with endogenously activated SHP-2, leading to induction of the IRS-1 and Grb2/Sos interaction in differentiated VSMCs. In dedifferentiated VSMCs, overexpressed myc-SHP-2DN markedly suppressed the formation of this complex, but myc-SHP-2DN did not. This negative regulation in dedifferentiated VSMCs may be due to dephosphorylation of the IRS-1 Tyr(P)-895 by the overexpressed cytosolic SHP-2wt.

We further examined the IGF-I-induced interaction between Grb2 and HA-tagged wild-type IRS-1 (HA-IRS-1wt) or its SHP-2- (Tyr-1172 and Tyr-1222 replaced with Phe; IRS-1-HA_F1172/F1222) or Grb2 (Tyr-895 replaced with Phe; IRS-1-HA_F895) binding site mutants (Fig. 5B). In differentiated VSMCs, IRS-1-HA_F1172/F1222 interacted with Grb2 in response to IGF-I stimulation, whereas IRS-1-HA wt and IRS-1-HA_F895 did not. Conversely, IRS-1-HA wt and IRS-1-HA_F1172/F1222 in dedifferentiated VSMCs formed a complex with Grb2 but IRS-
HAF895 did not. Grb2 that was associated with IRS-1-HAwt or IRS-1-HAF1172/F1222 also formed a stable complex with Sos (data not shown), indicating a tripartite complex formation among these signaling molecules. Thus, SHP-2 negatively regulates the IRS-1 and Grb2/Sos interaction in a VSMC phenotype-dependent and IRS-1 phosphorylation site-specific manner.

Negative Regulation of ERK and p38 MAPK by SHP-2—To confirm the negative regulation of ERK and p38 MAPK by SHP-2, we cotransfected both phenotypes of VSMCs with expression plasmids for myc-SHP-2 (wt or DN) or IRS-1-HA (wt or indicated mutants) and FLAG-ERK or FLAG-p38 MAPK, and we assayed the ERK and p38 MAPK activities in the anti-FLAG immunoprecipitates (Fig. 6). IGF-I activated ERK and p38 MAPK in differentiated VSMCs expressing myc-SHP-2wt (wt) but not in those expressing myc-SHP-2DN (DN) expressed in differentiated VSMCs. By contrast, IGF-I failed to activate ERK and p38 MAPK in differentiated VSMCs expressing myc-SHP-2wt or IRS-1-HAwt by guest on July 18, 2018http://www.jbc.org/Downloaded from

VSMC phenotype-dependent IGF-I-induced IRS-1 phosphorylation, SHP-2 activation, and their interaction. IGF-I-induced tyrosine phosphorylation of IRS-1 in VSMCs was detected by immunoprecipitation (IP) followed by immunoblot (IB) using indicated anti-IRS-1 antibodies (A). The PTPase activity in the anti-SHP-2 or anti-Myc immunoprecipitates was assayed (B). The values indicate the fold activation; the values obtained in dedifferentiated VSMCs stimulated with IGF-I (2 ng/ml) were set at 1.0. The open and closed bars indicate the SHP-2 activities in differentiated (D) and dedifferentiated (DD) VSMCs, respectively (left panel). IGF-I-induced PTPase activities of myc-SHP-2wt (wt) and myc-SHP-2DN (DN) expressed in differentiated VSMCs were compared (right panel). The PTPase activity of myc-SHP-2wt was set at 100%. The SHP-2 protein contents are shown below each graph. Progressive changes in the tyrosine-phosphorylated IRS-1 and SHP-2 interaction were characterized (C). The extracts from VSMCs stimulated with IGF-I (100 ng/ml) were immunoprecipitated by using an anti-SHP-2 or anti-IRS-1 antibody. The target proteins in the immunoprecipitates were detected by immunoblot (left panels). Double asterisks indicate tyrosine-phosphorylated IRS-1. The tyrosine phosphorylation (pTyr) of IRS-1 was quantified by densitometry (right graph). Open and closed bars indicate the results in differentiated and dedifferentiated VSMCs, respectively. These are representative results from three separate experiments, and each value represents the mean ± S.D. of three independent experiments. Progressive changes in the IGF-I (100 ng/ml)-induced tyrosine phosphorylation of Tyr-1172 of IRS-1 in both phenotypes of VSMC were characterized (D). Representative results from two separate experiments are shown.
ERK and p38 MAPK in dedifferentiated VSMCs expressing myc-SHP-2 wt or IRS-1-HA wt, but barely affected either MAPK in dedifferentiated VSMCs expressing myc-SHP-2DN, IRS-1-HAwt, or IRS-1-HAF1172/F1222. Thus, the VSMC phenotype-dependent interaction between IRS-1 and Grb2/Sos, which is negatively regulated by SHP-2, is critical for the IGF-I-induced activation of both MAPKs.

Ras as an Upstream Effector for the IGF-I-induced Activation of ERK and p38 MAPK—Although the Ras/Raf/MEK/ERK cascade is well known in various cell types (35), the pathway from Ras to p38 MAPK, however, remains unclear. Our present results suggest that Ras may be involved in the IGF-I-induced activation of both ERK and p38 MAPK in dedifferentiated VSMCs. We monitored the IGF-I-induced Ras activation with a

ERK and p38 MAPK in dedifferentiated VSMCs expressing myc-SHP-2 wt or IRS-1-HA wt, but barely affected either MAPK in dedifferentiated VSMCs expressing myc-SHP-2DN, IRS-1-HA wt, or IRS-1-HAF1172/F1222. Thus, the VSMC phenotype-dependent interaction between IRS-1 and Grb2/Sos, which is negatively regulated by SHP-2, is critical for the IGF-I-induced activation of both MAPKs.

Ras as an Upstream Effector for the IGF-I-induced Activation of ERK and p38 MAPK—Although the Ras/Raf/MEK/ERK cascade is well known in various cell types (35), the pathway from Ras to p38 MAPK, however, remains unclear. Our present results suggest that Ras may be involved in the IGF-I-induced activation of both ERK and p38 MAPK in dedifferentiated VSMCs. We monitored the IGF-I-induced Ras activation with a
 RAF-1 binding assay. Consistent with the kinase activities (Fig. 1C) and interactions between signaling molecules (Fig. 3), IGF-I dose-dependently activated Ras in dedifferentiated, but not differentiated, VSMCs (Fig. 7A). This activation was transient; Ras in dedifferentiated VSMCs was maximally activated at 5–10 min after IGF-I stimulation and the activation decreased thereafter. By contrast, IGF-I (2–100 ng/ml) maximally activated ERK and p38 MAPK for 10 min, and their activities remained high 4 h after stimulation (Fig. 7B). Thus, the transient activation of Ras induced by IGF-I led to rapidly rising and falling activities of both MAPKs, but the activities remained above the basal levels even 4 h after stimulation. To address the direct involvement of Ras in the IGF-I-induced MAPK activation in dedifferentiated VSMCs, we cotransfected differentiated and dedifferentiated VSMCs with ΔmSos (36), RasN17, or RasV12 (37), and FLAG-ERK or FLAG-p38 MAPK, and we assayed for the ERK and p38 MAPK activities in the anti-FLAG immunoprecipitates. ΔmSos or RasN17 markedly suppressed the IGF-I-induced activation of both MAPKs; RasN17 inhibited both MAPK activities to less than basal levels, whereas ΔmSos protein did them to nearly the basal levels (Fig. 7C). In contrast, their activities were greatly enhanced by RasV12 without IGF-I stimulation (Fig. 7C). Thus, the Grb2/Sos/Ras cascade induced by IGF-I activates both ERK and p38 MAPK in dedifferentiated VSMCs.

**Relationships between IGF-I Signaling Pathways and Biological Actions of IGF-I on Distinct Phenotypes of VSMCs**—To reveal the relationship between VSMC phenotype-dependent IGF-I signaling and its biological actions, we analyzed the IGF-I-induced cell proliferation and migration. Even under IGF-I-stimulated conditions, differentiated VSMCs never underwent cell proliferation, as monitored by BrdUrd incorporation (Fig. 8A) or migration (Fig. 8B), suggesting that IGF-I acts solely to maintain the differentiated phenotype but not as a mitogen or motogen. Conversely, IGF-I dose-dependently enhanced the proliferation and migration of dedifferentiated VSMCs (Fig. 8, A and B). Treatment with either PD98059 (30 μM) or SB203580 (20 μM) partially inhibited the BrdUrd incorporation and migration, but simultaneous treatment with both inhibitors completely suppressed them (Fig. 8, A and B), suggesting that...
these biological actions depend on the IGF-I-induced activation of ERK and p38 MAPK. We further analyzed the effects of the IGF-I-dependent interaction between IRS-I and Grb2/Sos on the BrdUrd incorporation and migration of dedifferentiated VSMCs by overexpressing IRS-1-HAwt, IRS-1-HA\textsubscript{F895}, or IRS-1-HA\textsubscript{P1172G/1222}. Dedifferentiated VSMCs expressing IRS-1-HA\textsubscript{F895} showed markedly less BrdUrd incorporation and migration compared with those expressing either IRS-1-HA\textsubscript{wt} or IRS-1-HA\textsubscript{P1172G/1222} (Fig. 9). These results indicate that the negative regulation of ERK and p38 MAPK by blockade of the IRS-1 and Grb2/Sos interaction results in inhibition of the proliferation and migration of dedifferentiated VSMCs.

**DISCUSSION**

Several studies have suggested that IGF-I is a potent mitogen and motogen for VSMCs, because IGF-I-induced activation of PI3K and/or MAPKs is involved in the proliferation and migration of VSMCs (12, 13). Conversely, we demonstrated by using our VSMC culture system that IGF-I plays a critical role in maintaining the differentiated phenotype of VSMCs via the PI3K/PKB(Akt), but not the ERK or p38 MAPK, pathway (19, 20). Here we demonstrate the distinct upstream signaling pathways, from IGF-IR to PI3K/PKB(Akt) and MAPKs, in different VSMC phenotypes and that the inconsistency regarding the distinct biological actions of IGF-I on VSMCs is because of the different VSMC phenotypes used. IGF-I potently activated ERK and p38 MAPK, via IRS-1-associated Grb2/Sos and Ras only in dedifferentiated VSMCs (Figs. 1 and 5–7), resulting in induction of the VSMC proliferation and migration (Figs. 8 and 9). Previous in vivo studies demonstrated that targeted overexpression of IGF-I in VSMCs (17) or IGF-I infusion into the circulatory system (18) enhances neointimal formation after vascular injury. Based on our present results, these in vivo observations might be explained by the idea that once VSMCs are primed by dedifferentiation stimuli such as vascular injury, IGF-I might further accelerate the proliferation and migration of dedifferentiated VSMCs via the signaling pathway shown in Fig. 10. In differentiated VSMCs, IRS-1-associated SHP-2 negatively regulates the activation of both ERK and p38 MAPK (Fig. 6). It has been reported that a variety of PTPases are involved in the
positive and negative regulation of signal transduction via receptor tyrosine kinases (38). For instance, CD45 positively regulates the T-cell antigen receptor signaling in lymphocytes (39). In hematopoietic cell lines, SHP-1 negatively regulates mitogenic signals through the colony-stimulating factor 1 and stem-cell factor receptors (40). SHP-2 can modulate receptor tyrosine kinase-mediated signaling in a positive or negative manner. Positive regulation by SHP-2 has been reported in mitogenic signaling through the insulin receptor (27, 29). However, SHP-2 also negatively regulates insulin receptor-mediated mitogenic signaling (31) and gp130-dependent transcriptional activation (30). Our present study demonstrates that SHP-2 is critical for the VSMC phenotype-dependent regulation of IGF-I signaling; in differentiated VSMCs, an IRS-1/SHP-2 interaction enhances the PTPase activity of SHP-2 (Fig. 4) and suppresses the recruitment of Grb2/Sos to IRS-1 by the dephosphorylation of IRS-1 Tyr-895, resulting in inhibition of the activation of both MAPKs (Figs. 5 and 6). In contrast, SHP-2 remains inactive in IGF-I-stimulated dedifferentiated VSMCs (Fig. 4). The quiescence of SHP-2 PTPase activity in dedifferentiated VSMCs is a specific feature of the IGF-I signaling, given that PDGF-BB markedly enhanced the SHP-2 activity in dedifferentiated VSMCs (Fig. 4), and SHP-2 never interacted with signaling molecules such as IRS-1 (Fig. 4), SHPS-1, Gab1, or Shc (data not shown) in the IGF-I-stimulated dedifferentiated VSMCs. Maile and Clemmons (41) have reported that SHP-2 in passaged porcine VSMCs associates with SHPS-1, IGF-IR, and IRS-1 in response to IGF-I stimulation, and these interactions depend on integrin-associated protein. It is reasonable to consider that the pathway via SHPS-1 might not function in differentiated VSMCs, given that no integrin-associated protein expression is detected in differentiated VSMCs in vivo (42). Our results using dedifferentiated VSMCs also showed this pathway to be silent. Presently, the reasons for this discrepancy remain unknown. The IRS-1 Tyr-1172 was significantly phosphorylated in IGF-I-stimulated dedifferentiated VSMCs. However, the IRS-1/SHP-2 interaction is not detected in dedifferentiated VSMCs despite such a tyrosine phosphorylation state of IRS-1 (Fig. 4). The IGF-I-induced interaction between IRS-1 and Grb2/Sos and the activation of ERK and p38 MAPK are markedly inhibited in dedifferentiated VSMCs overexpressing SHP-2wt (Figs. 5 and 6). These results suggest that in dedifferentiated VSMCs, the function of SHP-2 would be blocked by interacting with other tyrosine-phosphorylated proteins. Therefore, excess SHP-2wt might overcome such trapping of endogenous SHP-2 or dephosphorylate IRS-1 Tyr-895, resulting in blockade of the interaction between IRS-1 and Grb2/Sos.

Ras is a key transducer of mitogenic signaling in many cell types, including VSMCs, in vitro and in vivo. It is well documented that Ras activates ERK in a variety of cells, whereas stress-activated protein kinases, including p38 MAPK,
poorly activated by the Ras-coupled cascade (35). We now demonstrate that the activation of Ras functions as an upstream effector for both ERK and p38 MAPK in IGF-I-stimulated dedifferentiated VSMCs (Fig. 7). However, unlike the ERK pathway mediated through Ras/Raf/MEK, the p38 MAPK pathway might be indirectly affected by Ras, because IGF-I dose-responsive activation rates of both MAPKs were slightly different (Fig. 1, D and E). It has been reported that in some cell lines, including myoblast lines PKB(Akt), phosphorylate Raf, leading to suppression of the Ras/Raf/MEK/ERK axis (43). In our present study by using both phenotypes of VSMCs, treatment with PI3K inhibitor never increased the ERK activation by IGF-I in our preliminary experiment by using both phenotypes of VSMCs, treatment with PI3K inhibitor never increased the ERK activation by IGF-I (Fig. 1), suggesting that the PKB(Akt)-mediated negative regulation of the ERK pathway may not be involved. Our present study provides a novel insight into the signal transduction in VSMCs and into the pharmacological approaches that might be applicable for preventing vascular disorders such as atherosclerosis and restenosis. As described in the Introduction, IGF-I signal affects the mutually exclusive biological actions, such as proliferation and differentiation of hematopoietic cells, adipocytes, myoblasts, and osteoblasts. In these cells, the signaling pathways analogous to those of VSMCs (Fig. 10) may be involved in the distinct biological actions of IGF-I. In our preliminary experiments, we have identified the similar pathways regulating the IGF-I-induced proliferation and differentiation of myogenic cells. Thus, the IGF-I-induced pathways demonstrated here appear to represent a novel mechanism regulating the discriminating signals involved in cell proliferation and differentiation, not just in VSMCs, but in other cells as well.

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