0888-8809/05/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 19(1):1–11

doi: 10.1210/me.2004-0376

MINIREVIEW

Interaction between Insulin-Like Growth Factor-I Receptor and $\alpha V\beta 3$ Integrin Linked Signaling Pathways: Cellular Responses to Changes in Multiple Signaling Inputs

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Integrins are heterodimeric transmembrane proteins that mediate cell attachment to extracellular matrix, migration, division, and inhibition of apoptosis. Because growth factors are also important for these processes, there has been interest in cooperative signaling between growth factor receptors and integrins. IGF-I is an important growth factor for vascular cells. One integrin, $\alpha V\beta 3$, that is expressed in smooth muscle cells modulates IGF-I actions. Ligand occupancy of $\alpha V\beta 3$ is required for IGF-I to stimulate cell migration and division. Src homology 2 containing tyrosine phosphatase (SHP-2) is a tyrosine phosphatase whose recruitment to signaling molecules is stimulated by growth factors including IGF-I. If $\alpha V\beta 3$ ligand occupancy is inhibited, there is no recruitment of SHP-2 to $\alpha V\beta 3$ and its transfer to downstream signaling molecules is blocked. Ligand occupancy of $\alpha V\beta 3$ stimulates tyrosine phosphorylation of the $\beta 3$-subunit, resulting in recruitment of SHP-2. This transfer is mediated by an insulin receptor substrate-1-related protein termed DOK-1. Subsequently, SHP-2 is transferred to another transmembrane protein, SHPS-1. This transfer requires IGF-I receptor-mediated tyrosine phosphorylation of SHPS-1, which contains two YXXL motifs that mediate SHP-2 binding. The transfer of SHP-2 to SHPS-1 is also required for recruitment of Shc to SHPS-1. Ligand occupancy of $\alpha V\beta 3$ results in sustained Shc phosphorylation and enhanced Shc recruitment. Shc activation results in induction of MAPK. Inhibition of the Shc/SHPS-1 complex formation results in failure to achieve sustained MAPK activation and an attenuated mitogenic response. Thus, within the vessel wall, a mechanism exists whereby ligand occupancy of the $\alpha V\beta 3$ integrin is required for assembly of a multicomponent membrane signaling complex that is necessary for cells to respond optimally to IGF-I. (*Molecular Endocrinology* 19: 1–11, 2005)

The IGF-I receptor is ubiquitously present on multiple cell types, and IGF-I receptor numbers vary minimally among various cell types (*e.g.* 25–40,000 receptors per cell). These properties account for the balanced growth stimulation among tissues that occurs in response to increases in GH. Studies in mice have shown that in addition to functioning as a classic endocrine hormone (*e.g.* liver synthesized IGF-I is transported to the peripheral tissues and stimulates growth), IGF-I that is synthesized locally in peripheral tissues is also required for normal growth (1). In addition to its role in regulating normal systemic growth, autocrine/paracrine secreted IGF-I is an important component of the response to injury. Multiple studies in several different animal models have shown that after injury IGF-I is synthesized by the cell types that account for tissue regeneration, and that its synthesis is necessary for normal tissue repair (2, 3). Abnormal stimulation of secretion or altered tissue sensitivity to autocrine/paracrine IGF-I is believed to be involved in several disease processes, including atherosclerosis and angiogenesis (4, 5). The ability of cells to respond to this autocrine/paracrine secreted IGF-I is dependent not only on the amount of growth factor that is secreted but also on the state of cellular differentiation

First Published Online November 4, 2004

Abbreviations: ECM, Extracellular matrix; EGF, epidermal growth factor; FAK, focal adhesion kinase; FGF, fibroblast growth factor; IAP, integrin-associated protein; IGFBP, IGF binding protein; IRS-1, insulin receptor substrate-1; PDGF, platelet-derived growth factor; PI-3, phosphatidylinositol-3; PTB, phosphotyrosine binding; RGD, arginine-glycine-asparginine; SHP-2, Src homology 2 containing tyrosine phosphatase; SMC, smooth muscle cells; TS-1, thrombospondin-1; VEGF, vascular endothelial growth factor.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

and the abundance of other proteins in the extracellular environment that modulate cellular responses (6). In analyzing IGF-I-related autocrine/paracrine growth stimulation, major attention has been focused on the IGF binding proteins (IGFBPs) because of their capacity to bind to IGF-I with high affinity (7, 8).

In addition to IGFBPs, extracellular matrix (ECM) proteins have been shown to play a role in modulating cellular responses to IGF-I (9, 10). Thus, ECM proteins, such as types I and IV collagen, fibronectin, thrombospondin-1 (TS-1), and osteopontin have been shown to modulate the response of various cell types to IGF-I stimulation (9–12). Changes in the abundance of these proteins have been shown not only to modify cellular adherence that can alter IGF-I signaling but also to actively stimulate signal transduction through their binding to a specific class of cell surface receptors, termed integrins. Integrins are heterodimers that consist of one α- and one β-subunit (13). Vertebrates express 18 different α-subunits and eight β-subunits. These subunits assemble into 24 distinct integrin heterodimers. Generally, the types of heterodimers that are expressed and their relative abundance varies among different cell types. This review will focus on the response of vascular smooth muscle cells (SMC) to IGF-I and the role that ligand occupancy of smooth muscle cell integrins plays in modulating this response.

INTEGRIN ACTIVATION

Vascular smooth muscle has been shown to express $\alpha1\beta1$, $\alpha5\beta1$, $\alpha V\beta3$, $\alpha2\beta1$, $\alpha3\beta1$, $\alpha5\beta1$, $\alpha V\beta5$, and $\alpha1\beta6$ integrins (14). In SMC, integrins have been shown to be important mediators of cell adhesion, migration, ECM assembly, ECM contraction, and cellular replication (15). Integrin ligand occupancy can also regulate the state of cellular differentiation as well as the ability of cells to resist apoptosis (13, 16). Unlike tyrosine kinase-containing receptors, such as the IGF-I receptor, integrins do not contain intrinsic tyrosine kinase activity. In response to ligand occupancy, integrins signal through alternative mechanisms. These involve a conformational change in the integrin that results in a change in its activation state. The change in activation has been assessed by showing evidence of polymerization, clustering, or the surface exposure of different antibody binding epitopes (14, 15). Similarly, phosphorylation of key residues within the cytoplasmic domains of the β-subunit is often associated with binding of specific proteins to integrins in response to changes in ligand occupancy (17). Integrin cytoplasmic domains can bind constitutively to cytoskeletal components such as talin, so that changes in integrin conformation and activation can result in changes in cytoskeletal protein function. Changes in talin binding to integrin cytoplasmic tails results in a reorganization of actin and myosin filaments, and this leads to major changes in cell shape as well as locomotion (18). After conformational changes or phosphorylation of specific residues, integrin cytoplasmic domains bind to signaling molecules, and the display of signaling molecules can change as a function of integrin activation (19). Signaling molecules can assemble into multicomponent signaling complexes that have been shown to include small GTPase proteins such as, Rho, Rac, and Cdc42 as well as components of the MAPK pathway such as Shc and Grb-2 (20). Tyrosine kinases such as focal adhesion kinase (FAK) and Src and proteins that have been shown to modify the activity of these kinases, such as p130 CAS, also bind to activated integrins (21). The extracellular domains of integrins also have been shown to bind to specific proteins that can modulate integrin function. These include the urokinase type plasminogen activator and receptor, integrin-associated protein (IAP), and tetraspanin or CD9 (22–24). Direct integrin activation either by stimulatory ligands or changes in ion concentrations such as calcium or magnesium has been shown to activate protein complex assembly, which can result in activation of an integrin-linked signaling pathway (outside in signaling) (25). For example, the phosphatidylinositol-3 (PI-3) kinase pathway can be activated in this manner. It is important to point out, however, that most experiments wherein outside in signaling was used to stimulate integrin activation do not control for changes in ligand occupancy of the growth factor receptors so that the contribution role of growth factor receptor occupancy in regulating integrin activation is unknown.

DIRECT INTERACTIONS OF INTEGRINS AND GROWTH FACTOR RECEPTORS INFLUENCE SIGNALING

Although examples of activation of growth factor receptors by integrins in the absence of the growth factor ligands exist [*e.g.* direct activation of the $\alpha V\beta1$ integrin after cell attachment has been shown to result in activation of the epidermal growth factor (EGF) receptor in the absence of EGF binding] (26), the paradigm that has been most extensively evaluated is the direct association between integrins and growth factor receptors in response to growth factor stimulation. Stimulation of the $\alpha V\beta3$ integrin on endothelial and/or SMC showed that this integrin could associate with platelet-derived growth factor (PDGF), insulin, or vascular endothelial growth factor (VEGF) receptors (27, 28). Similarly, the EGF receptor has been shown to interact directly with $\alpha5\beta1$ and $\alpha6\beta4$ integrins (29). In general, direct activation of the EGF receptor by integrins (in the absence of EGF) requires integrin-mediated clustering such as that which occurs in response to integrin activation by ECM proteins after cell attachment. Direct physical association of IGF-I receptors and integrins has been demonstrated, although it is

cell-type specific. Coprecipitation of the IGF-I receptor and the α6 integrin after dual ligand stimulation was demonstrated for lens epithelial cells (30) and for $\alpha5\beta1$ or $\alpha1\beta1$ integrins and the IGF-I receptor in chondrocytes (31).

INDIRECT ACTIVATION OF INTEGRINS BY GROWTH FACTOR STIMULATION

Examples of growth factor receptor-induced activation of integrins via signaling intermediates also exist. Stimulation of the VEGF receptor has been shown to result in activation of signaling through $\alpha V\beta3$ and $\alpha V\beta5$ (32). Similarly, TGFβ binding to its receptor has been shown to result in enhanced expression of $\alpha5\beta1$ integrin on cell surfaces (33). IGF-I has been shown to enhance the affinity for $\alpha V\beta3$ integrin for ligands without change in receptor number (34). Ligand occupancy of the hepatocyte growth factor receptor results in phosphorylation of the cytoplasmic domain of β4, which results in Shc and PI-3 kinase recruitment (35). Hepatocyte growth factor receptor occupancy can also induce integrin clustering, thus resulting in changes in signaling complex assembly. Therefore, there are multiple ways in which dual activation of growth factor receptors and integrins may cooperatively interact to enhance cellular responses.

INTERACTIONS OF INTEGRIN AND GROWTH FACTOR RECEPTOR SIGNALING PATHWAYS

In addition to these direct interactions among membrane proteins, downstream components of integrin or growth factor receptor-linked signaling pathways have been shown to interact, resulting in dual activation of important intermediary signaling molecules. These interactions between downstream signaling pathway components have been shown to occur in the cytoplasm or within specifically localized membrane fractions. The latter interactions are often the result of binding to scaffolding proteins that are localized within membrane subdomains, and this results in the assembly of specific signaling complexes. It is the assembly of this specific group of proteins that results in transmission of a unique biological signal that is dependent on changes in integrin ligand occupancy as well as growth factor receptor ligand occupancy. One example of this type of signaling localization occurs within membrane rafts that are cholesterol-rich subdomains of the plasma membrane (36). Only proteins with specific modifications, such as palmytoylation, can enter rafts. Assembly of signaling complexes within rafts, therefore, is dependent on the transport of signaling intermediates into and out of rafts, a process that is both protein and domain specific. For example, PDGF receptor localization within lipid rafts of oligodendrocytes is dependent on ligand occupancy of the $\alpha6\beta1$ integrin by laminin (37). This localization is required for PDGF to stimulate cell proliferation because it allows the recruitment of PI-3 kinase, AKT and FAK to rafts after PDGF exposure. Alternatively, specific signaling complexes can be assembled in association with integrin cytoplasmic tails that are not dependent on their localization to specific membrane compartments. For example, FAK activation after changes in integrin conformation leads to the association of a Src family kinase, p130 CAS and PI-3 kinase, resulting in an enhancement in the ability of growth factors to activate PI-3 kinase when it is associated with this complex.

$\alpha V\beta3$ INTEGRIN AND GROWTH FACTOR RECEPTOR COOPERATIVE INTERACTION IN VASCULAR CELLS

Plating cells on various matrices has been the classic way of stimulating integrin activation, and it has been used to activate signaling proteins that are also activated by ligand-induced stimulation of growth factor receptors. Plating fibroblasts on fibronectin was shown to increase the expression of IRS-1 (38). Similarly, plating cells on vitronectin-rich matrix increased the association of FAK and IRS-1, resulting in enhanced IRS-1 phosphorylation (39). Plating chondrocytes on type I or type II collagen also resulted in association of FAK with $\beta1$ integrins and IGF-I-induced greater Shc expression in chondrocytes plated on type II collagen (27). Plating breast cancer cells on thrombospondin resulted in enhancement of $\alpha3\beta1$ integrin activity in response to IGF-I (40). Similarly, increased FAK activation in response to IGF-I has been shown in cells that are plated on specific combinations of integrins.

COOPERATIVE INTERACTION BETWEEN $\alpha V\beta3$ INTEGRIN AND IGF-I RECEPTOR SIGNALING

In stably attached cells, the $\alpha V\beta3$ integrin is capable of binding a variety of ligands that are present in vascular tissue such as osteopontin, thrombospondin, and vitronectin. Changes in ligand occupancy of $\alpha V\beta3$ have been shown to directly influence fibroblast growth factor (FGF) signaling in endothelial cells and IGF-I signaling in SMC. Disruption of the ligand occupancy of $\alpha V\beta3$ with a specific monoclonal antibody, LM609, has been shown to inhibit FGF-induced signaling in vascular endothelium and to inhibit FGF-stimulated angiogenesis (41). Similarly, exposure of human SMC to this antibody was shown to inhibit IGF-I-stimulated cell migration. The importance of $\alpha V\beta3$ ligand occupancy was further supported in studies in which exposure of SMC to disintegrin antagonists, such as echistatin, a small peptide that binds directly to $\alpha V\beta3$

and inhibits vitronectin-stimulated $\alpha V\beta 3$ functions, resulted in blocking IGF-I-stimulated SMC migration and division (34). The importance of these observations was reinforced in *in vivo* studies wherein it was shown that infusion of echistatin into developing atherosclerotic lesions in a pig model resulted in inhibition of IGF-I-stimulated IGFBP-5 synthesis and attenuation of lesion formation (42). That blocking $\alpha V\beta 3$ ligand occupancy was altering IGF-I signaling was shown by studies in which it was determined that exposure to echistatin resulted in a reduced ability of IGF-I to stimulate IGF-I receptor phosphorylation and attenuated activation of IRS-1 and PI-3 kinase by the IGF-I receptor (43). In contrast, antagonism of $\alpha 5\beta 1$ receptor ligand occupancy resulted in no change in these parameters. These findings were confirmed using the specific anti-$\alpha V\beta 3$ integrin monoclonal antibody. Unlike some other cell types, direct association between the $\alpha V\beta 3$ integrin and the IGF-I receptor in SMC has not been demonstrated after IGF-I stimulation.

$\alpha V\beta 3$ INTEGRINS AND SHP-2 PHOSPHATASE

In addition to its ability to enhance activation of IGF-I receptor tyrosine kinase in response to IGF-I, $\alpha V\beta 3$ integrin ligand occupancy has also been shown to regulate the activity of protein tyrosine phosphatases. These enzymes dephosphorylate tyrosine residues, and these dephosphorylation reactions can result in either activation or inactivation of growth factor signaling complexes. Blocking ligand occupancy of the $\alpha V\beta 3$ integrin was shown initially to result in aberrant transfer of Src homology 2 containing tyrosine phosphatase (SHP-2) to the IGF-I receptor (44). Transfer of this phosphatase to the receptor normally occurs 20 min after IGF-I stimulation, causing a progressive decrease in the amount of tyrosine receptor phosphorylation and subsequent attenuation of MAPK and PI-3 kinase activation. In the presence of the disintegrin echistatin, however, SHP-2 was transferred to the receptor 5 min after exposure to IGF-I causing premature dephosphorylation, and attenuation of IGF-I receptor mediated downstream signaling (45). Because others have shown that sustained growth factor receptor and MAPK activation is required for an optimal cell growth response (46, 47), this is one potential mechanism by which exposure to the disintegrin echistatin could result in attenuation of IGF-I signaling and biological actions. These findings strongly suggest that IGF-I signaling is modulated by the cooperative interaction of ligand-activated $\alpha V\beta 3$ and IGF-I receptor-linked signaling events that work in concert to extend the duration of IGF-I receptor activation by regulating the translocation of SHP-2 phosphatase.

To better understand how failure to transfer SHP-2 results in attenuated MAPK and PI-3 kinase signaling, it is useful to review the classical IGF-I receptor-linked signaling pathway. The IGF-I receptor is a heterotetramer that consists of two α-subunits that contain the ligand binding domains and two β-subunits that contain the tyrosine kinase activity. After ligand binding, the receptor undergoes a conformational change resulting in the activation of the tyrosine kinase, which results in transphosphorylation of the opposite β-subunit on specific tyrosine residues. These phosphotyrosines then bind to adaptor molecules such as Shc and IRS-1 (Fig. 1). Phosphorylation of these proteins has been shown to lead to activation of the PI-3 kinase and MAPK signaling pathways (48). After IRS-1 association and phosphorylation, it binds to the p85 regulatory subunit of PI-3 kinase, which recruits the p110 catalytic subunit to the plasma membrane resulting in activation of its enzymatic activity. MAPK activation can result from IRS-1 recruitment of Grb2 and son of sevenless to the plasma membrane resulting in activation of Ras/Raf signaling, which is followed by MAPK kinase (MEK) and MAPK activation or alternatively this pathway can be activated by phosphorylation of Shc, which then recruits Grb2, thus resulting in Ras activation. In SMC, inhibitor studies have shown that activation of PI-3 kinase is absolutely required for stimulation of cell migration and contributes to full activation of cellular proliferation (49). Similarly, although the role of MAPK is predominant for stimulating cellular proliferation, it also plays a role in activating cell migration principally through activating small GTPase proteins such as Rac or Rho that are necessary for full activation of cell motility (50).

INCREASES IN LIGAND OCCUPANCY OF $\alpha V\beta 3$ ENHANCE IGF-I SIGNALING AND ACTIONS IN SMOOTH MUSCLE CELLS

Under usual conditions, there is some constitutive activation of $\alpha V\beta 3$ due to the availability of ligands. At high culture density, there is abundant ligand available, and this is reflected in increased constitutive activation, *e.g.* tyrosine phosphorylation of the $\beta 3$-subunit of the $\alpha V\beta 3$ is enhanced (51). The addition of an increased amount of a soluble ligand such as vitronectin results in further enhancement of $\beta 3$ phosphorylation. If purified vitronectin is added to low-density cultures in which little basal $\beta 3$ phosphorylation can be detected, phosphorylation of the $\beta 3$-subunit is immediately stimulated and this is sustained for at least 2 h after vitronectin exposure. Two potential tyrosine phosphorylation sites (*i.e.* 773 and 785) are located within the cytoplasmic domain of the $\beta 3$-subunit. One of the tyrosines, 773, is contained within an NPXY motif making it capable of binding to phosphotyrosine binding (PTB) domain-containing proteins (52). Activation of these tyrosines by ligand occupancy results in sustained MAPK activation and enhanced SMC growth in response to IGF-I. The importance of tyrosine phosphorylation of the $\beta 3$-subunit for IGF-I signaling was confirmed by preparing cells in which

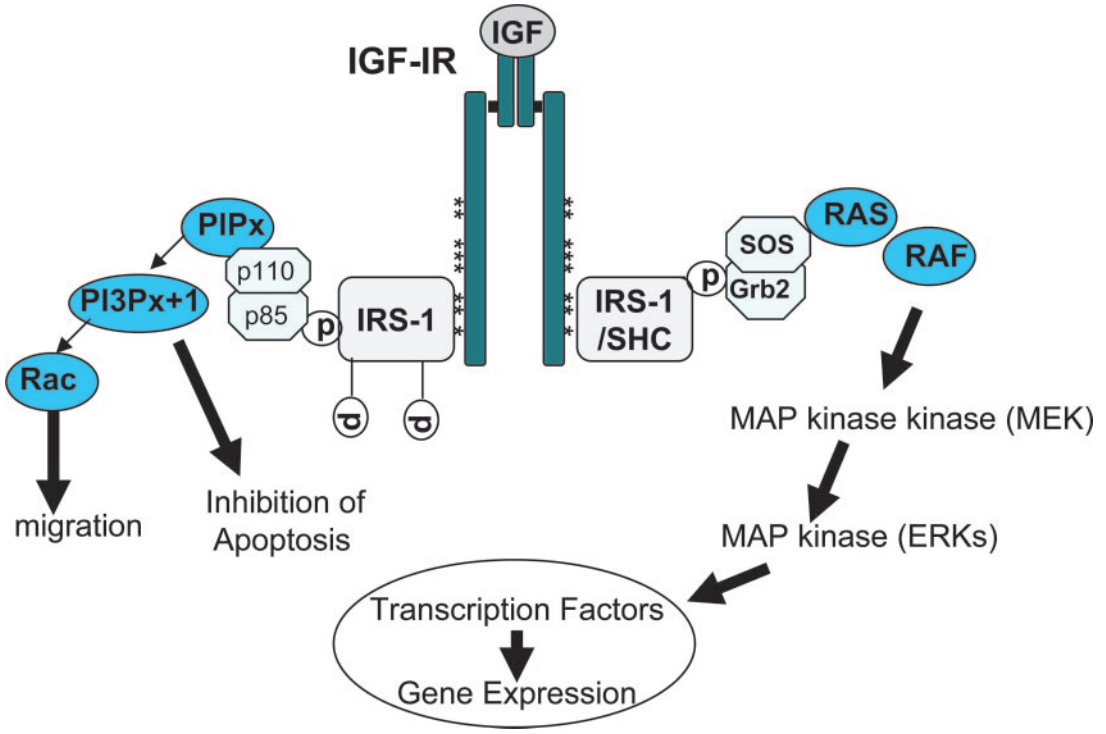

Fig. 1. IGF-I Receptor-Linked Signaling in SMC

Activation of PI-3 kinase pathway through IRS-1 is required for optimal stimulation of cell migration. Activation of the MAPK pathway proceeds through Shc, not IRS-1, in dedifferentiated SMC in response to IGF-I. MAPK activation is required for optimal stimulation of cell division.

these two tyrosines were mutated to phenylalanine. Cells expressing the mutant did not recruit SHP-2 to the plasma membrane or to downstream signaling molecules (51). Furthermore, these cells did not proliferate or migrate in response to IGF-I. MAPK was activated, but the response to IGF-I was not sustained. This effect is specific for tyrosine 773 because blocking SHP-2 transfer to that specific site resulted in impaired IGF-I activation of MAPK (53).

The importance of SHP-2 transfer to β3 for IGF-I signaling was confirmed by exposure of cells to a SHP-2/β3 blocking peptide. Inhibition of SHP-2 transfer resulted in attenuation of IGF-I stimulated PI-3 and MAPK activation as well as inhibition of IGF-I stimulated SMC activation and proliferation. Therefore, ligand-induced stimulation of β3 phosphorylation leads to SHP-2 transfer to the plasma membrane, and this event is dependent on β3 tyrosine phosphorylation.

Binding of Activated SHP-2 to αVβ3 Is Mediated by the Adapter Protein DOK-1

The transfer of SHP-2 to the β3-subunit also requires the activation of αVβ3 or the IGF-I receptor. SHP-2 binds to phosphorylated β3. It is bound to β3 basally in high-density cultures that have constitutive β3 phosphorylation. In low-density cultures, the addition of either vitronectin or IGF-I results in stimulation of β3 phosphorylation and subsequent recruitment of SHP-2 to phosphorylated β3 (51). Although SHP-2 binding to β3 is tyrosine phosphorylation-dependent, SHP-2 does not contain a PTB domain, and therefore it is incapable of binding directly to β3. Moreover, stimulation of β3 phosphorylation alone is not adequate to confer SHP-2 transfer to β3. An intracellular protein that contains a PTB domain, DOK-1, has been shown to bind directly to the tyrosine phosphorylated NPXY sequence in β3 (52). In addition to its PTB domain, DOK-1 also contains multiple YXXL motifs within its C-terminal domain. When these tyrosines are phosphorylated, they are capable of binding SH-2 domain containing proteins. We have determined that one of these motifs (Y^{337}XXL) mediates SHP-2 association with DOK-1 (53). After exposure of SMC cultures to IGF-I, DOK-1 is tyrosine phosphorylated and binds to SHP-2. Exposure of SMC to a cell-permeable peptide that contained this Y^{337}XXL sequence inhibited SHP-2 binding to DOK-1. Similarly, expression of a mutant DOK-1 that had tyrosine 337 substituted with phenylalanine eliminated SHP-2 binding. More importantly, each of the manipulations inhibited SHP-2 transfer to β3, suggesting that DOK-1 was mediating SHP-2/β3 association (Fig. 2A). To confirm that DOK-1 could mediate SHP-2 transfer to β3, we prepared a mutant form of DOK-1 in which arginines 207 and 208 (the residues that had been shown to mediate β3 binding) were substituted with alanines. The mutant DOK-1 did not associate with β3 and did not transfer SHP-2 to β3 after IGF-I stimulation. Therefore, inhibi-

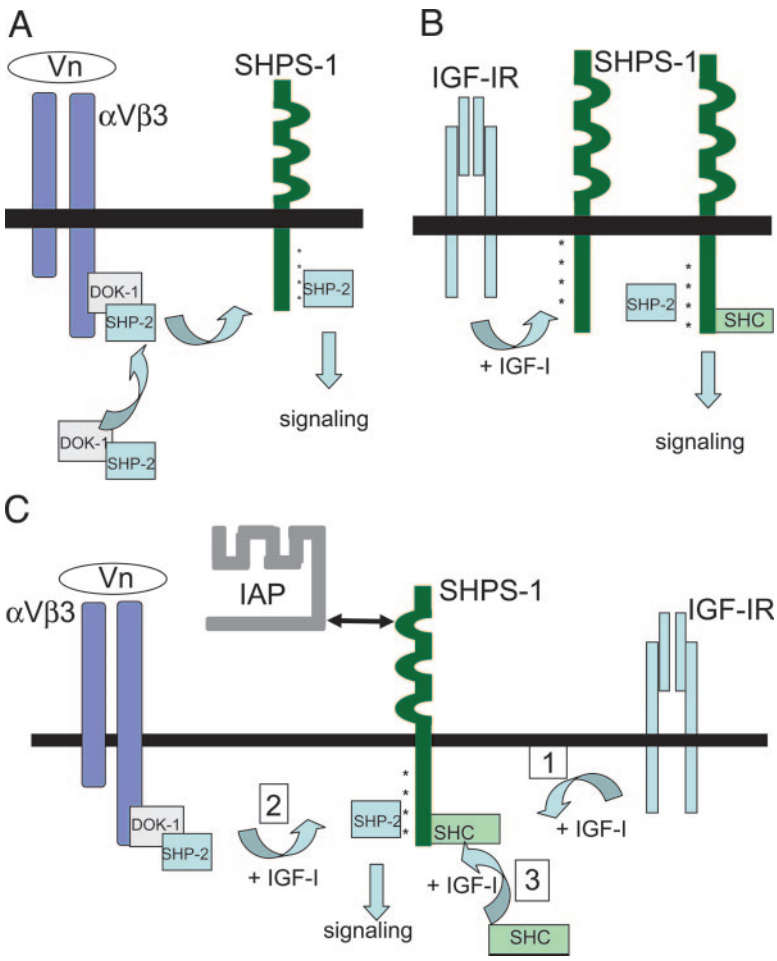

Fig. 2. IGF-I Receptor Signaling Requires Cooperative Interactions among Several Transmembrane Proteins

A, $\alpha V\beta 3$ integrin regulates the response of SMCs at least in part by its ability to regulate the recruitment of the tyrosine phosphatase SHP-2 to the cell membrane. Recruitment of SHP-2 to the cell membrane is necessary for its subsequent transfer to the transmembrane docking protein, SHPS-1, after SHPS-1 phosphorylation in response to IGF-I. Transfer of SHP-2 to SHPS-1 and other signaling molecule complexes is necessary for full activation of downstream signaling events including the PI-3 and MAPK pathways in response to IGF-I. B, SHPS-1 phosphorylation in response to IGF-I recruits SHP-2, which is necessary for IGF-I signaling. Recruitment of Shc to SHPS-1 is necessary for sustained activation of MAPK in response to IGF-I. SHP-2 recruitment to SHPS-1 is necessary for Shc recruitment to SHPS-1 but is not sufficient. C, After activation of the IGF-I receptor in response to ligand binding, SHPS-1 is phosphorylated. C1, This is dependent on the association of SHPS-1 with IAP. C2, Phosphorylation of SHPS-1 creates a high-affinity binding site for the transfer of SHP-2 from the cytoplasmic domain of $\beta 3$ to SHPS-1. Recruitment of SHP-2 to SHPS-1 is necessary for the recruitment of Shc to SHPS-1 and its subsequent phosphorylation. C3, Recruitment and phosphorylation of Shc is required for sustained activation of MAPK and therefore mitogenic signaling in response to IGF-I.

tion of either SHP-2 binding to DOK-1 or DOK-1 binding to $\beta 3$ results in failure of SHP-2 to bind to $\beta 3$ in response to IGF-I. Thus, DOK-1 is an important linker protein that can mediate the transfer of SHP-2 to the plasma membrane after stimulation by either ligand occupancy of $\beta 3$ or IGF-I receptor stimulation.

SHP-2 Is Recruited from the $\alpha V\beta 3$ Membrane-Associated Complex to SHPS-1 after IGF-I Stimulation

The question remained whether SHP-2 was transferred directly from $\alpha V\beta 3$ to the IGF-I receptor or whether other molecules whose activity could be modified by the IGF-I receptor were involved. Initial studies showed that blocking SHP-2 transfer to IRS-1 had no effect on MAPK activation. A second possibility was that the single chain transmembrane protein SHPS-1 was involved. SHPS-1 contains three Ig-like motifs in its extracellular domain and a cytoplasmic tail with four YXXL/I/V motifs. When two of these motifs are tyrosine phosphorylated, SHP-2 can bind to them through its SH-2 domains. Stimulation of SHPS-1 phosphorylation had been shown to recruit SHP-2 to SHPS-1, and when SHP-2 transfer to SHPS-1 was blocked, MAPK activation by insulin was attenuated

(54). Therefore, our studies focused on the role of SHPS-1 in mediating SHP-2 transfer and its role in MAPK activation. SHPS-1 phosphorylation and SHP-2 transfer to SHPS-1 were stimulated when SMC cultures were exposed to IGF-I (45). If SHP-2 transfer to β3 was blocked, however, either by inhibiting β3 tyrosine phosphorylation or blocking DOK1 transfer of SHP-2 to β3, there was no SHP-2 transfer to SHPS-1 although SHPS-1 phosphorylation was maintained (51). Instead, SHP-2 was aberrantly transferred to IGF-I receptor. Thus, transfer of SHP-2 to SHPS-1 requires that SHP-2 is first recruited to $\alpha V\beta 3$ and that SHPS-1 is phosphorylated in response to IGF-I. Disruption of SHP-2 transfer to the plasma membrane by pretreating cells with disintegrin antagonists or anti $\alpha V\beta 3$ antibodies also impairs SHP-2 transfer SHPS-1 despite IGF-I stimulation (44). In all cases wherein SHP-2 transfer to SHPS-1 is blocked, this is associated with failure to properly activate MAPK and PI-3 kinase in response to IGF-I. Other investigators have shown that PI-3 kinase activation can be attenuated after failure to appropriately transfer SHP-2 to downstream signaling molecules; therefore our observations regarding SHP-2 transfer are consistent with previously published data (55, 56).

SHPS-1 Provides a Scaffold for Localizing SHP-2 and Shc and Formation of This Complex Is Necessary for IGF-I Induced MAPK Activation

One potential role of SHPS-1 is that of a scaffolding protein for the assembly of multicomponent signaling complexes. Because our studies had shown that Shc activation was required to achieve optimal MAPK activation, we asked whether Shc localized to SHPS-1 after IGF-I stimulation. This was demonstrated by showing that IGF-I stimulated Shc association with SHPS-1. We further determined that in the presence of a specific peptide that blocked Shc association with SHPS-1, the Shc phosphorylation in response to IGF-I was markedly inhibited. This was accompanied by reduced MAPK activation and an attenuated cell growth response to IGF-I. Importantly, this peptide did not block SHP-2 association with SHPS-1, indicating that although SHP-2 association with SHPS-1 was also required for signaling, it was not sufficient. When SHP-2 transfer is blocked however, Shc binding to SHPS-1 cannot be detected. Therefore, at least two roles for SHPS-1 have been defined by our studies. The first is that in response to ligand occupancy of the IGF-I receptor, SHPS-1 undergoes tyrosine phosphorylation and binds to SHP-2 (Fig. 2B). SHP-2 is then transferred to appropriate downstream signaling molecules such as PI-3 kinase, which is required for their activation. Second, the coassociation of SHP-2 and Shc with SHPS-1 appears to be required for normal Shc activation in response to IGF-I receptor activation. This activation of Shc is necessary for sustained MAPK activation and an optimal mitogenic response of SMC to IGF-I. The molecular mechanism by which SHP-2 and Shc interact cooperatively while localized on SHPS-1 to form this signaling complex requires further study. The identity of the tyrosine kinase that is phosphorylating Shc within this complex is unknown. Preliminary data indicate that it may be a Src family kinase. Exposure of SMC to Src family kinase inhibitors results in attenuation of MAPK signaling and Shc activation in response to IGF-I. Similarly, use of a mutant form of Shc that does not bind to c-Src results in attenuation of Shc activation in response to IGF-I as well as reduced MAPK activation. SHP-2 is also required for optimal Src activation because expression of mutant forms of SHP-2 that do not bind to c-Src results in decreased Src and Shc activation (57, 58). Thus, SHP-2 binding to Src, which occurs in response to IGF-I, may be required for Shc and subsequently MAPK activation, and SHP-2 transfer to SHPS-1 may be required to localize Src within the SHPS-1 signaling complex.

MECHANISM OF $\alpha V\beta 3$ ACTIVATION

To further delineate the role of ligand occupancy of $\alpha V\beta 3$ in mediating IGF-I signaling, two types of experimental approaches have been used. First, the relative importance of different binding domains on $\alpha V\beta 3$ and on the vitronectin molecule itself in mediating positive signaling through $\alpha V\beta 3$ has been identified. Specifically, we have determined that the heparin binding domain of vitronectin, which had been reported to bind to the $\alpha V\beta 5$ integrin, also mediates vitronectin binding to $\alpha V\beta 3$ (59). ECM proteins that contain RGD (arginine-glycine-asparginine) sequences are known to bind to integrin receptors through this recognition domain. Vitronectin contains an RGD sequence that mediates its binding to $\alpha V\beta 3$. To determine the relative importance of each of these two domains in mediating the cooperative signaling between $\alpha V\beta 3$ and IGF-I receptor, we prepared synthetic peptides that each contained one of these regions of vitronectin. Addition of each of the peptides to SMC cultures with IGF-I gave quite different results. Exposure of cells to the heparin binding domain peptide resulted in activation of $\alpha V\beta 3$ phosphorylation and recruitment of SHP-2 to the plasma membrane (60). More importantly, however, it resulted in sustained IGF-I receptor phosphorylation in response to IGF-I as well as sustained MAPK activation and an enhanced mitogenic response. These responses were similar to the response of SMC to exposure to intact vitronectin. In contrast, addition of a synthetic peptide that bound to the RGD binding domain on $\alpha V\beta 3$ did not enhance these properties. Furthermore, it resulted in premature recruitment of SHP-2 to the receptor and premature receptor dephosphorylation, similar to the pSMC response to echistatin. Addition of the RGD peptide with the heparin binding domain peptide resulted in a partial attenuation of the ability of the heparin binding peptide to

enhance IGF-I stimulated receptor phosphorylation and sustained MAPK activation, suggesting that this site might be playing a partially antagonistic role.

To confirm the importance of the heparin binding domain within the intact protein, SMC were exposed to vitronectin and an antibody that had been prepared against the domain of the β3-subunit that binds to the heparin binding domain of vitronectin. In the presence of this antibody, the duration of IGF-I receptor phosphorylation and MAPK activation were attenuated and were similar in intensity and duration to cells that had not been exposed to exogenously added vitronectin. This strongly suggests that binding through this domain is necessary for these enhanced responses. To further delineate the role of the RGD domain, cells were exposed to echistatin and/or the RGD peptide or synthetic molecules known to bind to the RGD binding site on $\alpha V\beta 3$. Exposure of each of these ligands for periods greater than a 7-h exposure resulted in reduced β3 phosphorylation and SHP-2 recruitment to $\alpha V\beta 3$. This decrease in β3 phosphorylation was not due directly to activation of a phosphatase but rather to proteolytic cleavage. Binding of ligand to the RGD binding site on $\alpha V\beta 3$ activates calpain, which then cleaves the β3-subunit, resulting in failure to recruit SHP-2 to the membrane on IGF-I stimulation (61). Because the binding site for the DOK-1/SHP-2 complex has been eliminated, SHP-2 transfer to β3 and subsequently to SHPS-1 is impaired, resulting in failure to appropriately transfer this protein to the IGF-I receptor and to downstream signaling molecules; thus disintegrin antagonists and RGD binding site ligands appear to modulate the effect of heparin binding domain ligands by accelerating β3 cleavage and inhibiting SHP-2 transfer. Whether they impair other markers of β3 activation such as integrin clustering and/or recruitment of signaling molecules to specific membrane microdomains in response to IGF-I stimulation remains to be investigated.

An additional tool for studying the role of extracellular proteins in regulating IGF-I actions has come from the analysis of the role of integrin binding partners that bind to $\alpha V\beta 3$ on integrin signaling. One important binding partner of $\alpha V\beta 3$ is IAP, a five-transmembrane domain protein that modulates the ability of IGF-I to induce a 3- to 4-fold increase in the affinity of $\alpha V\beta 3$ for its ligands (62). IAP has been shown to enhance $\alpha V\beta 3$ affinity for ligands. After a 12-h exposure to IGF-I (63), the amount of IAP associated with $\alpha V\beta 3$ increased 6-fold. Expression of a mutant form of IAP that did not bind to $\alpha V\beta 3$ was associated with failure of IGF-I to stimulate an increase in $\alpha V\beta 3$/IAP association and, more importantly, failure of IGF-I to stimulate an increase in $\alpha V\beta 3$ affinity for vitronectin and to increase cell migration. These results were confirmed by using a monoclonal antibody (B6H12) that has been shown to inhibit IAP/$\alpha V\beta 3$ association. This monoclonal antibody also inhibited the ability of IGF-I to stimulate cell migration.

IGF-I stimulated the association of IAP with $\alpha V\beta 3$ by changing its membrane microdomain compartmentalization. In the basal state, IAP is associated almost exclusively with membrane rafts. After IGF-I exposure, IAP is slowly translocated from raft domains to nonraft domains where most of the $\alpha V\beta 3$ resides. Thus, IGF-I modulates integrin avidity for ligands by stimulating the translocation of IAP to the nonraft membrane compartment where it can associate with $\alpha V\beta 3$.

To further elucidate the role of IAP on IGF-I action, we determined whether it interacted with SHPS-1. We were able to demonstrate that the extracellular domain of IAP associated with SHPS-1 and that exposure to the anti-IAP monoclonal antibody, B6H12, blocked this interaction. We demonstrated that IAP bound directly to SHPS-1 and that in quiescent SMC disruption of the IAP/SHPS-1 interaction using B6H12 resulted in prevention of IGF-I from stimulating SHPS-1 phosphorylation, and SHP-2 transfer to SHPS-1 (64). Despite sustained IGF-I receptor phosphorylation, there was minimal phosphorylation of Shc and MAPK as well as impaired cell migration and proliferation responses to IGF-I. Overexpression of a deletion mutant of IAP that could not bind to SHPS-1 also showed impaired SHP-2 transfer and impaired cell migration and cell division responses to IGF-I. Therefore, IGF-I stimulation of SHPS-1 phosphorylation and the subsequent transfer of SHP-2 to SHPS-1 requires IAP association with SHPS-1. Inhibition of this interaction with a monoclonal antibody or by mutagenesis inhibits SHP-2 transfer and is associated with failure of SMC to respond to IGF-I with increases in MAPK activation and cell division. This further emphasizes the important role of SHP-2 transfer to SHPS-1 in activation of downstream signaling molecules such as Shc that are necessary for normal IGF-I responsiveness. Data published for other growth factor receptor-linked signaling systems (65, 66) suggest that integrin clustering and the appropriate assembly of a signaling complex within microdomains of the plasma membrane might be involved in the mechanism by which IAP/SHPS-1 interaction modulates SHP-2 transfer and Shc activation.

TS-1 is a pericellular protein that interacts with multiple cell surface receptors. It binds to both $\alpha V\beta 3$ and IAP. In the presence of increased concentrations of TS-1 or a synthetic peptide that contains the region of TS-1 that binds to IAP, there is an alteration in the SHPS-1/IAP interaction, which results in a delay in SHP-2 transfer to the IGF-I receptor prolonging receptor phosphorylation and enhanced MAPK activation in response to IGF-I (67). Therefore, altering the timing of SHP-2 transfer to both SHPS-1 and to the IGF-I receptor by changes in the IAP/SHPS-1 interaction can result in enhanced activation of signaling molecules such as MAPK and potentially Shc and enhancement of IGF-I actions.

There has been a great deal of interest in actions of IGFBPs that are mediated through their direct interaction with cellular proteins rather than by binding to IGF-I. IGFBP-5 is a form of IGFBP that is secreted by

SMC and is abundant in ECM. Recently, we have shown that IGFBP-5 binds to thrombospondin and inhibits its interaction with IAP. This leads to a decrease in the ability of TS-1 to prolong IGF-I receptor phosphorylation and to enhance IGF-I-stimulated protein synthesis and cell migration (68). Therefore, TS-1 may be one of the cell surface proteins through which IGFBP-5 acts to alter cellular responses to IGF-I.

In summary, IGF-I receptor-linked signaling in arterial SMC is highly regulated by ligand occupancy of the $\alpha V\beta 3$ integrin. These two signaling systems intersect by controlling the ability of activated $\alpha V\beta 3$ to regulate SHP-2 transfer to the plasma membrane and thus alter the optimal assembly of signaling molecules on the transmembrane protein, SHPS-1 (Fig. 2C). Ligand occupancy of SHPS-1 by proteins such as IAP can also modulate SHP-2 transfer, and disruption of the SHPS-1/IAP interaction leads to attenuation of IGF-I signaling. $\alpha V\beta 3$ is a very versatile molecule for regulating IGF-I signaling because stimulation of its domain that binds to heparin binding motifs in ECM proteins such as vitronectin results in enhancement of IGF-I actions, whereas stimulation of RGD recognition domain results in their attenuation. Both of these mechanisms involve changes in the transfer of SHP-2 to SHPS-1 and to other downstream signaling molecules as well as their activation in response to IGF-I stimulation. Future studies should be able to build on these observations to construct a molecular map that will identify the multiple components of the membrane-associated signaling complexes that are assembled in response to $\alpha V\beta 3$ and IGF-I receptor activation and how differential activation of these two signaling systems alters their assembly and subsequent actions.

Acknowledgments

The authors thank Ms. Laura Lindsey for her help in preparing the manuscript.

Received September 22, 2004. Accepted October 29, 2004.

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This work was supported by grants from the National Institutes of Health (HL56850 and AG02331) and a postdoctoral fellowship to Laura A. Maile, Ph.D., from the American Heart Association MidAtlantic Affiliate (022-53770).

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