Identification of IGPR-1 as a novel adhesion molecule involved in angiogenesis

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ABSTRACT Angiogenesis—the growth of new blood vessels from preexisting vessels—is an important physiological process and is considered to play a key role in tumor growth and metastasis. We identified the immunoglobulin-containing and proline-rich receptor-1 (IGPR-1, also called TMIGD2) gene as a novel cell adhesion receptor that is expressed in various human organs and tissues, mainly in cells with epithelium and endothelium origins. IGPR-1 regulates cellular morphology, homophilic cell aggregation, and cell–cell interaction. IGPR-1 activity also modulates actin stress fiber formation and focal adhesion and reduces cell migration. Silencing of expression of IGPR-1 by small interfering RNA (siRNA) and by ectopic overexpression in endothelial cells showed that IGPR-1 regulates capillary tube formation in vitro, and B16F melanoma cells engineered to express IGPR-1 displayed extensive angiogenesis in the mouse Matrigel angiogenesis model. Moreover, IGPR-1, through its proline-rich cytoplasmic domain, associates with multiple Src homology 3 (SH3)–containing signaling proteins, including SH3 protein interacting with Nck (SPIN90/WISH), bullous pemphigoid antigen-1, and calcium channel β2. Silencing of expression of SPIN90/WISH by siRNA in endothelial cells showed that SPIN90/WISH is required for capillary tube formation. These features of IGPR-1 suggest that IGPR-1 is a novel receptor that plays an important role in cell–cell interaction, cell migration, and angiogenesis.

INTRODUCTION

Angiogenesis is a coordinated cascade of numerous complex cellular processes, which include endothelial cell migration, proliferation, sprouting, and lumen formation, which ultimately leads to the formation of new vessels. These coordinated cellular events are regulated by the function of various cell surface receptors and soluble ligands (Rahimi, 2006; Carmeliet and Jain, 2011a). The ability of endothelial cells to form capillary tubes is prerequisite for the establishment of a continuous vessel lumen that routes the blood flow. Several key receptor tyrosine kinases such as vascular endothelial growth factor (VEGF) receptor-1 (VEGFR-1), VEGFR-2, and VEGFR-2, and cell adhesion molecules (CAMs), including cadherins, integrins, selectins, and immunoglobulin (Ig) superfamily proteins, are all involved in angiogenesis (Bach et al., 1998; Barreiro et al., 2002; Bazzoni, 2003). The roles of proangiogenic molecules, such as VEGF and VEGFRs (i.e., VEGFR-1, VEGFR-2, and VEGFR-3), are well known in regulation of differentiation, survival, proliferation, and migration of endothelial cells (Gory-Faure et al., 1999; Rahimi, 2006). Studies using knockouts or blocking antibodies also demonstrated a key role for integrins in angiogenesis. Vascular endothelial cadherin (VE-cadherin), an endothelium-specific member of the cadherin family of adhesion proteins (Bach et al., 1998), and other CAM proteins, such as PECAM-1, ICAM-1, and JAM-A, are also linked to angiogenesis (Bach et al., 1998; Barclay, 2003; Bazzoni, 2003).

The Ig-containing adhesion molecules are known for their vital role in embryonic development and pathological conditions such as cancer and inflammation by modulating cell–cell adhesion and cell migration (Takai et al., 2008; Yamagata and Sanes, 2008; DeLisser et al., 2010). The Ig domains engage in protein–protein interactions, such as the homophilic (i.e., trans-dimerization) interaction of cell adhesion receptors, and protein–ligand interactions, such growth factor receptors and soluble growth factors (Barclay, 2003; Rahimi, 2006). The Ig-containing cell adhesion molecules,
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Searching the human genome sequence database for Ig-containing proteins, we identified an uncharacterized protein—transmembrane and immunoglobulin domain–containing protein 2 (TMIGD2)—that has a single Ig domain, a single transmembrane domain, and a stretch of 110–amino acid cytoplasmic region highly rich in proline residues (Figure 1A). The extracellular region of this protein also contains two possible glycosylation sites (Figure 1A). Because of the presence of an immunoglobulin domain in its extracellular region and a proline-rich motif in its cytoplasmic region, we named this protein immunoglobulin and proline-rich receptor-1, or IGPR-1, and refer to it as such through homophilic and/or heterophilic interactions, selectively contribute to the specificity of cell–cell recognition and cell adhesion (Barclay, 2003; Rahimi, 2006). At the intracellular compartment, they interact with various cytoplasmic signaling proteins, which are often linked to the cytoskeleton (Takai et al., 2008).

In this study we identified a previously uncharacterized receptor—immunoglobulin-containing and proline-rich receptor-1 (IGPR-1)—as a novel adhesion molecule with a broad expression in epithelial and endothelial cells. IGPR-1 regulates cellular morphology, cell–cell interaction, and cell migration. More importantly, IGPR-1 associates with several SH3-containing proteins and regulates angiogenesis in vivo and in vitro.

RESULTS
Identification of IGPR-1 as a novel cell surface receptor

Searching the human genome sequence database for Ig-containing proteins, we identified an uncharacterized protein—transmembrane and immunoglobulin domain–containing protein 2 (TMIGD2)—that has a single Ig domain, a single transmembrane domain, and a stretch of 110–amino acid cytoplasmic region highly rich in proline residues (Figure 1A). The extracellular region of this protein also contains two possible glycosylation sites (Figure 1A). Because of the presence of an immunoglobulin domain in its extracellular region and a proline-rich motif in its cytoplasmic region, we named this protein immunoglobulin and proline-rich receptor-1, or IGPR-1, and refer to it as such.
protein (Figure 1E), suggesting that IGPR-1 is highly glycosylated in vivo and that glycosylation is responsible for its apparent high molecular weight. Of interest, treatment of cells with tunicamycin, a commonly used inhibitor of N-linked glycosylation in vivo, which is known to cause ER stress, also caused a rapid degradation of IGPR-1, suggesting that perhaps glycosylation of IGPR-1 is important for its stability and agents that induce ER stress promote degradation of IGPR-1 (unpublished data). Because IGPR-1 is predicted to be a plasma membrane protein, we also analyzed its membrane localization in PAE cells. The immunofluorescence microscopy assessment of cells expressing IGPR-1 showed that IGPR-1 is localized in the plasma membrane (Figure 1F). Additional analysis, including cell surface biotinylation, further demonstrated cell surface localization of IGPR-1 (Figure 1G). Taken together, the data establish IGPR-1 as a novel immunoglobulin containing membranous glycoprotein.

To gain insight into the tissue distribution of IGPR-1, we initially examined its expression in normal human tissues by analyzing the transcript of IGPR-1 by quantitative PCR (qPCR). The qPCR analysis using primers designed for exons 2 and 3 of IGPR-1 showed that IGPR-1 transcript was highly present in artery, vein, and brain. IGPR-1 transcript was also moderately detected in bone marrow, liver, and lung. IGPR-1 transcript, however, was relatively low in kidney, ovary, and throughout this article. The orthologue of human IGPR-1 is found only in eukaryotes, including primates, the guinea pig, canines, felines, dolphins, bovines, the llama, bats, the common shrew, and horses (Figure 1B). Of interest, the IGPR-1 gene is absent in mouse and rat genomes. The immunoglobulin domain of IGPR-1 was predicted to be Ig V (variable) fold and was found to be highly similar to the Ig domain of myelin-associated glycoprotein (MAG; Breithaupt et al., 2003). Using the Ig domain of MAG as a template, we constructed a structural model of IGPR-1. IGPR-1 seems to adapt a typical Ig V-like fold consisting of a sandwich of two antiparallel β-sheets (Figure 1C).

To examine its cellular and biochemical properties, IGPR-1 was cloned into a retroviral expression vector and expressed in porcine aortic endothelial (PAE) cells. Moreover, to detect expression of IGPR-1, we developed a polyclonal anti-IGPR-1 antibody against its cytoplasmic domain, which specifically recognizes IGPR-1 (Figure 1D). The predicted molecular weight of IGPR-1 protein is 31 kDa; however, the apparent molecular weight of IGPR-1 ectopically expressed in PAE cells as detected by Western blot analysis was ~55 kDa (Figure 1D). We reasoned that the higher molecular weight of IGPR-1 could be associated with its potential glycosylation at its extracellular region. Treatment of cell lysates derived from PAE cells with N-glycosidase F (PNGase), which is known to hydrolyze nearly all types of N-glycan chains from glycoproteins, generated a 31-kDa protein (Figure 1E), suggesting that IGPR-1 is highly glycosylated in vivo and that glycosylation is responsible for its apparent high molecular weight. Of interest, treatment of cells with tunicamycin, a commonly used inhibitor of N-linked glycosylation in vivo, which is known to cause ER stress, also caused a rapid degradation of IGPR-1, suggesting that perhaps glycosylation of IGPR-1 is important for its stability and agents that induce ER stress promote degradation of IGPR-1 (unpublished data). Because IGPR-1 is predicted to be a plasma membrane protein, we also analyzed its membrane localization in PAE cells. The immunofluorescence microscopy assessment of cells expressing IGPR-1 showed that IGPR-1 is localized in the plasma membrane (Figure 1F). Additional analysis, including cell surface biotinylation, further demonstrated cell surface localization of IGPR-1 (Figure 1G). Taken together, the data establish IGPR-1 as a novel immunoglobulin containing membranous glycoprotein.

**IGPR-1 is expressed in various human organs and cells**

To gain insight into the tissue distribution of IGPR-1, we initially examined its expression in normal human tissues by analyzing the transcript of IGPR-1 by quantitative PCR (qPCR). The qPCR analysis using primers designed for exons 2 and 3 of IGPR-1 showed that IGPR-1 transcript was highly present in artery, vein, and brain. IGPR-1 transcript was also moderately detected in bone marrow, liver, and lung. IGPR-1 transcript, however, was relatively low in kidney, ovary,
IGPR-1 activity regulates angiogenesis in vivo and in vitro

Adhesion molecules are known to regulate capillary tube formation of endothelial cells and angiogenesis (Gory-Faure et al., 1999; DeLisser et al., 2010). IGPR-1 is expressed by endothelial cells (Figure 2D), suggesting a possible role for IGPR-1 in angiogenesis. To examine the role of IGPR-1 in angiogenesis, we subjected PAE cells expressing IGPR-1 to an in vitro Matrigel-based angiogenesis assay. Our analysis showed that PAE cells expressing IGPR-1 undergo increased capillary tube formation (Figure 3A). Together, the data demonstrate that expression of IGPR-1 in endothelial cells is necessary for capillary tube formation.

Expression of IGPR-1 and PAE cells expressing empty vector were preincubated with GST protein and both adhered to the plate in a similar manner. However, incubation of PAE cells expressing IGPR-1 with the soluble extracellular domain of IGPR-1 totally inhibited adhesion/spreading. Because overexpression of IGPR-1 increased the adhesive phenotype of PAE cells, we examined the possible role of IGPR-1 in cell–cell interaction. To this end, we generated PAE cell lines expressing IGPR-1 and ΔN-IGPR-1 (where the extracellular domain of IGPR-1 was deleted) and subjected them to an aggregation assay. Expression of IGPR-1 and ΔN-IGPR-1 in PAE cells is shown in Figure 4, C and D. PAE cells expressing wild-type IGPR-1 but not ΔN-IGPR-1 or empty vector formed large aggregates of cells (Figure 4, E–G), suggesting that IGPR-1 mediates homophilic cell–cell interaction and that the extracellular domain is necessary for its function to mediate this. To examine the role of IGPR-1 in cell adhesion and the role of its Ig-containing extracellular domain in this process, we generated a recombinant glutathione S-transferase (GST)–Ig containing the extracellular domain of IGPR-1 (Figure 4H). PAE cells expressing IGPR-1 and PAE cells expressing empty vector were preincubated with GST protein and both adhered to the plate in a similar manner. However, incubation of PAE cells expressing IGPR-1 with the soluble extracellular domain of IGPR-1 totally inhibited adhesion/spreading of these cells to the plate (Figure 4I). As noted, preincubated cells with soluble IGPR-1 only loosely adhered, with no spreading (Figure 4I), and they come off the tissue plate (unpublished data). Quantification of adherent cells also is shown (Figure 4J). Taken together, the data suggest that IGPR-1 activity regulates cell–cell interaction and adhesion, and its extracellular domain is critically important for its ability to mediate these cellular events.

IGPR-1 regulates focal adhesion and cell migration

To further investigate biological responses associated with IGPR-1 activity in PAE cells, we analyzed focal adhesion formation in PAE cells. Immunofluorescence staining of PAE cells with vinculin demonstrated that expression of IGPR-1 in PAE cells markedly increases focal adhesion as measured by vinculin localization with respect to focal contact in these cells (Figure 5A). Of note, IGPR-1 expression in PAE cells increased both number and size of focal adhesions (Figure 5A). Of interest, adhesions in PAE cells expressing IGPR-1 were negative for IGPR-1 (unpublished data). Beyond epithelial cells that were positive for IGPR-1 across the tissue microarray staining, endothelial and epithelial cells, urothelium of the bladder, skin epidermis, epithelium of gastrointestinal, and rectum (Figure 2C). Moreover, endometrial glands of the uterus, the ureter, fallopian tube epithelium, colonic epithelium, small bowl epithelium, stomach epithelium, including both chief and parietal cells, trophoblastic epithelium of placenta, and pancreatic acinar cells were all positive for IGPR-1 (unpublished data). Of note, thyroid, cerebellum, cerebral cortex, and thymus were negative for IGPR-1 (unpublished data). Beyond epithelial cells that were positive for IGPR-1 across the tissue microarray staining, endothelial cells present in vein and arteries also consistently were positive for IGPR-1, as shown in tissue sections derived from ureter, esophagus, skin, skeletal muscle, gastrointestinal, and cervix (Figure 2D). As noted, IGPR-1 transcript was also highest in vein and artery (Figure 2A). Together, the data demonstrate that IGPR-1 is expressed in various organs; however, its expression is mainly in epithelial and endothelial cell types.
played a distinct actin stress fiber formation mainly in central regions of the cells, with a significantly reduced level of membrane ruffles (Figure 5B; compare with Figure 5E). Taken together, the data suggest that IGPR-1 regulates focal adhesion and actin stress fiber remodeling.

Because IGPR-1 activity regulates focal adhesion and reduced membrane ruffles, which are linked to cellular migration, we decided to analyze the possible function of IGPR-1 in cell migration. The activity of various proteins, including paxillin, an adaptor protein that localizes to focal adhesion, was implicated in the regulation of different steps of cell migration (Abou Zeid et al., 2006), and so were higher at the cell periphery, and consistent with their observed morphology, they were aligned along each other with a tight contact (Figure 5C; compare with Figure 5F). Quantitative analysis based on the number of focal adhesions showed that IGPR-1 expression in PAE cells resulted in an increased number of focal adhesions by 62% (Figure 5G). Staining of cells for actin with FITC-labeled phalloidin also demonstrated that expression of IGPR-1 in PAE cells alters actin stress fibers and cytoskeleton remodeling. PAE cells expressing empty vector form stress fibers primarily at the periphery of cells, with membrane ruffling, which is reflective of actin filament assembly (Figure 5F). PAE cells expressing IGPR-1, however, displayed a distinct actin stress fiber formation mainly in central regions of the cells, with a significantly reduced level of membrane ruffles (Figure 5B; compare with Figure 5E). Taken together, the data suggest that IGPR-1 regulates focal adhesion and actin stress fiber remodeling.
IGPR-1 associates with SH3-containing cytoplasmic signaling proteins

The cytoplasmic region of IGPR-1 is an emblematic proline-rich motif (Figure 1A). The proline-rich motif is known to interact with SH3-containing signaling proteins (Kaneko et al., 2008). To identify signaling proteins that interact with IGPR-1 and potentially mediate its cellular function, we used an SH3 protein array consisting of 34 SH3 domains derived from 34 individual proteins. The blotting of the SH3 array membrane with purified recombinant GST–proline-rich motif of IGPR-1 identified four distinct SH3 domains, including SH3 protein interacting with Nck (SPIN90/WISH; 90 kDa), calcium channel β2 (CACNB2), bullous pemphigoid antigen-1 (BPAG1), and melanoma inhibitory activity (MIA; Figure 6B).

SPIN90 is known to play a key role in cell adhesion and in actin cytoskeleton reorganization (Lim et al., 2001; Takenawa and Suetosu, 2007). CACNB2 is one of the subunits of L-type voltage-pen dent calcium channels. Voltage-dependent calcium channels are oligomeric proteins composed of α1, α2δ, β(1–4), and γ subunits, in which they control calcium entry into cells (Yamagata and Sanes, 2008). The β subunit is a member of membrane-associated guanylate kinase–like proteins with a guanylate kinase domain and an SH3 domain. BPAG1 is a member of plakin family proteins, comprising...
cytoskeleton-binding proteins (Fuchs and Yang, 1999; Fuchs and Karakesisoglou, 2001; Fuchs et al., 2004). BPAG1 is involved in anchoring keratin intermediate filaments to the cytoplasmic side of hemidesmosomes (Matsumura et al., 1997). MIA is a small, secreted protein that interacts with extracellular matrix proteins (Bosserhoff et al., 1999). To further validate the array data, we created a GST recombinant SH3 domain of SPIN90, BPAG1, and CANCB2 and tested their binding to IGPR-1 in a GST pull-down assay. The MIA binding to IGPR-1 was not considered for further analysis due to the fact that it is a secreted protein. The data showed that IGPR-1 strongly binds to SH3 domains of SPIN90, BPAG1, and CANCB2 (Figure 6E). The binding of SPIN90 and BPAG1 was stronger than that of CANCB2, suggesting that perhaps these SH3-containing proteins interact with IGPR-1 more readily than with CANCB2. We further validated the in vivo binding of SPIN90 with IGPR-1 by immunoprecipitation in PAE cells expressing IGPR-1. Cell lysates derived from PAE cells expressing IGPR-1 immunoprecipitated with anti-IGPR-1 selectively coprecipitated with endogenous SPIN90, as detected with anti-SPIN90 antibody (Figure 6F).

**SPIN90 activity is required for angiogenesis**

Identification of SPIN90 as an IGPR-1–interacting protein suggests that SPIN90 might play a role in IGPR-1–mediated angiogenesis. To further determine the biological importance of SPIN90 in angiogenesis, we initially overexpressed SPIN90 in PAE cells and examined its potential to stimulate capillary tube formation of PAE cells. Expression of SPIN90 in PAE cells increased capillary tube formation (Figure 7A), indicating that SPIN90 activity is linked to angiogenic events in endothelial cells. Quantification of capillary tube formation and expression of IGPR-1 and SPIN90 in PAE were also found (Figure 7, B and C). To address the biological importance of endogenous SPIN90 in angiogenesis, we silenced expression of SPIN90 by
IGPR-1 regulates angiogenesis

In this study we identified IGPR-1 as a novel cell surface protein expressed in various human organs and tissues, predominantly in cells of epithelial and endothelial origins. On the basis of the observed characteristics of IGPR-1, such as promoting cellular aggregation, cell–cell interaction, morphological change of cells, and increase in focal adhesion, we propose IGPR-1 as a novel CAM. Various classes of CAMs have been described, including, cadherins, mucins, integrins, selectins, and the Ig superfamily (Takai et al., 2008). CAMs mediate homophilic (like-binds-like) adhesion between cells of a single type and heterophilic adhesion between cells of different types, and the cytoplasmic regions of these proteins are usually connected to the cytoskeleton (Takai et al., 2008). Consistent with its observed effect on cell adhesion, transfer of IGPR-1 into PAE cells significantly increases the expression of vinculin. Vinculin is a major constitutive component of focal adhesion and adherens junctions, where it binds to several cytoskeletal proteins, linking microfilaments to CAMs (Pawlak and Helfman, 2001). Consistent with CAM-mediated phenotypic characteristics such as focal adhesion and cellular morphology, introducing IGPR-1 to PAE cells resulted in the acquisition of cubical morphology and elevated focal adhesion. These cells also were resistant to trypsin/EDTA-mediated detachment from cell culture plates and acquired increased cell adhesion properties. The function of IGPR-1 is highly correlated with those of other well-known CAMs. Cadherins are known to stabilize cell–cell contacts to form adherent junctions in epithelial cells, fibroblasts, and endothelial cells (Matsumura et al., 1997; Bach et al., 1998; Gory-Faure et al., 1999).

In agreement with a possible role of IGPR-1 in cell adhesion and cell–cell interaction, expression of IGPR-1 in PAE cells and B16F cells inhibits cell migration. Of interest, the reduced migration of these cells by IGPR-1 also correlates with the inhibition of phosphorylation of paxillin at tyrosine 118 (Y118). Phosphorylation of Y118 of paxillin is linked to inhibition of cell migration (Abou Zeid et al., 2006; Zaidel-Bar et al., 2007), suggesting that IGPR-1 inhibits cellular migration, in part by stimulating dephosphorylation of paxillin either by increasing protein phosphatase activity or preventing its phosphorylation by inhibiting tyrosine kinases such as Src family kinases and focal adhesion kinases, which are involved in the phosphorylation of paxillin. The cytoplasmic domain of IGPR-1 contains a proline-rich motif that recognizes SH3-containing signaling proteins. The data presented in this article demonstrate that the cytoplasmic domain of IGPR-1 interacts with several SH3-containing signaling proteins, including SPIN90. SPIN90 activity is known to regulate cell

siRNA and examined the biological consequence of depletion of SPIN90 in HUVECs. Depletion of SPIN90 significantly reduced capillary tube formation of HUVECs (Figure 7E). Quantification of capillary tube formation of HUVECs transfected with control siRNA or SPIN90 siRNA is shown in Figure 7F. The effect of SPIN90 siRNA in silencing the expression of SPIN90 in HUVECs is shown in Figure 7G. Taken together, the data indicate that SPIN90 associates with IGPR-1 through its SH3 domain, and its activity regulates angiogenesis.

DISCUSSION

In this study we identified IGPR-1 as a novel cell surface protein expressed in various human organs and tissues, predominantly in cells of epithelial and endothelial origins. On the basis of the observed characteristics of IGPR-1, such as promoting cellular aggregation, cell–cell interaction, morphological change of cells, and increase in focal adhesion, we propose IGPR-1 as a novel CAM. Various classes of CAMs have been described, including, cadherins, mucins, integrins, selectins, and the Ig superfamily (Takai et al., 2008). CAMs mediate homophilic (like-binds-like) adhesion between cells of a single type and heterophilic adhesion between cells of different types, and the cytoplasmic regions of these proteins are usually connected to the cytoskeleton (Takai et al., 2008). Consistent with its observed effect on cell adhesion, transfer of IGPR-1 into PAE cells significantly increases the expression of vinculin. Vinculin is a major constitutive component of focal adhesion and adherens junctions, where it binds to several cytoskeletal proteins,
adhesion through actin cytoskeleton reorganization (Lim et al., 2001; Takenawa and Miki, 2001), suggesting that the proline-rich cytoplasmic domain of IGPR-1, through recruitment of SPIN90, modulates cell adhesion and cellular morphology. Our observation indicates that SPIN90 regulates capillary tube formation of endothelial cells, demonstrating a significant role for SPIN90 in IGPR-1–mediated biological responses, particularly in angiogenesis.

In general, CAMs interact with distinct signaling proteins whose activities are linked to the cytoskeleton. For example, cadherins are known to interact with β-catenin (Harris and Tepass, 2010), nectins interact with the filamentous (F)-actin–binding protein afadin and PAR3 (Takai et al., 2008), ICAM-1 associates with α-actinin and ERM proteins (Barreiro et al., 2002), and JAMs (junctional adhesion molecules) bind to ZO1 (Bazzoni, 2003). Unlike the known CAMs, IGPR-1 distinctively interacts with SH3-containing proteins, including SPIN90, BPAG1, and CANCB2, which might differentiate IGPR-1 from the already known CAMs.

Another interesting and important aspect of this work is that IGPR-1 is mainly expressed in cells of epithelial and endothelial cell origin. For example, epithelium and endothelium of various organs/tissues were positive for IGPR-1, suggesting that IGPR-1 as a novel adhesion molecule could play a significant role in the pathobiology of endothelial and epithelial cells. Angiogenesis—the growth of new blood vessels from preexisting blood vessels—is an important physiological process in the body and is required for normal wound healing and female reproductive activity. Pathological angiogenesis, either excessive or insufficient, is now recognized as a common denominator underlying a number of deadly and debilitating human diseases such as cancer, age-related macular degeneration, diabetic retinopathy, and cardiovascular diseases (Carmeliet and Jain,
Acquisition of angiogenesis by tumor cells is considered the most critical step in tumor growth and metastasis. To grow beyond 2 mm in diameter, a tumor needs to undergo angiogenesis, which is often established by hypoxia-induced expression of VEGF and other hypoxia-induced growth factors (Carmeliet and Jain, 2011b). The present study suggests an important role for IGPR-1 in angiogenesis. Modulation of expression of IGPR-1 by ectopic expression or silencing in endothelial cells significantly altered the angiogenic phenotype of endothelial cells in culture, and introducing IGPR-1 to tumor cells increased tumor angiogenesis.

In conclusion, we identified IGPR-1 as a novel adhesion molecule involved in cell–cell interaction and regulation of cell migration. IGPR-1 associates with several SH3-containing signaling proteins, including SPIN90, and regulates angiogenesis, suggesting a significant role for IGPR-1 in angiogenesis-associated diseases such as cancer. Further studies should illuminate the mechanisms by which IGPR-1 regulates cell–cell interaction and its biological importance in human diseases, in particular in angiogenesis-associated diseases such as cancer.

**MATERIALS AND METHODS**

**General reagents**

BS3 was purchased from Pierce Biotechnology (Rockford, IL). SYBR green qPCR reagents were purchased from Applied Biosystems (Carlsbad, CA). Immunohistochemistry/immunofluorescence microscopy reagents included a Dako primary staining kit, the EnVision System-HRP (DAB) for rabbit primary antibodies (K4010), antibody diluent, background reducer (S3022), citrate buffer (S1966), and Protein Block Serum-Free (X0909), all from Dako (Carpinteria, CA). Hematoxylin (AMH100907) was purchased from Thermo Scientific (Waltham, MA). The proliferation kit was purchased from Promega (Madison, WI). PNGase F was purchased from BioChain (Newark, CA).

**Plasmids, primers, siRNAs, and antibodies**

The cDNA corresponding to IGPR-1 (MGC:23244, IMAGE:4811204), which was purchased from Open Biosystems, Thermo BioSystems, Huntsville, AL, was PCR amplified and cloned into retroviral vector pMSCV.puro (Invitrogen, Carlsbad, CA) via XhoI and EcoRI restriction sites. IGPR-1 was cloned in frame of the pcDNA3.1.His.Myc vector (Invitrogen), and the identity of IGPR-1 was confirmed by sequencing the plasmids. All plasmids were transfected into baculovirus-infected insect cells (S1966), and Protein Block Serum-Free (X0909), all from Dako (Carpinteria, CA). Hematoxylin (AMH100907) was purchased from Thermo Scientific (Waltham, MA). The proliferation kit was purchased from Promega (Madison, WI). PNGase F was purchased from New England BioLabs (Ipswich, MA). Total human RNA was purchased from BioChain (Newark, CA).

**Aggregation assay**

Single-cell suspensions were obtained by detaching cells from a plate with 1 mM EDTA in phosphate-buffered saline (PBS) solution. The cells were then washed twice in 10% DMEM medium. The cells were resuspended in DMEM. Approximately 5 x 10⁵ cells per 2.5 ml were incubated in a six-well plate (precoated with 1% bovine serum albumin at 37°C for 2 h) with gentle shaking at 37°C for 1 h, followed by no shaking for 1 h. Cells were viewed under a light microscope, and images were taken.

**Cell adhesion assay**

For cell adhesion assay, cells expressing IGPR-1 were detached from plates incubated in suspension for 2 h with GST or the GST-Ig domain of IGPR-1 and replated onto tissue culture dishes and allowed to adhere for 2 h at 37°C. Nonadherent cells were removed by washing twice with PBS, after which the adherent cells were fixed for 5 min in methanol and counted under a microscope.

**RT-qPCR analysis**

RT-qPCR (reverse transcription quantitative PCR) was performed as recommended by the manufacturer (Applied Biosystems) and 18S used as internal controls.

**Mouse Matrigel angiogenesis assay**

Mice (six animals for each experimental group) were injected with Matrigel (10 mg/ml) plus B16 melanoma cells (1 x 10⁵) that were engineered to express IGPR-1 or empty vector. Before injection, the animals were sedated with Avertin (0.3 ml per 20-g mouse). A 25-gauge needle was used to inject 0.3 ml of Matrigel mixture subdermally into mice. After 8 d, animals were sacrificed, and tumor-induced angiogenesis and Matrigel plugs were removed for further analysis as described (Meyer et al., 2011a). The tumor plugs were homogenized in 1 ml of deionized H₂O on ice and cleared by centrifugation at 10,000 rpm for 5 min at 4°C. The supernatant was collected and used in duplicate to measure hemoglobin content with Drabkin’s reagent along with hemoglobin standards as suggested by the manufacturer (Sigma-Aldrich, St. Louis, MO). The absorbance was read at 540 nm.

**Endothelial cell capillary tube formation and migration assays**

Endothelial cells were seeded on Matrigel with endothelial cell growth medium (Clonetics, San Diego, CA), and capillary tube formation was viewed under microscope and photographed after 24 h as described (Meyer et al., 2008). Quantification of capillary tube formation was established by ImageJ (National Institutes of Health, Bethesda, MD). Migration assay was performed basically by creating a “wound” in a cell monolayer by scratch using the tip of the tissue culture pipette. After 10 h, images were captured under microscope and documented.

**Site-directed mutagenesis**

All the site-directed mutagenesis was performed using a PCR-based, site-directed mutagenesis strategy (Meyer et al., 2006). The identities of deletions were confirmed by sequencing the plasmids. All the cDNAs were either cloned into pcDNA3.1His.Myc vector or into the retroviral vector pMSCV.puro (Clontech, Mountain View, CA). In some cases the PCR products were cloned into pGEX2T vector (Pharmacia, GE Healthcare, Piscataway, NJ) and used to make GST-fusion protein in Escherichia coli.

**Virion production and transient transfection**

For virus production pMSCV.puro vector containing IGPR-1 or other cDNA of interest was transfected into 293-GP cells, and viral supernatants were collected for 5 d as previously described (Rahimi et al., 2000).
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