The Adaptor Protein Gab1 Couples the Stimulation of Vascular Endothelial Growth Factor Receptor-2 to the Activation of Phosphoinositide 3-Kinase*

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Phosphoinositide 3-kinase (PI3K) mediates essential functions of vascular endothelial growth factor (VEGF), including the stimulation of endothelial cell proliferation and migration. Nevertheless, the mechanisms coupling the receptor VEGFR-2 to PI3K remain obscure. We observed that the Grb2-bound adapter Gab1 is tyrosine-phosphorylated and relocated to membrane fractions upon VEGF stimulation of endothelial cells. We could detect the PI3K regulatory subunit p85 in immunoprecipitates of endogenous Gab1, and vice versa, and measure a Gab1-associated lipid kinase activity upon VEGF stimulation. Furthermore, transfection of the Gab1-YF3 mutant lacking all p85-binding sites strongly repressed PI3K activation measured in vitro. Moreover, Gab1-YF3 severely decreased the cellular amount of phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) generated in response to VEGF. Furthermore, adenoviral expression of Gab1-YF3 suppressed both Akt phosphorylation and recovery of wounded human umbilical vein endothelial cell monolayers, a VEGF-dependent process involving cell migration and proliferation under PI3K control. Transfection of other Gab1 mutants, lacking Grb2-binding sites or the pleckstrin homology (PH) domain, also prevented Akt activation, further demonstrating Gab1 involvement in PI3K activation. These mutants were also used to show that interactions with both Grb2 and PtdIns(3,4,5)P3 mediate Gab1 recruitment by VEGFR-2. Importantly, Gab1 mobilization was impaired by (i) PI3K inhibitors, (ii) deletion of Gab1 PH domain, (iii) PTEN (phosphatase and tensin homolog deleted on chromosome 10) overexpression to repress PtdIns(3,4,5)P3 production, and (iv) overexpression of a competitor PH domain for PtdIns(3,4,5)P3 binding, which altogether demonstrated that PI3K is also an upstream regulator of Gab1. Gab1 thus appears as a primary actor in coupling VEGFR-2 to PI3K/Akt, recruited through an amplification loop involving PtdIns(3,4,5)P3 and its PH domain.

Gene deletion experiments have shown that vascular endothelial growth factor (VEGF)* and its receptor VEGFR-2 (Flk-1/KDR) are key regulators of angiogenesis and vasculogenesis, controlling the proliferation, survival, migration, and permeability of endothelial cells. Various intracellular signaling pathways have been proposed to mediate these biological activities of VEGF, including the phospholipase Cγ (PLCγ)/protein kinase C pathway and the Ras/mitogen-activated protein kinases pathway (see Ref. 1 for review). More recently, VEGFR-2 was found to activate phosphoinositide 3-kinase (PI3K) and its major downstream target, the kinase Akt/protein kinase B (PKB). This PI3K/Akt-PKB pathway seems to mediate major biological responses of VEGF in endothelial cells, including the stimulation of their proliferation and migration (2–5). Further supporting these findings, studies based on gene targeting strategies demonstrated that PI3K/Akt constitutes a major axis of regulation of vascular homeostasis and angiogenesis (6–8). Akt also relays VEGF in the control of vascular permeability, by regulating the endothelial nitric-oxide synthase (9, 10). In addition, mice deficient for Foxo1, a transcription factor target of Akt, show fatal vascular remodeling due to alterations in the endothelial cell response to VEGF (11).

Despite the amount of data sustaining a role for the PI3K/Akt pathway in VEGF signaling, the mechanisms coupling VEGFR-2 to this pathway remain unclear. It is well established that PI3K activation is highly dependent upon the phosphorylation of a typical Tyr-X-X-Met sequence present in an “upstream” protein and specifically recognized by the SH2 domains of p85, the regulatory subunit of the growth factor-regulated PI3K subgroup. This motif is usually encountered within the sequence of receptors or docking proteins rapidly mobilized and phosphorylated by receptors (12, 13). In the case of VEGF-2, its sequence does not display this motif, and its

The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor-2; EGFR, EGF receptor; HUVEC, human umbilical vein endothelial cell; MBD, Met-binding domain; PAEC, porcine aortic endothelial cell; PH, pleckstrin homology; PI3K, phosphoinositide 3-kinase; PLCγ, phospholipase Cγ; PtdIns, phosphatidylinositol; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10; SH, Src homology; WT, wild-type; GST, glutathione S-transferase; CMV, cytomegalovirus; PKB, protein kinase B.

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‡ The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor-2; EGFR, EGF receptor; HUVEC, human umbilical vein endothelial cell; MBD, Met-binding domain; PAEC, porcine aortic endothelial cell; PH, pleckstrin homology; PI3K, phosphoinositide 3-kinase; PLCγ, phospholipase Cγ; PtdIns, phosphatidylinositol; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10; SH, Src homology; WT, wild-type; GST, glutathione S-transferase; CMV, cytomegalovirus; PKB, protein kinase B.
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known downstream adapters also fail to contain authentic p85 binding sites. This strongly suggests that an unidentified docking protein couples VEGFR-2 to PI3K activation.

Gab1 (Grb2-adaptor binder 1) is the prototype of a subfamily of large multiadapter proteins sharing an N-terminal PH domain, two proline-rich regions involved in constitutive binding to Grb2, and multiple tyrosine phosphorylation sites. This family includes Gab1, Gab2, and Gab3 in mammals, DOS in Drosophila, and Soc-1 in Caenorhabditis elegans. Whereas Gab1 is widely distributed, Gab2 and Gab3 are more specific of the hematopoietic lineage (see Refs. 14 and 15 for reviews). Gab1 plays a critical role in the early development of multiple organs in mice, because it is a crucial signaling intermediate of major regulators of cell fate in solid tissues represented by the epidermal growth factor receptor (EGFR) and the hepatocyte growth factor receptor c-Met (16, 17). Indeed, Gab1 supplies to these receptors the indispensable tyrosine phosphorylation motifs necessary to mobilize essential effectors, including PI3K, the tyrosine phosphatase Shp2, and the PLCγ (18–20). These signaling functions result in Gab1 involvement in multiple cell responses mediated by the EGFR and/or c-Met, including apoptosis control, transformation, and morphogenesis (21–23). Other studies have suggested a role for Gab1 downstream of other growth factor or cytokine receptors, including the platelet-derived growth factor receptor or the insulin receptor (14, 15, 18, 24). However, these latter receptors can directly activate PI3K and Shp2 through intrinsic binding motifs or well known adapter proteins, respectively, and thus Gab1 function is considered much less essential for these receptors (14, 15). The Gab1 sequence also displays a novel phosphotyrosine-binding domain called the Met-binding domain (MBD) that can recognize c-Met and possibly the EGFR (25, 26). Moreover, PI3K can be also involved in Gab1 recruitment through its lipid product phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) that can associate with Gab1 PH domain (26, 27).

Considering the pending question of the coupling of VEGFR-2 to PI3K and the broad tissue distribution of Gab1, we tested its involvement in VEGF signaling in endothelial cells. We observed that Gab1 is rapidly phosphorylated and relocated in response to VEGF stimulation, which led us to conduct a series of experiments indicating that Gab1 is most likely the primary mediator of PI3K activation downstream of VEGFR-2.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant VEGF (catalog no. V7259), monoclonal anti-β-actin (catalog no. A5441), and anti-FLAG tag (catalog no. F3165) antibodies were from Sigma. Polyclonal anti-p85 (catalog no. 06-195) antibody was from Upstate. The monoclonal anti-Gab1 (catalog no. sc-9049) and monoclonal anti-Myc epitope tag (clone 9E10) were from Santa Cruz Biotechnology Inc., the monoclonal anti-T7 tag was from Novagen (catalog no. 69522–3), and the monoclonal anti-HA epitope tag (clone 12CA5) from Roche Applied Science. The monoclonal anti-pan-Ras antibody (catalog no. OP40) was from Oncogene Research Products. The monoclonal 4G10 anti-phosphotyrosine antibody was produced as previously described (28). The polyclonal anti-phosphoAkt (catalog no. 9271S), anti-phospho-Gab1-Tyr-627 (catalog no. 3231), and -Tyr-307 (catalog no. 3234) antibodies were from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated anti-mouse (catalog no. 7076) and -rabbit (catalog no. W4011) antibodies were from Cell Signaling Technology and Promega, respectively. Wortmannin was from Sigma, LY294002 from BIOMOL, and SU1498 and PP2 from Calbiochem. Cell culture reagents were from Invitrogen.

Expression Plasmids and Adenoviruses—The pcDNA3 plasmid encoding Myc-tagged Gab1-WT, Gab1-YF3 (mutated on the three PI3K-binding sites) and Gab1ΔPH (deleted of its PH domain), and the pCIneo constructs encoding the PH domain of Tec and its R29C mutant were already described (29). The constructs encoding HA-tagged PTEN and its G129E mutant were kindly provided by Prof. N. Tonks (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) (30). The pBatt-FLAG plasmids encoding Gab1 lacking the binding site to Grb2 SH3 domains (Gab1Grb2) and Gab1 with an invalidated MBD (Gab1ΔMet) were kindly provided by Dr. U. Schaeper (Berlin, Germany) (20). For adenovirus production, the sequences encoding Gab1-WT or Gab1-YF3 were subcloned into the shuttle plasmid pTrackCMV. Recombinant adenoviruses were then obtained according to the pAdEasy homologous recombination system (31).

Cell Culture, Stimulation, Transient Transfection, and Adenoviral Infection—Human umbilical vein endothelial cells (HUVECs) were grown in M199 supplemented with 10% fetal bovine serum, 20 mm l-glutamine, 10 μg/ml hydrocortisone, 2 ng/ml basic fibroblast growth factor, 1 ng/ml epidermal growth factor, 22.5 μg/ml heparin, and antibiotics as previously described (32). Before stimulation, cells were incubated overnight in M199 supplemented with 0.5% bovine serum albumin. Porcine aortic endothelial cells (PAECs) stably expressing VEGFR-2 (33) (kindly provided by Dr. M. Trombe, Toulouse, France) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, antibiotics, and 0.4 mg/ml G418 (Invitrogen, catalog no. 11811-031). Before stimulation, cells were incubated overnight in serum-free medium. Stimulations were performed using 50 ng/ml VEGF for 5 min, unless otherwise indicated.

For transient transfections of PAECs, subconfluent 100-mm plates were treated with a mixture containing 6 μl of FuGeneTM 6 reagent (Roche Applied Science) with 2 μg of the indicated DNA construct, according to the manufacturer’s instructions. The mixture was then added to cells incubated in normal culture medium for 24 h, before serum deprivation and stimulation. For adenoviral infection of PAECs or HUVECs, subconfluent plates were incubated with the indicated concentrations of adenoviruses during 24 h, then serum-starved overnight and stimulated or not with VEGF as indicated.

Cell Lysis, Immunoprecipitations, and Immunoblotting—Cells were scrapped off in lysis buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% glycerol, 1% Nonidot P-40, 10 μg of each aprotinin and leupeptin, and 1 mM orthovanadate. After shaking for 15 min at 4 °C followed by a 13,000 × g centrifugation for 15 min, soluble material was incubated with the appropriate antibody for 1 h at 4 °C. The antigen-antibody complexes were incubated with protein A-Sepharose (Amersham Biosciences) for 1 h, then collected by
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Analysis of Phosphatidylinositol Polyphosphate—Subconfluent cells grown in 100-mm plates were serum-starved for 16 h upon reaching 80–90% confluence and then labeled for 5 h with 100 \( \mu \text{Ci} \) of \( ^{32}P \)H\(_2\)PO\(_4\) (Amersham Biosciences) per milliliter in phosphate-free Dulbecco’s modified Eagle’s medium (Sigma). Cells were then stimulated for the indicated time and washed once with ice-cold phosphate-buffered saline before addition of 3.75 ml of 2.4 \( \mu \)Ci H\(_2\)PO\(_4\) solution. Then lipid extraction was performed as described previously (34, 36). Briefly, lipids were extracted by addition of 3 ml of methanol and 4.5 ml of chloroform followed by intense vortexing. After centrifugation, the lower phase containing the lipids was collected, and the upper phase was re-extracted with 4.5 ml of chloroform. The lower phases were then combined and evaporated under nitrogen, and lipid extracts were solubilized in 150 \( \mu \)l of chloroform/methanol (v/v) and first resolved by TLC using chloroform/acetone/methanol/acetic acid/water (80/30/26/24/14, v/v). After autoradiography, the TLC spots corresponding to the PtdIns(4,5)P\(_2\)/PtdIns(3,4,5)P\(_3\) area were scraped off, then deacylated in a solution containing methylene 40%/water/methanol/\( \mu \)-butanol (26.8/16.1/45.7/11.4, v/v), and finally analyzed by high-performance liquid chromatography on a Whatman Partisphere 5 SAX column as described (37).

RESULTS

VEGF Stimulates Gab1 Tyrosine Phosphorylation and Relocalization in Endothelial Cells—To identify a possible role of Gab1 in VEGF signaling, we examined if Gab1 was tyrosine-phosphorylated in stimulated HUVECs. Gab1 immunoprecipitations were thus performed from control or VEGF-treated cells, followed by anti-phosphotyrosine immunoblotting analysis. Fig. 1A shows that VEGF induced the tyrosine phosphorylation of Gab1. We confirmed this result using two antibodies recognizing Gab1 only if phosphorylated on Tyr-307 or Tyr-627. Fig. 1 (B and C) shows that both residues are phosphorylated in response to VEGF stimulation of HUVECs. Thus, Gab1 undergoes tyrosine-phosphorylation under VEGF stimulation, suggesting a role for Gab1 in VEGFR-2 signaling. To test this implication, we turned to PAECs stably expressing the human VEGFR-2, the only cell line that can be selectively activated through this receptor upon incubation with VEGF (33). PAECs express a low level of VEGFR-2, which is hardly detectable using immunoblotting (data not shown), and the strength of VEGF-induced signals is comparable in HUVECs and PAECs (see below). Fig. 1D shows that VEGF also stimulates Gab1 tyrosine phosphorylation in PAECs. In addition, VEGF was found to induce a rapid enrichment of Gab1 in membrane fractions prepared from this cell line, as displayed in Fig. 1E. Moreover, this redistribution seems to occur earlier than...
the VEGF-induced Akt phosphorylation. Altogether, these results indicate that Gab1 is mobilized following VEGFR-2 stimulation and suggest that this could mediate PI3K/Akt activation. In addition, as a first approach to determine whether Gab1 was phosphorylated directly by VEGFR-2 or by downstream signaling relays such as Src family kinases, we studied Gab1 phosphorylation in the presence of SU1498 or PP2, pharmacological inhibitors of the tyrosine kinase activity of VEGFR-2 and Src family kinases, respectively. As shown in Fig. 1 (F and G), each inhibitor suppressed the VEGF-induced Gab1 phosphorylation, both in HUVECs or PAECs. This suggests that VEGFR-2 kinase activity is not entirely accountable for Gab1 phosphorylation but requires a significant participation of Src family kinases.

VEGF Induces the Physical Association of Gab1 with p85 and with PI3K Activity, through Gab1 Tyr-X-X-Met Motifs—To determine if Gab1 is actually involved in PI3K activation, we searched for evidence of a physical interaction between Gab1 and PI3K. This was tested in PAECs using coimmunoprecipitation experiments between endogenous Gab1 and p85, the PI3K regulatory subunit. Firstly, using immunoblotting, p85 was found to be strongly enriched in Gab1 immunoprecipitates prepared from VEGF-treated cells in comparison with unstimulated control cells (Fig. 2A). In addition, we performed the reciprocal coimmunoprecipitation of Gab1 with p85 to further evaluate the association between these proteins. As displayed in Fig. 2B, Gab1 immunoprecipitation of p85 immunoprecipitates shows that the cell stimulation with VEGF induces a rapid increase of Gab1 amount in these fractions. Next, we determined if Gab1 immunoprecipitates contained a lipid kinase activity. An in vitro lipid kinase assay was thus performed on these immunoprecipitates using phosphatidylinositol (PtdIns) and [γ-32P]ATP as substrates. Following the reaction, lipids were extracted and analyzed by TLC and autoradiography. The results displayed in Fig. 2C show that PtdIns was converted into PtdIns 3-phosphate when Gab1 immunoprecipitates were prepared from VEGF-treated cells but not from unstimulated cells. We observed that PtdIns 3-phosphate production was suppressed when the cells were treated with wortmannin (W) before stimulation, which confirmed the kinase activity as being PI3K. Taken together, these results indicate that a physical interaction between endogenous Gab1 and p85 is strongly reinforced, if not created, by VEGF-2 stimulation.

The Gab1 sequence displays three Tyr-X-X-Met motifs that are potential phosphorylation sites recognized by p85 SH2 domains (18). To determine if these motifs are involved in the association between Gab1 and p85, we took advantage of a coimmunoprecipitation assay using a Gab1 plasmid expressing a presequence tag at its N terminus and Gab1 immunoprecipitation. As expected, the plasmid expressing the Gab1-Myc-WT fusion protein was immunoprecipitated by Myc antibody (Fig. 2D). In contrast, the Gab1-Myc-Xaa-WT plasmid did not precipitate Gab1 (Fig. 2E). These results indicated that Tyr-X-X-Met motifs are involved in Gab1 interaction with Gab1 and PI3K. This was confirmed by the fact that Gab1 immunoprecipitates precipitated Gab1-Myc-WT but not Gab1-Myc-Xaa-WT plasmids (Fig. 2F). These data suggest that Gab1 interaction with Gab1 and PI3K is actually involved in PI3K activation, since the Gab1-YF3 mutant was transiently transfected in PAECs, and its ability to interact with p85 was determined in comparison with that of wild-type (WT) Gab1. For convenience, this interaction assay was performed using a
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Gab1-YF3 Exerts a Dominant Negative Activity on PtdIns(3,4,5)P₃ Production in Intact Cells, on p85-associated Lipid Kinase Activity, and on Akt-PKB Phosphorylation in Response to VEGF—To investigate Gab1 importance in coupling VEGF-2 to the PI3K/Akt pathway, we used Gab1-YF3 as a dominant negative mutant. Because this protein does not bind p85 (Fig. 2D), its overexpression must prevent any Gab1-mediated PI3K activation by complementing all the functions of endogenous Gab1 except PI3K mobilization. We thus measured PI3K activation under VEGF stimulation in cells transiently transfected with Gab1-YF3. We first determined the cellular level of the major PI3K lipid product PtdIns(3,4,5)P₃ using high-performance liquid chromatography analysis of metabolically labeled phosphoinositides, which was achieved as follows: after transfection, cells were serum-starved overnight, then labeled with [³²P]H₃PO₄ before stimulation with VEGF. Lipids were then extracted, followed by TLC purification, and then phosphoinositides were deacylated and finally resolved by high-performance liquid chromatography. Fig. 3A displays the elution profiles of radioactive PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ from cells treated as indicated. They show that the generation of PtdIns(3,4,5)P₃ induced by VEGF is strongly reduced in cells expressing Gab1-YF3 in comparison with cells transfected with Gab1-WT or with the empty vector. The quantification of profiles obtained from several experiments showed that Gab1-YF3 expression leads to a significant decrease in PtdIns(3,4,5)P₃ production compared with cells transfected with empty vector or with Gab1-WT, whereas the level of PtdIns(4,5)P₂ was not

Fig. 2. VEGF induces a physical association of endogenous Gab1 with p85 and a PI3K activity. A, resting PAECs were stimulated with VEGF as indicated, then processed for Gab1 immunoprecipitation followed by anti-p85 (top) and anti-Gab1 immunoblotting (bottom). Lane -Ab, mock immunoprecipitation performed without adding the primary antibody. B, p85 immunoprecipitates were performed from resting or stimulated cells, then immunoblotted with anti-Gab1 (top) and anti-p85 (bottom) antibodies. C, in vitro lipid kinase assay: cells were stimulated or not with VEGF as indicated. Following cell lysis, cleared lysates were subjected to Gab1 immunoprecipitation, followed by an in vitro lipid kinase assay using PtdIns and [γ-³²P]ATP as substrates. After reaction, phospholipids were extracted then separated by TLC and visualized with a PhosphorImager. A representative TLC from three independent experiments is shown (top). Lane +W, cells were treated with 100 nm wortmannin before stimulation. Bottom, immunoprecipitate aliquots were subjected to anti-Gab1 immunoblotting to observe that each contained an equal amount of Gab1. D, cells were transfected with constructs encoding Myc-tagged Gab1, either wild-type (WT) or mutated on the three p85-binding sites (YF3), or FLAG-tagged Gab1 mutated on the Grb2-binding site (ΔGrb2), as indicated. After stimulation, cells were lysed and incubated with beads bound to a GST fusion protein containing, or not, the p85 SH2 domains, as shown. The amount of Gab1 associated with the beads was determined by anti-Myc immunoblotting (IB Myc, top). Bottom, anti-Myc immunoblotting of corresponding lysates to control the expression level of the Gab1-Myc constructs. E, cells were transfected with constructs encoding Myc-tagged Gab1, either wild-type (WT) or mutated on the three p85-binding sites (YF3), or FLAG-tagged Gab1 mutated on the Grb2-binding site (ΔGrb2), as indicated. After stimulation, cells were lysed and incubated with beads bound to a GST fusion protein containing, or not, the Grb2 protein, as shown. The amount of Gab1 associated with the beads was determined by anti-Myc or anti-FLAG immunoblotting (IB Myc or IB flag, top). Middle and bottom, respectively, anti-Myc and anti-FLAG immunoblotting of corresponding lysates to control the expression level of the Gab1 constructs.

GST-p85 fusion protein. Fig. 2D shows that Gab1-WT from VEGF-treated cells was efficiently pulled down by GST-p85, which confirms that Gab1 can interact with p85 in response to VEGF stimulation. Moreover, in sharp contrast with Gab1-WT, Gab1-YF3 has completely lost the ability to interact with p85. To verify that this construct retained the ability to bind Grb2, we used a GST-Grb2 fusion protein in this assay instead of GST-p85. Fig. 2E shows that Gab1-YF3 was pulled down by GST-Grb2 almost as efficiently as Gab1-WT, whereas, as a negative control, Gab1 specifically mutated on the Grb2-binding site (ΔGrb2) was not precipitated. Altogether, these results indicate that Gab1-YF3 has lost the ability to associate with p85 but not with Grb2. As a whole, the results shown in Figs. 1 and 2 indicate that VEGF-R2 stimulation leads to Gab1 phosphorylation and to its association with p85/PI3K through Tyr-X-Met motifs. These data also suggest that Gab1 could be an important mediator of PI3K activation downstream of VEGF-2, because none of its known signaling intermediates displays these features.
significantly modified (Fig. 3B). Taking into account that, under these experimental conditions of biochemical transfection, the percentage of transfected cells was ~50% (data not shown), these data suggest that Gab1 plays a major role, if not a critical one, in PI3K activation downstream from VEGFR-2.

To confirm Gab1 function in this pathway, we also measured PI3K activation in p85 immunoprecipitates prepared from cells transfected or not with Gab1-YF3, using the in vitro lipid kinase assay described in Fig. 2B. As shown in Fig. 3C, Gab1-YF3 expression strongly decreased the amount of PI3K activity in p85 immunoprecipitates, in comparison with cells transfected with empty vector or with Gab1-WT. These data also support the view of a major role for Gab1 in VEGFR-2-mediated PI3K activation.

To further establish Gab1 function in this pathway, we designed adenoviruses expressing Gab1-WT or its YF3 mutant derivative. These viruses also encode green fluorescent protein and can infect >90% of cells in PAEC or HUVEC monolayers (data not shown). The effect of these adenoviruses on PI3K signaling was measured using Akt phosphorylation as a readout. As shown in Fig. 4A, infection of PAECs with Gab1-YF3 adenoviruses totally abolished Akt phosphorylation induced by VEGF. As a control, cells infected with the Gab1-WT adenoviruses sometimes displayed a reduced Akt phosphorylation, but this effect was not statistically significant, as shown by the graph representing the quantitative analysis of Akt phosphorylation (Fig. 4A). We conclude that Gab1-YF3 has a potent dominant negative effect on VEGF-induced Akt activation, which further designates Gab1 as an essential signaling intermediate between VEGFR-2 and PI3K.

Adenoviral Expression of Gab1-YF3 Prevents the VEGF-induced Recovery of Wounded HUVEC Monolayers—The above data demonstrating a role for Gab1 in coupling VEGF-2 to PI3K suggest that Gab1 must be significantly involved in the biological activities of VEGF. To test this assumption, we examined whether expression of the Gab1-YF3 mutant influences the recovery promoted by VEGF of a mechanically wounded endothelial cell monolayer, a process involving cell migration and proliferation under the control of PI3K (2–6). To perform this assay, an area of a HUVEC monolayer was denuded by mechanical scraping, and then the
recolonization of this area by the cells was monitored by microscopy. To study Gab1 involvement in this process, HUVECs were infected with adenoviruses encoding Gab1-WT or -YF3 24 h before the intervention. As displayed in Fig. 4B, observation of the wound 48 h after the aggression shows that Gab1-YF3 dramatically impaired the VEGF-induced recolonization of the denuded area, in comparison with cells expressing Gab1-WT. As shown in the graph collecting data from similar experiments, Gab1-YF3 inhibited the recolonization by ~50% in comparison with cells expressing Gab1-WT. Moreover, Gab1-YF3 abolished the effect of VEGF on the recolonization, because the free area when cells were treated with Gab1-YF3 and VEGF was not different from that of unstimulated cells (Fig. 4B, graph). This indicates that the role of Gab1 in coupling VEGF-2 to PI3K has functional implications for VEGF biological activity by mediating its ability to promote wound healing.

VEGF-2 Recruits Gab1 through Grb2 and PI3K Lipid Products—Several Gab1 domains can potentially mediate its recruitment by VEGF-2, including proline-rich regions constitutively bound to Grb2, the phosphotyrosine-binding domain MBD, or the PH domain (18). To define how VEGF-2 mobilizes Gab1, we used Gab1 constructs mutated on each of these domains. These mutants were transfected in PAECs, and their “recruitability” was monitored by studying their ability to associate with membrane fractions in response to VEGF. As shown in Fig. 5 (A and B), whereas Gab1-WT or Gab1 deleted of the MBD (ΔMet) were readily enriched in membrane fractions prepared from VEGF-treated cells, the Gab1 mutants deficient for binding to Grb2 (ΔGrb2) or deleted of the PH domain (ΔPH) were not redistributed, suggesting that VEGF-2 mobilizes Gab1 essentially through Grb2- and PH domain-mediated interactions.

To further define the role of these domains in Gab1 function and recruitment, we studied the influence of their corresponding Gab1 mutants on Akt activation. This was achieved by the cotransfection of these Gab1 constructs with HA-tagged Akt, to monitor Akt activation only in transfected cells. Following cell stimulation, HA-Akt was immunoprecipitated, and its phosphorylation was studied using immunoblotting with a phosphospecific antibody. Fig. 5C shows that transfection of Gab1 constructs lacking the PH domain or the Grb2-binding sites suppressed Akt phosphorylation stimulated by VEGF, which also indicates that...
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**FIGURE 5.** Role of Gab1 domains in Akt activation and Gab1 recruitment mediated by VEGFR-2. A, PAECs were transfected with constructs encoding Gab1-Myc wild type (WT) or deleted of its PH domain (ΔPH). After stimulation with VEGF when indicated, cells were scrapped off and membrane fractions were prepared as above. Fractions were then analyzed by anti-Myc immunoblotting (top) and an anti-Ras antibody to verify that equal amounts of membrane proteins were present in each sample (middle). Bottom, lysate aliquots were analyzed by anti-Myc immunoblotting to control Gab1-Myc constructs expression. B, PAECs were transfected with a FLAG-tagged Gab1 construct mutated on its binding sites to Grb2 (ΔGrb2) or to c-Met (ΔMet). The experiment was then processed as in A, except that immunoblots were revealed with anti-FLAG instead of anti-Myc antibody. C, PAECs were cotransfected or not with a vector encoding HA-tagged Akt and the indicated Gab1 construct. Following stimulation, cells were processed for HA-Akt immunoprecipitation, followed by anti-phospho-Akt immunoblotting (top). Aliquots of corresponding lysates were also analyzed by anti-HA, anti-Myc, or anti-FLAG immunoblotting to control the expression of the different constructs (middle) and bottom. D, PAECs were transfected with the Gab1-Myc-WT plasmid. Before stimulation, cells were treated for 15 min with 100 nM wortmannin (+W) or 20 μM LY294002 (+LY) when indicated. After stimulation, cells were lysed, then membrane fractions were prepared and analyzed as in A, E, resting PAECs transfected with the Gab1-Myc-WT plasmid were stimulated or not by VEGF, then lysed and processed for Gab1-Myc immunoprecipitation (IP Myc) followed by anti-phosphotyrosine immunoblotting (IB pTyr, top). Before stimulation, cells were treated for 15 min with 100 nM wortmannin (+W) or 20 μM LY294002 (+LY) as indicated. The immunoprecipitates were also revealed by anti-Myc immunoblotting (IB Myc, bottom). Lane -Ab, mock immunoprecipitation was performed from a 5-min-stimulated PAEC lysate without adding anti-Myc antibody. F, PAECs expressing Gab1-Myc-WT were cotransfected or not with a construct encoding HA-tagged PTEN wild type (WT) or mutated on its catalytic site (G129E). Following transfection, cells were stimulated or not (−) with VEGF and then scrapped off, and membrane fractions were prepared as above. Fractions were then analyzed by anti-Myc (top) and an anti-Ras (middle) immunoblotting to verify that equal amount of membrane proteins were present in each sample. Bottom, lysate aliquots were analyzed by anti-Myc and anti-HA immunoblotting to control expression of Gab1 and PTEN constructs. G, PAECs expressing Gab1-Myc-WT were cotransfected or not (−) with a construct encoding the T7-tagged PH domain of Tec (WT) or its derivative mutated on Arg-29 (R29C). Cells were then processed as in F, except that we used an anti-T7 instead of an anti-HA antibody.

both Grb2 and the PH domain are essential for Gab1 recruitment and function in PI3K signaling. Because Gab1 PH domain has binding affinity for PtdIns(3,4,5)P₃ (27), these data also suggested that PI3K could be an upstream regulator of Gab1 recruitment by VEGFR-2, in addition to being a downstream Gab1 effector. To test this hypothesis, we examined the role of PI3K in Gab1 mobilization and phosphorylation during VEGF stimulation. This was first achieved by treating cells with PI3K pharmacological inhibitors. Fig. 5D shows that wortmannin or LY294002 totally abolished the membrane redistribution of Gab1 induced by VEGF. In agreement with this, the VEGF-stimulated Gab1 tyrosine phosphorylation was also suppressed by PI3K inhibitors (Fig. 5E), suggesting that Gab1, in addition to its association with Grb2, is recruited by an interaction between its PH domain and PtdIns(3,4,5)P₃.

To further test this conclusion, we used two other experimental strategies to interfere with PI3K activation. Firstly, the lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) was overexpressed in PAECs to increase the degradation of PI3K lipid products. As shown in Fig. 5F, PTEN overexpression prevented Gab1 membrane relocation. As a control, overexpression of the PTEN G129E mutant unable to recognize PtdIns(3,4,5)P₃ as a substrate (30) did not block this translocation and even stimulated it. These data thus further confirm that PI3K lipid products are essential for Gab1 recruitment, because an increase of their degradation by PTEN overexpression blocks Gab1 mobilization.

We then used another strategy to interfere with PI3K lipid products to further validate their role in Gab1 recruitment. The PH domain of the kinase Tec binds PtdIns(3,4,5)P₃ with high affinity, and it can be used as a competitor for binding to PtdIns(3,4,5)P₃ when overexpressed in transfected cells (29, 39). As shown in Fig. 5G, transfection of Tec-PH completely abrogated the VEGF-induced membrane redistribution of Gab1. We observed that the overexpression of Tec-PH mutated on Arg-29, a residue essential for interaction with PtdIns(3,4,5)P₃ (40), did not produce any modification of Gab1 recruitment compared with cells transfected with the empty vector. These results thus indicate that PtdIns(3,4,5)P₃ is essential for Gab1 recruitment downstream of VEGFR-2. Taken together, the Fig. 5 results demonstrate that Grb2 is essential for Gab1 recruitment and that PI3K is an upstream
regulator of Gab1 by promoting its mobilization through its PH domain.

DISCUSSION

Although PI3K is a major intracellular signaling relay of the biological activity of VEGF, the mechanisms coupling VEGFR-2 stimulation to PI3K activation are unclear. Several groups reported experiments suggesting that the receptor binds p85 (41) or that adapter proteins such as VEGF receptor-associated protein (VRAP/TSAd) and Shb promote PI3K activation downstream of VEGFR-2 (42–44). However, none of these proteins display a single consensus motif required to bind p85 SH2 domains, implying that their participation in PI3K activation is an indirect consequence of their ability to induce the formation of large signaling complexes. Within these complexes, at least one other partner must be involved in a direct and phosphorylation-dependent binding to p85, which represents a critical step in PI3K activation by most growth factors (12, 13).

This study reports several data demonstrating that Gab1 is a strong candidate for this function. Firstly, Gab1 is rapidly tyrosine-phosphorylated and relocated to membrane fractions upon VEGF stimulation of endothelial cells. Secondly, we were able to detect upon VEGF stimulation an enrichment of p85 in immunoprecipitates of endogenous Gab1 and reciprocally. We could also measure a Gab1-associated lipid kinase activity stimulated by VEGF. This was already a strong indication that Gab1 plays a significant role in PI3K activation downstream of VEGFR-2, because the identification of interactions using coimmunoprecipitation of endogenous protein is not a sensitive method. In addition, pull-down experiments with a GST-p85 fusion protein confirmed the existence of an interaction promoted by VEGF between p85 and wild-type Gab1 but not with a Gab1-YF3 mutant lacking all p85-binding tyrosines.

We next used Gab1-YF3 as a dominant negative mutant to define the relative importance of Gab1 in VEGFR-2-mediated PI3K activation. Indeed, it was reported that, when overexpressed, Gab1-YF3 exerts a potent dominant negative activity on Gab1-mediated PI3K activation by displacing endogenous Gab1 from signaling complexes (26, 29). This was taken as evidence that Gab1 plays a primary role in EGF signaling, a finding that was later confirmed by a gene knock-out/knock-in approach (45). We thus choose to overexpress Gab1-YF3 in endothelial cells to study Gab1 function in VEGFR-2 signaling.

Using this strategy to block endogenous Gab1, we monitored its effect on PI3K activation through different methods. By performing lipid kinase assays on p85 immunoprecipitates, Gab1-YF3 expression was found to strongly repress the stimulatory activity of VEGF on PI3K. Moreover, we were able to study directly the level of PI3K activation in intact cells by isolating the major PI3K lipid product PtdIns(3,4,5)P3 from metabolically labeled cells. Although this requires a complex methodology, it is the only technique that allows for direct monitoring or PI3K activation in intact cells. Using this method, we observed that transfection of Gab1-YF3 reduced by half the generation of PtdIns(3,4,5)P3 induced by VEGF. Taking into account that no more than 50% of the cells express the transfected construct in these transient transfection experiments (data not shown), the extent of PtdIns(3,4,5)P3 inhibition by Gab1-YF3 implies that Gab1 plays a critical role in VEGFR-2-mediated PI3K activation.

To circumvent the issue of partial transfection, we produced adenoviruses expressing both green fluorescent protein and either Gab1-WT or Gab1-YF3. These vectors indeed allow us to observe green fluorescent protein expression in >90% of cells in PAEC or HUVEC monolayers (data not shown). When cells were infected with adenoviruses expressing Gab1-YF3, this resulted in the total suppression of VEGF-induced Akt phosphorylation, which fully supports the conclusions drawn from transient transfection experiments. Moreover, adenoviral expression of Gab1-YF3 strongly altered the recovery of wounded HUVEC monolayers, a process promoted by VEGF and requiring cell proliferation and migration under the control of PI3K (2–6). This allowed us to verify that Gab1 involvement in VEGFR-2 signaling has important functional consequences for endothelial cells.

We also started to decipher how VEGFR-2 recruits Gab1. Several Gab1 domains could be involved, including the prolinerich regions bound to Grb2, the phosphotyrosine-binding domain MBD, or the PH domain (18). We thus used Gab1 constructs mutated on each of these domains to apprehend their function in Gab1 recruitment. We did not find evidence of an involvement of the MBD, which indicated that, unlike c-Met and the EGF receptor, the VEGFR-2 does not contain motifs prone to be recognized by this phosphotyrosine-binding domain. In contrast, the Gab1 Grb2-binding region and PH domain were found to be essential, because Gab1 constructs altered in one of these regions were not redistributed to membrane fractions upon VEGF stimulation. Moreover, these mutants exerted a potent inhibition of Akt activation, further demonstrating the role of Gab1 in PI3K/Akt activation. The observation that Grb2 mediates Gab1 recruitment is not unexpected considering that the Shc/Grb2 pathway seems to be activated by VEGF, at least in PAECs (46). More interestingly, the apparent critical role of the PH domain in Gab1 recruitment suggested that PI3K may also be an upstream activator of Gab1, because its particular PH domain has binding affinity for PtdIns(3,4,5)P3 (27). In support of this hypothesis, we observed that PI3K inhibitors completely blocked both Gab1 redistribution to membrane fractions and its tyrosine phosphorylation induced by VEGF stimulation.

We then used other strategies to interfere with PI3K activation and thereby define its participation in Gab1 recruitment. Overexpression of the phosphatase PTEN that degrades specifically PI3K lipid products was found to prevent Gab1 mobilization. In contrast, the PTEN G129E mutant, unable to recognize PtdIns(3,4,5)P3 (30), somewhat overstimulated Gab1 recruitment, most likely through a dominant negative effect of this mutant on endogenous PTEN, resulting in a more sustained PI3K activation. Therefore, these experiments fully support the notion that PI3K promotes Gab1 mobilization by VEGFR-2. Significant evidence supporting concept was the overexpression of Tec kinase PH domain as a competitor for PtdIns(3,4,5)P3 bind-
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FIGURE 6. Model illustrating Gab1 involvement downstream from VEGFR-2. See “Discussion” for details.

of these studies (48), using Gab1-YF3 as a dominant negative mutant, that Gab1 mediates this activation downstream of tyrosine kinases activated by the flow. In agreement with this report, our study designates Gab1 as an essential regulator of the PI3K/Akt pathway in endothelial cells.

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