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Gab1 Contributes to Cytoskeletal Reorganization and Chemotaxis in Response to Platelet-derived Growth Factor*

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Gab1 is a scaffolding/docking protein that has been suggested to play a role in signal transduction downstream of certain plasma membrane receptors, including platelet-derived growth factor (PDGF) receptors. We found that PDGF induced a rapid Gab1 phosphorylation, which depended on the recruitment of Grb2, indicating that Grb2 acts as a bridge between Gab1 and the PDGF β-receptor. PDGF also enhanced the binding of Gab1 to the phosphatase SHP-2, but not to p85. To further study the role of Gab1 in PDGF signaling, we transfected porcine aortic endothelial cells with a doxycycline-inducible Gab1 construct. Increased Gab1 expression enhanced the recruitment and activation of SHP-2, as well as the phosphorylation of the mitogen-activated protein kinases Erk and p38 by PDGF. Gab1 expression also enhanced the formation of lamellipodia and cellular protrusions. In Gab1-deficient mouse embryonic fibroblasts, the same phenotype was induced by restoring the expression of wild-type Gab1, but not a mutant Gab1 that was unable to associate with SHP-2. These effects of PDGF on the actin cytoskeleton were not altered by the inhibition of p38 or Erk, but could be blocked by a dominant-negative form of Rac (Asn^{17}). Finally, Gab1-deficient fibroblasts showed a decreased chemotactic response toward gradients of PDGF as compared with wild-type cells. In conclusion, Gab1 plays a selective role in the regulation of the mitogen-activated protein kinases Erk and p38 downstream of the PDGF β-receptor, and contributes to cytoskeletal reorganization and chemotaxis in response to PDGF.

Platelet-derived growth factor (PDGF),[1] a major mitogen for connective tissue cells, exerts its cellular effects by binding to PDGF α- and β-receptors, which belong to the receptor tyrosine kinase family. Upon ligand binding, PDGF receptors are phosphorylated and interact with at least 11 different types of Src homology (SH)2 domain-containing signaling molecules (reviewed in Ref. 1). These signaling proteins include the SH2-containing phosphatase 2 (SHP-2), phospholipase Cγ, the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K), the tyrosine kinase Src, the transcription factor Stat5, and the adaptor proteins Grb2 and Shc. In addition, many of these mediators also interact with large scaffolding proteins, which are recruited to the receptor complex and phosphorylated on multiple tyrosines.

One such scaffolding protein is Gab1, which contains a PH-domain that binds to phosphatidylinositol-trisphosphate in the plasma membrane and is important for correct subcellular localization of Gab1 (2–7). Gab1 binds to several receptor tyrosine kinases, either directly, as in the case of hepatocyte growth factor (HGF) receptor (c-Met) (8, 9), or indirectly by means of another adaptor protein, Grb2, *e.g.* to epidermal growth factor (EGF) receptor and nerve growth factor (NGF) receptor (reviewed in Ref. 5). This interaction allows the tyrosine phosphorylation of Gab1, which is required for the recruitment of down-stream SH2-domain-containing proteins. The most prominent down-stream interaction partners described for Gab1 so far are p85 and SHP-2 (10, 11), both of which are also directly associated with and activated by the PDGF receptors (reviewed in Ref. 1). The association of p85 with Gab1 plays an important role in the activation of PI3K and its downstream effectors by EGF (10) and HGF (12). It has also been reported that mutations of the p85 binding sites of Gab1 decrease Akt activation and apoptosis inhibition by NGF (13). Recruitment of the tyrosine phosphatase SHP-2 by Gab1 enhances activation of the Erk mitogen-activated protein kinase (MAPK) pathway (4).

Gab1-null mice die *in utero* and show a phenotype reminiscent of the those of mice lacking functional HGF, PDGF, and EGF signaling pathways and with developmental defects in heart, placenta, and skin (14, 15). These observations, and the fact that not much is known about the importance of Gab1 downstream of PDGF receptors, led us to investigate the role of Gab1 in PDGF signaling. We observed that Gab1 was activated by PDGF stimulation, which induced the association of Gab1 with SHP-2, but not with p85, in contrast to what was reported in response to HGF and EGF. Using cells in which Gab1 ex-

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[1] The abbreviations used are: PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; SH2, Src homology 2; SHP-2, SH2-containing phosphatase 2; HGF, hepatocyte growth factor; EGF, epidermal growth factor; NGF, nerve growth factor; MAPK, mitogen-activated protein kinase; FBS, fetal bovine serum; MEF, mouse embryonic fibroblasts; DMEM, Dulbecco's modified Eagle's medium; PAE, porcine aortic endothelial; BSA, bovine serum albumin; PDGFRβ, PDGF β-receptor; WGA, wheat germ agglutinin; FITC, fluorescein isothiocyanate; AMCA, 7-amino-4-methylcoumarin-3-acetic acid; TRITC, tetramethyl rhodamine isothiocyanate; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride.

pression is inducible, we could demonstrate that Gab1 phosphorylation is linked to the activation of Erk-1, Erk-2, and p38. Our data point to a role for Gab1 in cell motility, but not in the mitogenic response of cells to PDGF.

MATERIALS AND METHODS

Cell Culture—PAE TetON cells were grown in Ham's F-12 medium supplemented with 10% Tet system approved FBS (Clontech) and L-glutamine. Mouse embryonic fibroblasts (MEF) were grown in F-12 medium supplemented with 10% calf serum and L-glutamine, and COS1 cells were grown in DMEM supplemented with 10% FBS. For starvation, both PAE and MEFs were washed in plain F-12 medium and then incubated in F-12 medium containing 0.1% of essentially fatty acid-free bovine serum albumin (BSA) and L-glutamine for 24 h. COS1 cells were washed in DMEM and then starved in DMEM supplemented with 0.1% BSA.

Antibodies and Reagents—Antibodies toward phosphotyrosine ($pTyr^{99}$), PDGFRβ (958), Gab1 (H-189), SHP-2 (C-18 and N-16), Stat5 (N-20), and Akt1/2 (H-136) were obtained from Santa Cruz Biotechnology; anti-HA antibodies were from Roche Diagnostic Corporation; anti-phosphorylated ($pThr^{202}/pTyr^{204}$) Erk-1 and −2, anti-phosphorylated ($pSer^{473}$) Akt, anti-p38, anti-phosphorylated ($pTyr^{694}$) Stat5, anti-phosphorylated ($pTyr^{705}$) Stat3, and anti-Stat3 were from Cell Signaling Technology; anti-phosphorylated ($pThr^{180}/pTyr^{182}$) p38 was from BIOSOURCE. Antiserum recognizing Erk-2 was raised as described previously (16). For immunofluorescence also, anti-Myc and anti-HA antibodies from Santa Cruz Biotechnology were used, together with secondary fluorescein isothiocyanate (FITC)-conjugated anti-rabbit and anti-mouse antibodies (Dako), 7-amino-4-methylcoumarin-3-acetic acid (AMCA)-conjugated anti-mouse antibodies (Jackson ImmunoResearch) and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated phalloidin (Sigma). Doxycycline hydrochloride was purchased from Sigma. U0126 and SB203580 were obtained from Calbiochem.

DNA Constructs and Cell Transfection—Wild-type N-terminally HA-tagged Gab1 in pcDNA3 has been described elsewhere (17). The HA-tagged Gab1 insert was transferred to the pUHD10.3 vector, and the construct was used for the establishment of a doxycycline-dependent, Gab1 over-expression system in PAE cells stably transfected with PDGF β-receptor (PAE/Rβ/Gab1); the procedure described previously (18, 19) was followed except that LipofectAMINE PLUS (Invitrogen) was used for transfection. Clones were screened for doxycycline-regulated Gab1 expression by Western blotting with HA-antibodies.

The Y627F/Y659F mutant HA-Gab1 in pcDNA3 was created using the QuikChange™ mutagenesis protocol (Stratagene). The oligonucleotides used were GGA GAC AAA CAG GTG GAA TTC TTA GAT CTC GAC TTA GAT and ATC TAA GTC GAG ATC TAA GAA TTC CAC CTG TTT GTC TCC for the Y627F mutation. For the Y659F mutant, the oligonucleotides AGA TGA GAG AGT GGA TTT TGT TGT TGT TGA CCA AC and GTT GGT CAA CAA CAA CAA CAA AAT CCA CTC TCT CAT CT were used. For the creation of the ΔGBS1 mutant, Gab1 oligonucleotides GCA GAC TGT GAA CCA AAC CTC AAG CCA GAC and GTC TGG CTT GAG GTT TGG TTC ACA GTC TGC were used, and for the ΔGBS2 mutant, CTA TTC CAG ATA TTC CTA AAC CAC ATC CAG CTC A and TGA GCT GGA TGT GGT TTA GGA ATA TCT GGA ATA G were used. Myc-tagged, dominant-negative Asn^{17}-Rac was a kind gift from Ninna Richnau and Pontus Aspenström. All transfections were performed using LipofectAMINE Plus™ (Invitrogen), according to the manufacturer's instructions.

Immunoprecipitation and Western Blotting—After starvation in 0.1% BSA overnight, cells were treated with 50 ng/ml of PDGF-BB for different time periods or left untreated. Cells were then washed once with ice-cold PBS and lysed in 1% Triton X-100, 10% glycerol, 100 mM NaCl, 5 mM EDTA, 20 mM Hepes (pH 7.4), 1% Trasylol, 1 mM phenylmethylsulfonyl fluoride, and 500 μM Na_3VO_4. Incubations with antibodies were performed overnight in the cold, protein A-Sepharose beads were added, and incubation was prolonged with end-over-end mixing for 2 h. The beads were washed three times in lysis buffer and once in distilled H_2O, boiled with sample buffer containing dithiothreitol, and eluates were separated by SDS-PAGE. To isolate the PDGFRβ, wheat germ agglutinin (WGA)-Sepharose (Amersham Biosciences) was added to the lysates, and samples were incubated end-over-end in the cold for 2 h. The samples were then washed and treated as described for immunoprecipitates.

For Western blotting, samples were electro-transferred to polyvinylidene difluoride (PVDF) membranes, which were blocked in 5% milk powder (anti-phospho-site-specific antibodies) or 5% BSA (other antibodies) in Tris-buffered saline solution containing 0.1% Tween 20. Primary antibodies were used at concentrations recommended by the suppliers in the same buffer and in overnight incubations in the cold. After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (both from Amersham Biosciences), and proteins were visualized using ECL Western blotting detection systems from either Roche Applied Science or Santa Cruz Biotechnology on a cooled charge-coupled device camera (Fuji). Quantifications were done using Aida image analyzer software, version 3.10.

GST Pull-down Experiments—For GST pull-down experiments, lysates from COS1 cells were incubated with 3 μg of each GST fusion protein, glutathione-Sepharose beads were then added, and tubes were incubated end-over-end in the cold. After extensive washing, the beads were treated like protein A-Sepharose beads during immunoprecipitation, as described above. In Figs. 3–5, lanes marked "lysate" were loaded with a volume of lysate equal to 5% of the amount taken for each pull-down.

Phosphatase Assay Using 4-Nitrophenyl-phosphate—After starvation and PDGF stimulation, PAE cells were lysed as above (without Na_3VO_4 and in the presence of 2 mM dithiothreitol), and SHP-2 was immunoprecipitated using an antibody raised against the N-terminal part of the protein. Protein A-Sepharose was added and the beads were washed extensively in lysis buffer. The beads with bound SHP-2 were then incubated in 50 mM sodium 4-nitrophenyl-phosphate (Fluka Chemie AG) for 15 min at 30 °C. The reaction was terminated by the addition of 0.2 M NaOH, and the optical density (OD) of the solution was read at 410 nm. Data are shown as fold increase of OD after PDGF stimulation.

Immunofluorescence—Cells were seeded on cover slips and starved, then pretreated and stimulated as described in legends to Figs. 1–6. For transient transfection of cells with different forms of Gab1 and Asn^{17}-Rac, LipofectAMINE Plus™ (Invitrogen) was used according to manufacturer's instructions. After stimulation, cells were fixed in PBS containing 2.7% formaldehyde for 20 min at room temperature. Cells were then washed three times in ice-cold PBS and permeabilized in 0.2% Triton X-100 diluted in PBS for 5 mins. After washing in PBS, the cells were blocked in 5% FBS in PBS for 1 h. The coverslips were then exposed consecutively to primary and secondary antibodies diluted in PBS containing 5% FBS, overnight and for 1 h, respectively, at 4 °C. Coverslips were washed and mounted using Fluoromount-G (Southern Biotechnologies Associated, Inc) and photographed with a Hamamatsu ORCA charge-coupled device digital camera, using the QED imaging system software with a Zeiss Axioplan 2 microscope.

RESULTS

Gab1 Associates with SHP-2 and Grb2, but Not with p85, upon PDGF Stimulation—To study the role of Gab1 in PDGF signaling, we first analyzed the association of Gab1 with other proteins in PAE cells expressing PDGFRβ at a physiological level. By immunoprecipitation, we observed a consistent phosphorylation of Gab1 on tyrosine residues in response to PDGF-BB stimulation as monitored by immunoblotting with anti-phosphotyrosine antibodies (Fig. 1*A*). PDGF stimulation also caused a shift in the apparent molecular mass of the protein as detected by immunoblotting with Gab1 antibodies (Fig. 1*A*). These observations are in line with previously published work using other cell lines (14). SHP-2 was also phosphorylated in response to PDGF, and both SHP-2 and Grb2 co-precipitated with Gab1 upon PDGF stimulation, but p85 and PDGFRβ were not detectable in Gab1 immunoprecipitates (Fig. 1*A*). Instead, p85 strongly associated with phosphorylated PDGFRβ, as shown by immunoprecipitations of either of these two proteins. These findings suggest that activated PDGFRβ interacts directly with p85, as described before (6, 20), and indirectly with Gab1. SHP-2 also bound to Gab1 and to a phosphorylated component of a size corresponding to PDGFRβ (see phosphotyrosine blot, Fig. 1), which is consistent with previous work showing that SHP-2 interacts with PDGFRβ (21).

Grb2 Binding Is Required for Gab1 Phosphorylation and Association with PDGFRβ—Previous reports indicated that Grb2 binds constitutively to Gab1. Using pull-down experiments with immobilized recombinant GST-Grb2 chimeras and

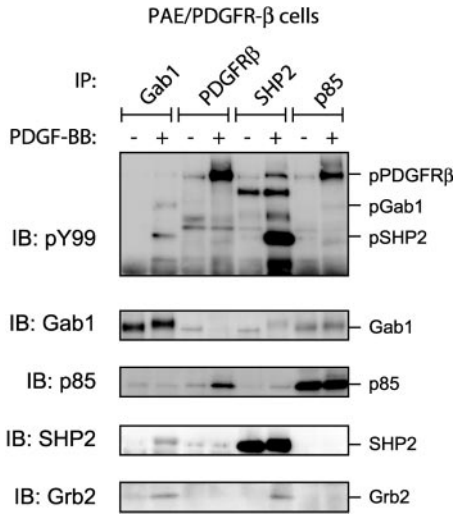

FIG. 1. **PDGF induces the association of Gab1 with SHP-2 and Grb2, but not p85.** PAE cells stably transfected with the human PDGFRβ (PAE/Rβ cells) were starved in medium containing 0.1% BSA and stimulated with 50 ng/ml PDGF-BB for 8 mins or left untreated. Immunoprecipitations were performed with antibodies toward Gab1, PDGFRβ, SHP-2, or p85, and precipitated proteins were separated by SDS-PAGE. After electrotransfer to a PVDF membrane, immunoblotting using antibodies against phosphotyrosine (pTyr^{99}), Gab1, p85, SHP-2, and Grb2 was performed.

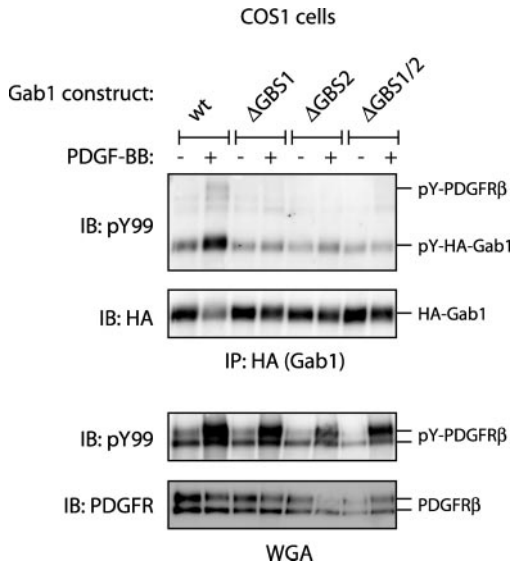

FIG. 2. **The Gab1-Grb2 interaction is required for PDGF-induced Gab1 phosphorylation.** COS1 cells were transfected with PDGFRβ and either wild-type or mutant HA-Gab1, as indicated. After starvation, cells were either stimulated with 50 ng/ml PDGF-BB for 8 min or left untreated, and then lysed. One fraction of the lysates was used for immunoprecipitation with anti-HA antibodies and the other was used for WGA precipitation. After SDS-PAGE separation and electrotransfer, the PVDF-membranes were probed sequentially with anti-phosphotyrosine and anti-HA antibodies (HA immunoprecipitates) or with anti-phosphotyrosine and anti-PDGFRβ antibodies (WGA precipitates).

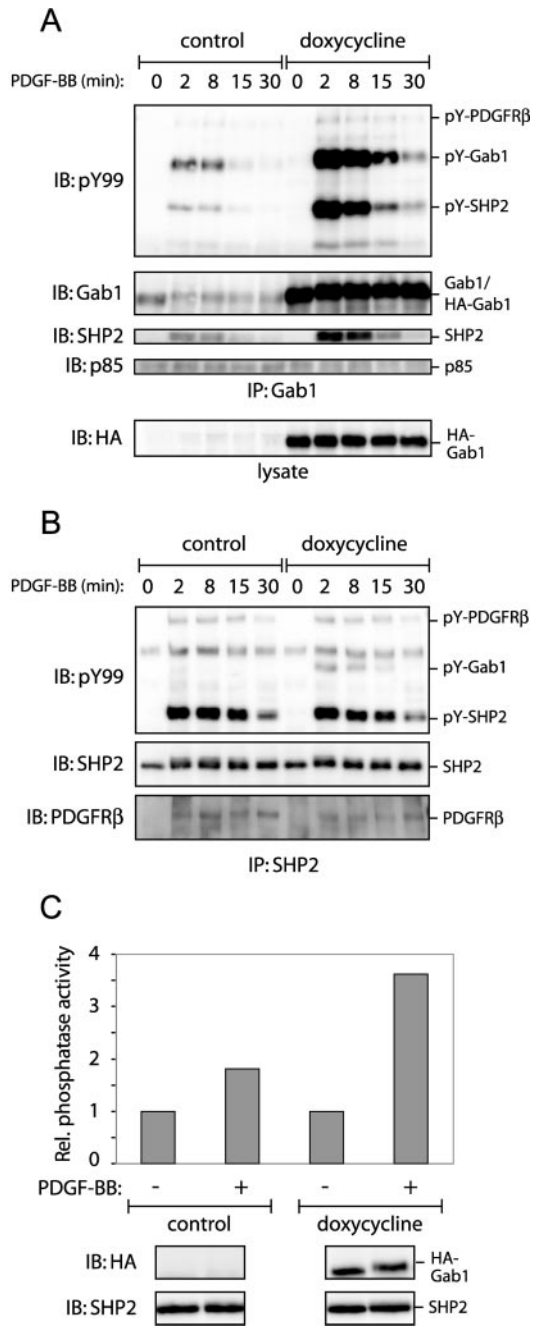

FIG. 3. **Gab1 over-expression causes increased PDGF-induced association between Gab1 and SHP-2, and increased SHP-2 phosphatase activity.** *A*, PAE/Rβ/Gab1 cells were treated or not with 200 ng/ml doxycycline for 48 h and kept in starvation medium with 0.1% BSA for the last 24 h. Cells were then stimulated with 50 ng/ml PDGF-BB for 0, 2, 8, 15, and 30 min, washed in PBS, and lysed. Gab1 was immunoprecipitated and analyzed by Western blotting with anti-phosphotyrosine, anti-Gab1, anti-SHP-2, or anti-p85 antibodies. Total cell lysates were analyzed by Western blotting using anti-HA antibodies. *B*, SHP-2 was immunoprecipitated from lysates of PAE/Rβ/Gab1 cells treated as above, and samples were analyzed by Western blotting with antibodies against phosphotyrosine, SHP-2, and PDGFRβ. *C*, PAE/Rβ/Gab1 cells were incubated with or without doxycycline and stimulated with PDGF-BB (50 ng/ml) for 15 min, or left untreated. Cells were then lysed, and SHP-2 was immunoprecipitated. The SHP-2 immunoprecipitates bound to protein A-Sepharose beads were used for phosphatase activity measurements using a 4-nitrophenyl-phosphate assay. As a control, lysates from each condition in the same experiment were analyzed for HA-Gab1 and SHP-2 levels by Western blotting.

lysates from COS cells transfected with HA-tagged Gab1 and PDGFRβ, we showed that Gab1 interacted with the C-terminal SH3 domain of Grb2 but not with its SH2 domain or the N-terminal SH3 domain (data not shown), as described by others (8).

To determine the role of Grb2 in Gab1 activation, we investigated the ability of PDGF to induce phosphorylation of Gab1 mutants in which one or both of the Grb2 binding sequences (GBS1 and GBS2) were deleted. As analyzed by transfection in COS cells, deletion of either of the Grb2 binding sites decreased the effect of PDGF on Gab1 phosphorylation and abolished the co-precipitation of Gab1 and phosphorylated PDGFRβ, which

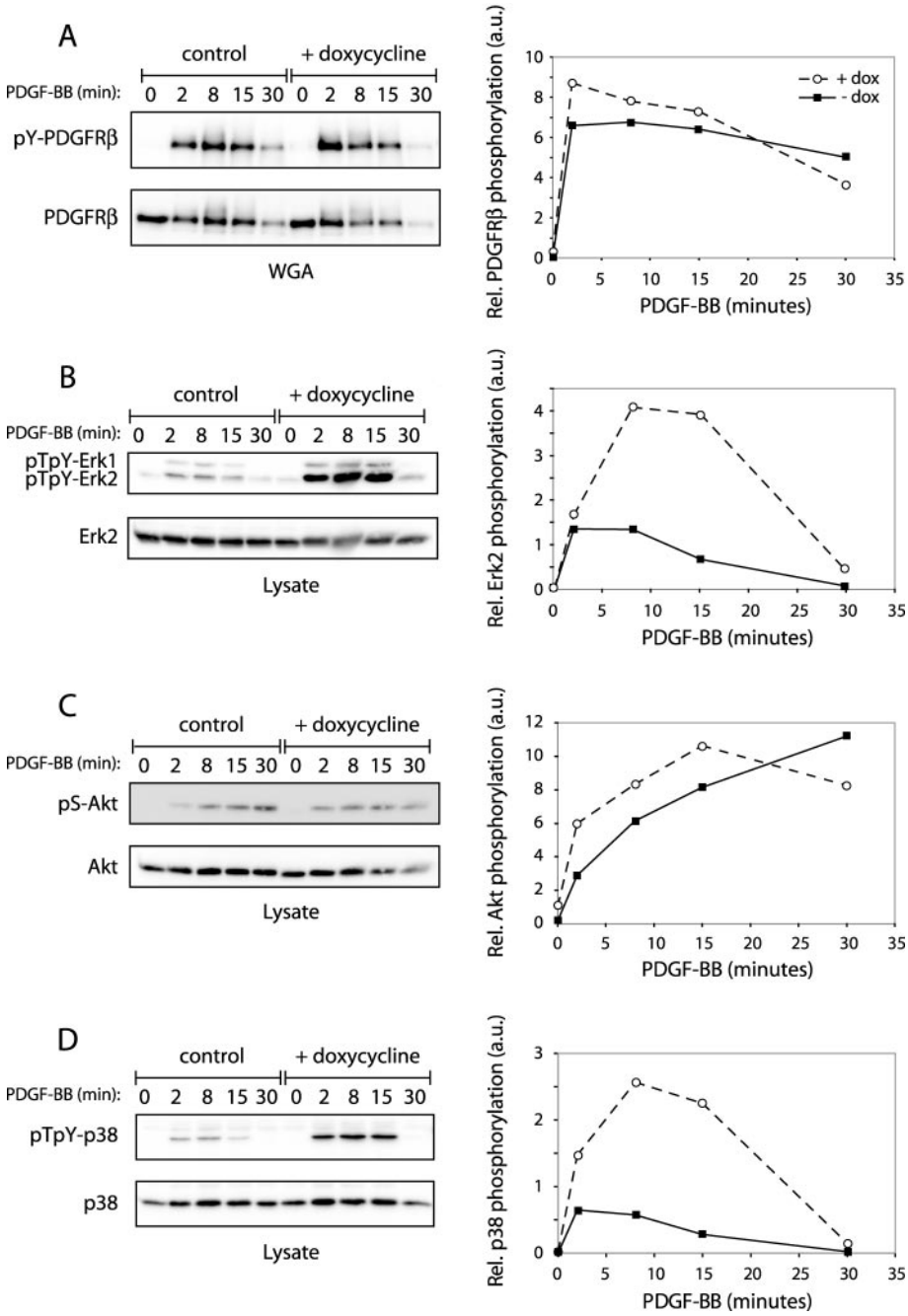

FIG. 4. **Gab1 over-expression enhances Erk and p38 phosphorylation in response to PDGF.** *A*, WGA-Sepharose was used to isolate PDGFRβ from PAE/Rβ/Gab1 cells treated as described for Fig. 3*A*. Proteins were eluted from the beads after extensive washing and analyzed by Western blotting with anti-phosphotyrosine and anti-PDGFRβ antibodies. Total cell lysates were also analyzed with antibodies against phosphorylated ($pThr^{202}/pTyr^{204}$) Erk-1/2, and Erk-2 (*B*); against phosphorylated ($pSer^{473}$) Akt1/2, and Akt1/2 (*C*); or against phosphorylated ($pThr^{180}/pTyr^{182}$) p38 and p38 (*D*). For each panel, a quantification of the amount of phosphorylated protein divided by the total amount of that protein at a given time point is shown to the *right*.

was occasionally seen in both transfected COS cells and cells expressing endogenous levels of proteins (Fig. 2). Removal of both Grb2 binding sites abolished the effect of PDGF on Gab1 phosphorylation. These observations indicate that Grb2 is required for Gab1 recruitment to the PDGFRβ complex and its phosphorylation on tyrosine residues.

Gab1 Expression Leads to Increased SHP-2 Recruitment and Activation of SHP-2 Phosphatase Activity—To study the effect of Gab1 in PDGF signaling, we established a Gab1-inducible system in PAE cells stably expressing human PDGFRβ. Several different clones of cells expressing inducible Gab1 were selected, and one was chosen for further use, from now on referred to as PAE/Rβ/Gab1. Treatment of the cells with 200 ng/ml doxycycline for 2 days induced expression of exogenous HA-Gab1 to about five times the endogenous levels as determined by densitometric analysis of the Gab1 immunoblot in Fig. 1. At this concentration, doxycycline had no effect on control cells.

PAE/Rβ/Gab1 cells grown in the absence or presence of doxycycline were stimulated with PDGF for different time periods, and the kinetics of Gab1 phosphorylation were studied after immunoprecipitation with Gab1 antibodies (Fig. 1*A*, *upper panels*). Both endogenous Gab1, as well as induced, exogenous HA-Gab1, were rapidly tyrosine-phosphorylated in response to PDGF. The amount of PDGF-induced co-immunoprecipitation with SHP-2 was increased in doxycycline-treated cells, whereas the association between Gab1 and the regulatory subunit p85 of PI3K remained unaffected by both ligand stimulation and doxycycline treatment (Fig. 3*A*, *middle panels*). The amount of HA-Gab1 expression in the absence of doxycycline was not detectable, as seen in the immunoblot using anti-HA antibody (Fig. 3*A*, *lower panel*).

It is known that the PDGFRβ interacts directly with SHP-2 (21). To examine whether the increased association between Gab1 and SHP-2 has any effect on this interaction, we immunoprecipitated SHP-2 from PAE/Rβ/Gab1 cells and analyzed

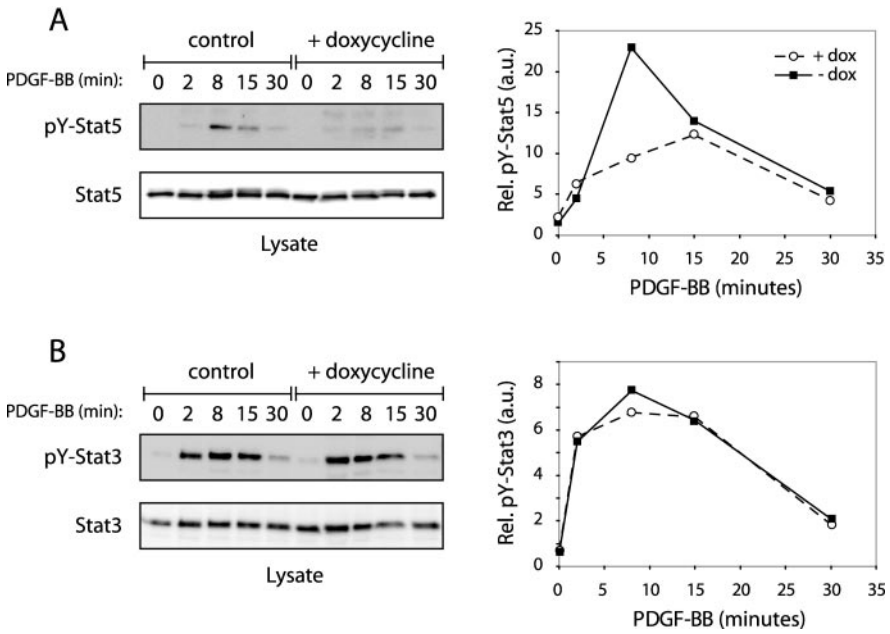

FIG. 5. **Gab1 over-expression causes a decreased phosphorylation of Stat5, but not Stat3.** Lysates from PAE/Rβ/Gab1 were prepared as described for Figs. 3*A* and 4, *B–D*. After SDS-PAGE separation and electro-transfer, the PVDF membranes were probed with antibodies against phosphorylated (pTyr^{694}) Stat5 and total Stat5 (*A*), or antibodies against phosphorylated (pTyr^{705}) Stat3 and total Stat3 (*B*). Graphs showing the amount of phosphoprotein normalized against protein levels are shown to the *right*.

the precipitates for the presence of PDGFRβ. As shown in Fig. 3*B*, there was no change in the amount of co-immunoprecipitating PDGFRβ in response to Gab1 expression, as seen by anti-phosphotyrosine or anti-PDGFRβ immunoblotting. It has been proposed that one major function of Gab1 is to bring SHP-2 to the membrane, where it then can be activated and exert its function (4). We used the PAE/Rβ/Gab1 cells treated with and without doxycycline and with and without PDGF-BB, in a phosphatase assay, to elucidate whether PDGF-induced SHP-2 activity was elevated by Gab1 over-expression. As seen in Fig. 3*C*, the phosphatase activity doubled upon doxycycline treatment. In a control experiment, no effect of Gab1 over-expression was seen on phosphorylation and degradation of the PDGFRβ in PAE/Rβ/Gab1 cells (Fig. 4*A*), which argues that the differences caused by doxycycline treatment are indeed downstream of the receptor itself. In conclusion, SHP-2 recruitment to Gab1 increased when the expression of the latter was induced, resulting in increased SHP-2 activity.

Expression of Gab1 Increases both PDGF-induced Erk and p38 Activation—We next tested whether increasing Gab1 expression affected PDGF signaling. As shown in Fig. 4, Gab1 induction caused a pronounced increase in Erk-1 and Erk-2 phosphorylation in response to PDGF, but did not change the phosphorylation of Akt, a downstream effector of PI3K, in agreement with our observations that PDGF did not promote the association of p85 with Gab1. We also investigated whether Gab1 regulated the activity of other MAPK family members besides Erk. Using an antibody specific for activated p38, we found that Gab1 over-expression also caused an increased activation of this MAPK in response to PDGF.

Stat3 and Stat5 have previously been reported to be activated during PDGF signaling (reviewed in Ref. 1). In addition, Stat5 has been shown to be a SHP-2 substrate (22). Analysis of PDGF-induced Stat3 and Stat5 tyrosine phosphorylation in PAE/Rβ/Gab1 cells revealed that Gab1 over-expression had no effect upon Stat3 activation (Fig. 5) but decreased Stat5 phosphorylation, which is in line with the observations that Stat5 is a substrate for SHP-2 (23), whose phosphatase activity was elevated under these conditions (Fig. 3).

Gab1 Contributes to Membrane Ruffling and Cellular Protrusions in Response to PDGF—Gab1 has been proposed to be required for HGF-induced branching tubulogenesis and changes in cell morphology (24). To see if there is any effect of Gab1 on PDGF-induced actin reorganization and cell morphology, we studied PAE/Rβ/Gab1 cells grown in the presence or absence of doxycycline. Fixation and actin staining of the cells revealed that the doxycycline-treated cells displayed a much more prominent reorganization of the actin cytoskeleton after PDGF stimulation, as compared with control cells which did not over-express Gab1 (Fig. 6*A*). It should be noted that Gab1 overexpression did not induce any change in the actin cytoskeleton in the absence of PDGF (data not shown).

Next, we transiently transfected Gab1$^{-/-}$ MEFs with either wild-type or a mutant form of Gab1, Y627F/Y659F, which was shown to be unable to bind to SHP-2 (Fig. 6*B* and Ref. 25). Reconstituting wild-type Gab1 in these cells caused an increased responsiveness to PDGF in terms of formation of lamellipodia and cellular protrusions, whereas the Y627F/Y659F mutant gave no such effect. Also, expression of PDGFRβ together with wild-type Gab1 in COS1 cells showed enhanced actin reorganization, whereas expression of the Y627F/Y659F gave no similar effect in response to PDGF treatment (Fig. 6*C*).

The formation of lamellipodia is dependent upon Rac activity (3), and hence we wanted to investigate the role of Rac in PDGF-induced Gab1 signaling. PAE/Rβ/Gab1 cells, therefore, were transiently transfected with the dominant-negative (Asn^{17}) form of Rac1 (Fig. 6*D*). Among cells over-expressing Gab1 in response to doxycycline, those transfected with Asn^{17}-Rac showed no sign of membrane ruffling and formation of protrusions in response to PDGF stimulation, whereas cells not transfected with Asn^{17}-Rac showed protrusions with lamellipodia as expected.

Because Gab1 enhanced p38 activation in response to PDGF, we investigated whether p38 activity was required for the effect seen on the cytoskeleton and cell morphology of PAE/Rβ/Gab1 cells. Incubation in the presence of the p38 inhibitor SB203580 caused no major effect on the degree of lamellipodia formation and cellular protrusions in response to PDGF-BB (data not shown), implying that p38 is not essential for PDGF-induced actin reorganization.

Gab1 Levels do Not Influence Mitogenicity, but Gab1$^{-/-}$ MEFs Show Decreased Chemotactic Response to PDGF—To elucidate whether Gab1 has any effect upon the mitogenic response to PDGF, we subjected PAE/Rβ/Gab1 cells to a [^{3}H]thymidine incorporation assay (Fig. 7). Neither the response to PDGF nor to serum was changed by the over-expres-

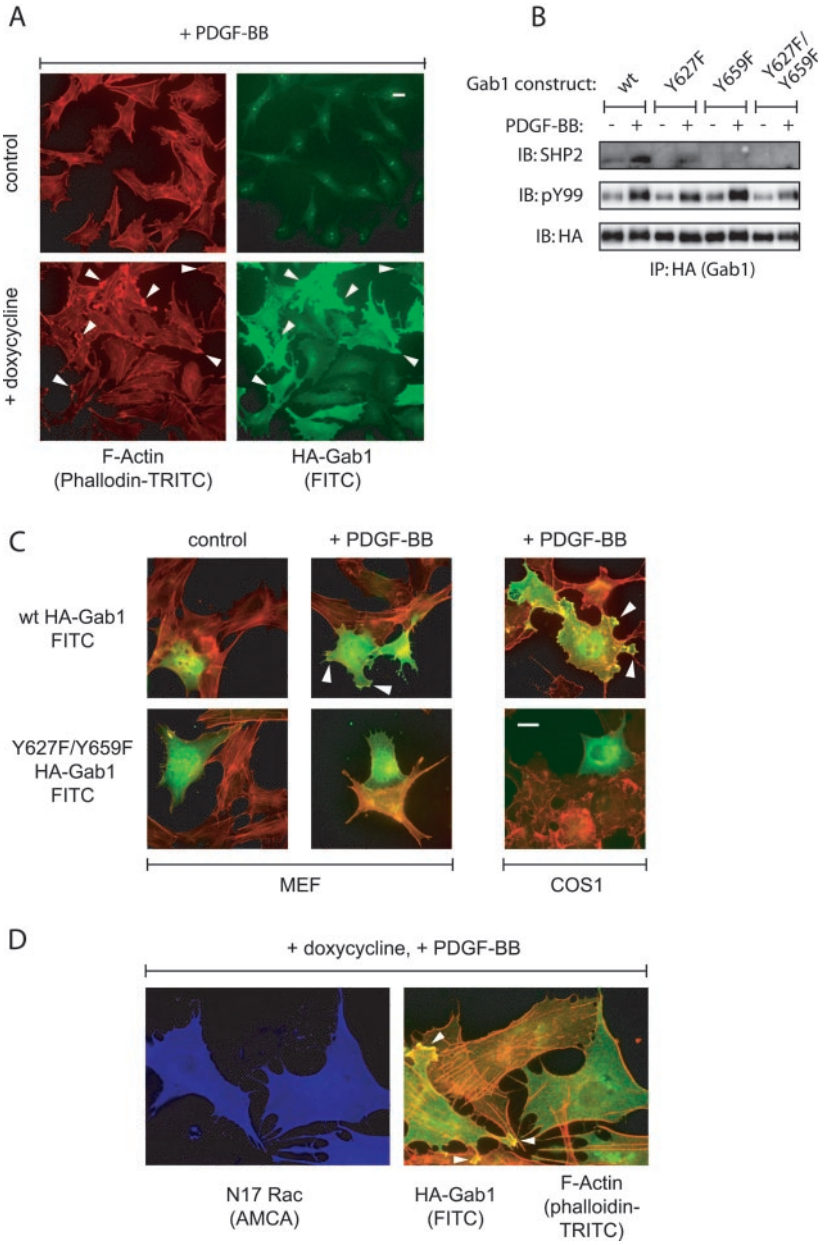

FIG. 6. **Expression of wild-type Gab1 causes an increase in lamellipodia formation and cellular protrusions in response to PDGF.** *A*, PAE/Rβ/Gab1 cells on coverslips were treated or not with doxycycline for 48 h, starved and stimulated with 50 ng/ml PDGF-BB for 8 mins, or left untreated, and then fixed. Cells were stained with anti-HA-FITC antibodies (*green*) for induced HA-Gab1 and with phalloidin-TRITC (*red*) for F-actin. *Arrowheads* point at parts of the Gab1 over-expressing cells which show strong lamellipodia formation (200× magnification; *bar*, 20 μm). *B*, COS1 cells were transfected with PDGFRβ and either wild-type, Y627F, Y659F, or Y627F/Y659F HA-Gab1. After starvation, cells were stimulated or not with 50 ng/ml PDGF-BB for 8 mins and lysed. Gab1 was immunoprecipitated with anti-HA antibodies, and analyzed by Western blotting with anti-phosphotyrosine, anti-HA, or anti-SHP-2 antibodies. *C*, Gab1$^{-/-}$ MEFs and COS1 cells were transiently transfected with either wt Gab1 or a mutant Gab1 unable to bind SHP-2 (Y627F/Y659F) on coverslips. Cells were starved and then stimulated with 50 ng/ml PDGF-BB for 8 mins, or left untreated, and then fixed. The cells were then stained using anti-HA-FITC antibodies and phalloidin-TRITC. *Arrowheads* show cellular protrusions where both F-actin (*red*, in the form of lamellipodia) and HA-Gab1 (*green*) localize at the plasma membrane (630× magnification; *bar*, 20 μm). *D*, PAE/Rβ/Gab1 cells were transiently transfected with Myc-tagged Asn^{17}-Rac1 on coverslips and treated with 200 ng/ml doxycycline for 48 h. After starvation, cells were stimulated with 50 ng/ml PDGF-BB for 8 mins and then fixed. Cells were stained with anti-HA antibodies coupled to FITC (*green*), phalloidin-TRITC (*red*), and anti-Myc-AMCA (*blue*). *Arrowheads* point at protrusions with accumulated Gab1 and F-actin at the membrane of a cell over-expressing HA-Gab1 but not expressing Asn^{17}-Rac1, whereas none of the Asn^{17}-Rac-positive cells show any sign of these protrusions (630× magnification; *bar*, 20 μm).

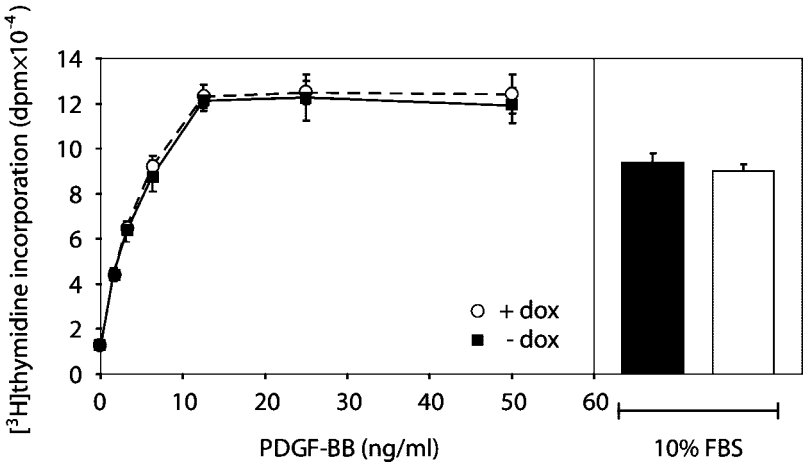

FIG. 7. **Over-expression of Gab1 does not cause any change in the mitogenic response to PDGF or serum.** PAE/Rβ/Gab1 cells were grown in the absence or presence of 200 ng/ml doxycycline for 48 h, during which time the cells were subjected to a [^{3}H]thymidine incorporation assay. The mitogenic responses of the cells to different concentrations of PDGF (0–50 ng/ml PDGF-BB) and to 10% serum were tested and run in triplicate as described (29).

sion of Gab1. Thus, increased Gab1 levels do not increase mitogenicity in these cells.

The PDGF-dependent increase in cytoskeletal reorganization after Gab1 induction caused us to inquire whether the chemotactic response to PDGF could also be affected by Gab1. Gab1$^{-/-}$ MEFs showed a reduced migratory response in the

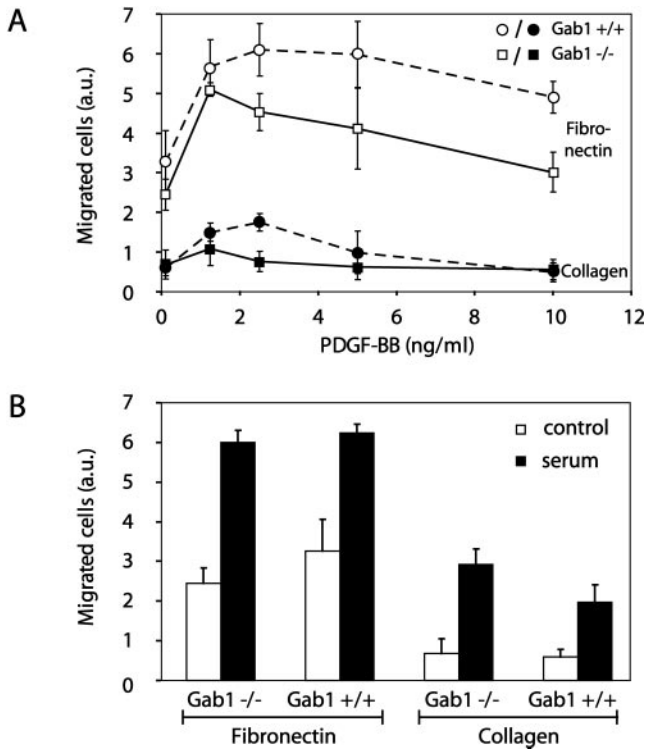

FIG. 8. **Gab1$^{-/-}$ MEFs show a lower chemotactic response toward PDGF.** Starved Gab1$^{-/-}$ and Gab1$^{+/+}$ control cells were seeded on wells coated with either fibronectin or collagen and were allowed to migrate through the filter toward gradients of PDGF (*A*) or toward 10% serum (*B*). After 4 h of incubation, the chamber was disassembled, and cells were fixed and stained as described (29). Cells remaining on the upper, application side of the filter were removed, and the number of cells which had migrated toward ligand were evaluated by measuring the optical density with a cooled charge-coupled device camera. The results are shown as the mean of quadruplicates with standard deviations. *White symbols* depict the cells seeded on fibronectin, and *black symbols* depict cells seeded on collagen; *circles* are wt MEFs, and *boxes* are Gab1$^{-/-}$ MEFs.

presence of PDGF compared with Gab1$^{+/+}$ cells, both on fibronectin and collagen substrates (Fig. 8*A*). The chemotactic response of these two cell lines to serum was comparable on both substrates (Fig. 8*B*), indicating that Gab1 deficiency caused a specific decrease in PDGF-induced chemotaxis.

DISCUSSION

In this study, we analyzed the interactions of Gab1 with Grb2 and SHP-2 downstream of the PDGFRβ, and investigated the role of Gab1 in PDGF signaling. We first focused on the role of Grb2 in the Gab1 complex. As described by others (8), Grb2 binding to Gab1 depended upon the C-terminal SH3 domain of Grb2 and upon two proline-rich motifs in Gab1. We showed that mutation of these two Grb2-binding motifs prevented the co-immunoprecipitation of phosphorylated PDGFRβ with Gab1. Because the SH2 domain of Grb2 is known to interact directly with the PDGFRβ, and indirectly via Shc and SHP-2 (reviewed in Ref. 1), it is likely that Grb2 acts as a bridge between PDGFRβ and Gab1. Interestingly, we observed that the PH domain of Gab1 was also required for Gab1 phosphorylation upon PDGF stimulation (data not shown), possibly because that domain is necessary for proper Gab1 localization.

Besides Grb2, SHP-2 was also associated with Gab1 in response to PDGF treatment. This interaction required the two SH2 domains of SHP-2 (data not shown) and Tyr^{627} and Tyr^{659} of Gab1. By contrast to what was shown in different models, mutation of Tyr^{627} alone was not enough to disrupt Gab1 binding to SHP-2 (26). Recruitment of SHP-2 to Gab1 was reported to increase SHP-2 phosphatase activity, which, in turn, enhances the activation of Erk (25). In line with this report, we observed that Gab1 over-expression increased the activation of SHP-2, Erk-1, and Erk-2 by PDGF. In addition, p38 phosphorylation was also increased upon PDGF treatment. The co-regulation of Erk and p38 was not surprising, because both seem to depend upon Ras activation in PAE cells (27). The importance of Gab1 as a regulator of MAPK activation downstream the PDGFRβ was confirmed by analyzing Gab1-deficient MEF clones, which showed a reduced activation of p38, Erk-1, and Erk-2 in response to PDGF, compared with wild-type cells[2] (14). Interestingly, Erk activation by PDGF also depends upon direct recruitment of SHP-2 to PDGFRβ (21). In conclusion, Gab1 may act as an enhancer of SHP-2 activity, and it potentiates the activation of Erk and p38 by PDGF.

We did not find any PDGF-dependent increase in the association between Gab1 and p85, as opposed to what was found in the context of NGF and HGF signaling. Consistent with this finding, Akt phosphorylation was not affected by Gab1 over-expression. The lack of connection between the PI3K pathway and Gab1 downstream of PDGFRβ might be explained by dephosphorylation of the p85 docking sites of Gab1 by SHP-2, as reported by Zhang *et al.* (28).

Gab1 over-expression in PAE cells did not increase the mitogenic activity of PDGF, but it dramatically enhanced cytoskeletal reorganization. Because of clonal variations, it was not possible to compare Gab1-deficient MEFs with wild-type controls. However, restoration of Gab1 expression in Gab1-deficient clones dramatically enhanced the formation of lamellipodia and cellular protrusions in response to PDGF. This effect was prevented by mutation of Tyr^{627} and Tyr^{659} of Gab1, pointing to the involvement of SHP-2. Surprisingly, blocking Erk and p38 activation by specific inhibitors did not affect actin reorganization by PDGF, in contrast to what had been shown by others in PAE cells (27). The formation of lamellipodia is known to be dependent upon the activation of the small Rho-GTPase Rac, and the introduction of dominant-negative Asn^{17}-Rac abolished the increased actin reorganization seen with Gab1 induction. It is not clear, however, whether Gab1 regulates the activation of Rac, or whether Rac takes part in a parallel pathway that is also required for lamellipodia formation upon PDGF treatment. Future studies will have to determine which is the molecular link between the Gab1-SHP-2 complex and actin reorganization.

Finally, we showed that Gab1-deficient fibroblasts exhibited a slightly decreased chemotactic response compared with wild-type cells in response to PDGF. This finding is consistent with the notion that Gab1 regulates the organization of the cell cytoskeleton, which plays a key role in cell migration. However, in PAE cells over-expressing Gab1, we saw no enhanced chemotaxis (data not shown), possibly because the endogenous level of Gab1 was sufficient for optimal migration, or because an exaggerated cytoskeletal reorganization disturbed the process of directed cell migration. Previously, we showed that mutation of SHP-2-binding sites in PDGFRβ decreased the chemotactic response toward gradients of PDGF (21), which supports the involvement of SHP-2 in this process.

In conclusion, we show that PDGF induces the phosphorylation of Gab1 in a Grb2-dependent manner, leading to increased activation of SHP-2 and Erk and p38. In addition, we provide evidence for a role for Gab1 in reorganization of the cytoskeleton and chemotaxis downstream of PDGFRβ.

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