Protein Kinase C-α and Protein Kinase C-ε are Required for Grb2-associated Binder-1 Tyrosine-phosphorylation in Response to Platelet-derived Growth Factor

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Summary

Grb2-associated binder-1 (Gab1) is an adapter protein related to the IRS-family. It is a substrate for the insulin receptor, as well as the epidermal growth factor (EGF) receptor and other receptor-tyrosine kinases. To investigate the role of Gab1 in signaling pathways downstream of growth factor receptors, we stimulated rat aortic vascular smooth muscle cells (VSMC) with EGF and platelet-derived growth factor (PDGF). Gab1 was tyrosine-phosphorylated by EGF and PDGF within 1 minute. AG1478 (EGF receptor kinase specific inhibitor) failed to block PDGF-induced Gab1 tyrosine-phosphorylation, suggesting that transactivated EGF receptor is not responsible for this signaling event. Because Gab1 associates with phospholipase C (PLC)γ, we studied the role of PLCγ pathway in Gab1 tyrosine-phosphorylation. Gab1 tyrosine-phosphorylation by PDGF was impaired in Chinese hamster ovary (CHO) cells expressing mutant PDGFβ receptor (Y977/989F: lacking the binding site for PLCγ). Pretreatment of VSMC with U73122 (specific PLCγ inhibitor) inhibited Gab1 tyrosine-phosphorylation as well, indicating the importance of PLCγ pathway. Gab1 was tyrosine-phosphorylated by phorbol ester to the same extent as PDGF stimulation. Studies using antisense PKC oligonucleotides and specific inhibitors showed that PKC-α and PKC-ε are required for Gab1 tyrosine-phosphorylation. Binding of Gab1 to the protein-tyrosine phosphatase SHP2 and phosphatidylinositol 3-kinase was significantly decreased by PLCγ and/or PKC inhibition, suggesting the importance of the PLCγ/PKC dependent Gab1 tyrosine-phosphorylation for the interaction with other signaling molecules. Since PDGF-mediated ERK activation is enhanced in CHO cells that overexpress Gab1, Gab1 serves as an important link between PKC and ERK activation by PDGFβ receptors in VSMC.
**Introduction**

The members of the insulin receptor substrate (IRS) family participate in signaling cascades activated by various cytokines (1,2). These proteins have multiple tyrosine-phosphorylation sites that act as docking sites for the Src homology (SH) 2 domains of various signaling proteins. These allow IRS proteins to function as multi-site docking proteins and link growth factor receptors to multiple signaling pathways. Gab1 is structurally similar to the Drosophila daughter of sevenless protein (DOS) and to IRS-1 with an amino-terminal pleckstrin homology (PH) domain, several proline-rich sequences, and multiple potential tyrosine-phosphorylation sites for binding of SH2 and SH3 containing proteins (3-5). Gab1 is rapidly phosphorylated on tyrosine residues upon stimulation of cells with EGF, insulin, hepatocyte growth factor (HGF), nerve growth factor, interleukin (IL)-3, IL-6, and erythropoietin (6-10).

To date, several signaling molecules, such as the adapter protein Grb2, the protein-tyrosine phosphatase SHP2, p85 phosphatidylinositol 3-kinase (PI3-K), and PLC\(\gamma\), have been found to associate with Gab1 in various cell lines (6,8,9,11,12). However, it has not yet been established what kinase is responsible for Gab1 tyrosine-phosphorylation or whether Gab1 is required for growth factor mediated signal transduction. In the present study, we demonstrated that Gab1 was rapidly tyrosine-phosphorylated by PDGF in VSMC. We then investigated the upstream mediators of Gab1 phosphorylation. Based on the association between Gab1 and PLC\(\gamma\) (11,12), we investigated the potential role of PLC\(\gamma\) for Gab1 tyrosine-phosphorylation. We used CHO cells expressing mouse wild type (WT) and mutant (Y977/989F: lacking the PLC\(\gamma\) binding site) PDGF\(\beta\) receptor and the specific PLC\(\gamma\) inhibitor U73122. Gab1 tyrosine-phosphorylation was decreased in both Y977/989F transfected CHO cell and VSMC pretreated with U73122.
PKC is a molecule activated downstream of PLCγ, and Gab1 tyrosine-phosphorylation by PDGF was totally blocked after inhibiting PKC activity. Experiments using phorbol esters suggested the involvement of conventional PKC (cPKC) and/or novel PKC (nPKC) for this process. We previously demonstrated the expression of PKC-α (cPKC), PKC-δ and PKC-ε (nPKC) in cultured VSMC (13). Studies with PKC inhibitors and antisense PKC oligonucleotides showed that PKC-α and PKC-ε, but not PKC-δ, are required for Gab1 tyrosine-phosphorylation by PDGF. Gab1 tyrosine-phosphorylation was inhibited by protein tyrosine kinase inhibitor genistein, showing importance of genistein-sensitive tyrosine kinases in this signaling event.

It has been suggested that tyrosine-phosphorylated Gab1 associates with other signaling molecules such as SHP2 and phosphatidylinositol 3-kinase (PI3-K), and subsequently regulates mitogen-activated protein kinase (MAPK) activity (9,14-18). In the present study, PDGF-induced Gab1 tyrosine-phosphorylation increased the association with SHP2 and PI3-K. The binding between Gab1 and SHP2 or PI3-K was decreased by PLCγ and/or PKC inhibition that was parallel to the abrogation of Gab1 tyrosine-phosphorylation. Since PDGF-mediated ERK activation was significantly enhanced in Gab1-overexpressed CHO cells, these results indicate the importance of the PLCγ/PKC dependent Gab1 tyrosine-phosphorylation for the interaction with other signaling molecules, which subsequently regulates ERK activation.
EXPERIMENTAL PROCEDURES

Reagents – Reagents and other supplies were obtained from the following sources: Cell culture media was from GIBCO-BRL (Gaithersburg, MD, USA). Rabbit polyclonal anti-cPKC-α and anti-nPKC-ε antibodies, mouse monoclonal anti-SH-PTP2 (SHP2) and Protein A/G PLUS-agarose were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-human PDGFβ receptor, rabbit anti-PI3-K p85 and mouse monoclonal anti-phosphotyrosine antibody (4G10) were from Upstate Biotechnology (Lake Placid, NY, USA). Rabbit polyclonal anti-phospho-specific ERK1/2 antibody was from New England Biolabs (Beverly, MA, USA). AG1295, AG1296 (PDGF receptor tyrosine kinase inhibitors), AG1478 (EGFR kinase inhibitor), phorbol-12-myristate-13-acetate (PMA), phorbol-12,13-dibutyrate (PDBU), chelerythrine (general PKC inhibitor), rottlerin (PKCδ inhibitor), herbimycin A, genistein (a general protein tyrosine kinase inhibitors) and PP2 (Src family kinase inhibitor) were from Calbiochem (La Jolla, CA, USA). Recombinant human PDGF-BB was from Sigma (St. Louis, MO, USA). Recombinant human EGF was from Clonetics (San Diego, CA, USA).

Cell Culture – Rat aortic VSMC were isolated from the thoracic aorta of 200-250 g male Sprague-Dawley rats and maintained in DMEM supplemented with 10% serum as described (19). VSMC of passage 8 to 14 at 70-80% confluence were growth-arrested by incubation in DMEM without serum for 48 hours before use. Mouse PDGFβ receptor wild type (WT) or mutant (Y977/989F) expressing Chinese hamster ovary (CHO) cells were a kind gift from Dr. Harlan E. Ives. CHO cells were maintained in HAM supplemented with 10% serum and 600 μg/ml G418. pcDNA3.1 vector or pcDNA3.1 Gab1 was co-transfected with pcDNA3.1 hemagglutinin (HA)-epitope tagged ERK2 into WT PDGF receptor expressing CHO cells.
Immunoprecipitation and Immunoblot Analyses – The immunoprecipitation and immunoblot analyses were performed following previously described methods (20). Growth-arrested VSMC were stimulated with PDGF-BB or EGF as indicated in each experiment. Cells were lysed in Triton/NP-40 lysis buffer (0.5% NP-40, 10 mM Tris pH 7.5, 2.5 mM KCl, 150 mM NaCl, 20 mM β-glycerolphosphate, 50 mM NaF, 1 mM Na3VO4, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM DTT), scraped off the dish and centrifuged. Lysates containing equal amounts (1mg) of protein were incubated with antibodies overnight at 4°C. After incubating with protein A/G-agarose for 2 hours, precipitates were washed 4 times with the lysis buffer and then resuspended in SDS-PAGE sample buffer. After heating at 100°C for 5 minutes, samples were separated by SDS-PAGE (6%-8%) and transferred to nitrocellulose membranes. After incubation in blocking solution (5% BSA, PBS pH 7.5, 0.1% Tween-20), membranes were incubated with primary antibodies (1:1000 dilution in blocking solution) for 2 hours at room temperature. After washing membrane 6 times (5 minutes each) with washing buffer (PBS pH 7.5, 0.1% Tween-20), the blots were incubated with the appropriate secondary antibodies (1:5000 dilution in blocking solution) for 1 hour at room temperature. The membranes were washed 6 times and proteins were detected by the ECL system (Amersham Inc, Buckinghamshire, England).

Transfection Protocol for PKC-isoform Antisense Oligonucleotides – CHO cells were grown to 50% confluence in 10-cm tissue culture dishes for transfection. Lipid 2012G (Sequitur, Inc.) was prepared in 5 µl/ml DMEM with10%-serum, containing 200 nM oligonucleotide. After 48 hour-incubation, cells were serum-starved for 24 hours and stimulated with PDGF for 5 minutes. Antisense and scramble oligonucleotides for mouse PKC-α and PKC-ε were generous gifts from Dr. Brett Monia (ISIS Pharmaceuticals, Carlsbad, CA).
Statistical Analysis – Tyrosine-phosphorylation levels were measured by densitometry of autoradiograms using NIH image. Results are corrected by the amount of loaded protein and presented as mean ± SEM from at least three separate experiments. Significant differences (p<0.01) were determined by Student’s t-test.
RESULTS

EGF and PDGF Stimulate Gab1 tyrosine-phosphorylation – To measure the effect of growth factors on Gab1 tyrosine-phosphorylation, VSMC were exposed to 10 ng/ml EGF or PDGF-BB for 1 to 20 minutes. After the preparation of cell lysates, Gab1 was immunoprecipitated and immunoblots were performed for phosphotyrosine. Tyrosine-phosphorylation of Gab1 occurred within 1 minute and peaked at 3 and 5 minutes after exposure to EGF and PDGF respectively (Fig. 1).

It has been reported that cross-talk (transactivation) exists among growth factor receptors (21,22). To determine the role of PDGF-induced EGF receptor transactivation in Gab1 tyrosine-phosphorylation, AG compound studies were performed (Fig. 2). VSMC were preincubated with 10 μM AG1295 and AG1296 (specific PDGF receptor kinase inhibitors), or AG1478 (specific EGF receptor kinase inhibitor) for 30 minutes before EGF and PDGF stimulation. We measured the inhibitor effects of AG compounds by their effect on tyrosine-phosphorylation in co-immunoprecipitated EGF receptor (170 kDa) or PDGF receptor (180 kDa). Gab1 tyrosine-phosphorylation induced by EGF was inhibited by AG1478, but not by AG1295 or AG1296. On the contrary, PDGF-induced Gab1 tyrosine-phosphorylation was inhibited by AG1295 and AG1296, but not by AG1478. These results show that EGF receptor kinase activity is required for Gab1 tyrosine-phosphorylation when VSMC were stimulated by EGF, but transactivated EGF receptor kinase activity is not required when stimulated by PDGF. Inhibition of PDGF-induced Gab1 tyrosine-phosphorylation with AG1295 and AG1296 suggests that PDGF receptor kinase activity is responsible for this signaling event.
Role of PLCγ in PDGF-induced Gab1 phosphorylation – Although PLCγ has been reported to be associated with Gab1 (11,12), the function of this association has not yet been elucidated. To investigate the role of PLCγ in Gab1 tyrosine-phosphorylation, we prepared CHO cells transfected with mouse PDGFβ receptor wild type (PDGFβR-WT) or mutant (PDGFβR-Y977/989F) which is unable to interact with PLCγ. In transfected CHO cells stimulated by PDGF (10 ng/ml, 5 minutes), both PDGFβR-WT and PDGFβR-Y977/989F were tyrosine-phosphorylated to the same extent (Fig. 3A). However, Gab1 tyrosine-phosphorylation by PDGF was significantly impaired in CHO cells transfected with PDGFβR-Y977/989F compared to PDGFβR-WT, demonstrating PDGFβ receptor kinase activity (Fig. 3B). PDGF-induced Gab1 tyrosine-phosphorylation was also abrogated after pretreatment with the PLCγ inhibitor 10 μM U73122 (Fig. 3C). These results indicate that PLCγ activity plays an important role in Gab1 tyrosine-phosphorylation induced by PDGF. The results further suggest that the PDGFβ receptor is not the Gab1 tyrosine kinase.

PKC activity is required for Gab1 tyrosine-phosphorylation and ERK activation – Because PLCγ stimulates diacylglycerol-dependent PKC isoforms, we further investigated the role of PKC in PDGF-stimulated Gab1 tyrosine-phosphorylation. To examine the signaling pathway downstream of PKC, we treated VSMC with the phorbol esters PMA and PDBU, which bind and activate PKC (cPKC and nPKC). Gab1 tyrosine-phosphorylation was stimulated by 200 nM PMA to the same extent as by PDGF (Fig. 4, compare lanes 2 and 3).

Longer exposure to phorbol ester is known to down-regulate PKC expression (13). VSMC were pretreated with 1 μM PDBU for 24 hours to down-regulate phorbol ester-responsive PKC isoforms, and stimulated with PDGF. As shown in Fig. 4 (lane 4), PDGF-mediated Gab1
tyrosine-phosphorylation was significantly inhibited in PDBU treated cells. These findings suggest a significant role for conventional and/or novel PKC isoforms for Gab1 tyrosine-phosphorylation.

It has been reported that PKC is essential for erythropoietin receptor tyrosine-phosphorylation and regulates Gab1 tyrosine-phosphorylation by erythropoietin (23). To investigate the possibility that PDGFβ receptor is positively regulated by PKC in this context, we treated VSMC with chelerythrine (PKC inhibitor acting on the catalytic domain). Although PDGF-induced Gab1 tyrosine-phosphorylation was significantly inhibited by chelerythrine pretreatment (Fig. 4B), tyrosine-phosphorylation in co-immunoprecipitated PDGFβ receptor was not inhibited, suggesting PKC regulates Gab1 tyrosine-phosphorylation downstream of PDGFβ receptor but not by regulating the tyrosine-phosphorylation status of PDGFβ receptor itself. It is also suggested that the binding between PDGFβ receptor and Gab1 is independent of the tyrosine-phosphorylation status of Gab1.

**PKC-α and PKC-ε are required for Gab1 tyrosine-phosphorylation** — We have previously characterized PKC isozyme expression in cultured VSMC, and showed that PKC-α, PKC-δ and PKC-ε are expressed among cPKC and nPKC (13). To determine the PKC isozyme specificity for Gab1 tyrosine-phosphorylation, we incubated VSMC with rottlerin, which is a specific PKC-δ inhibitor, and stimulated with PDGF. As shown in Fig. 5A, PDGF-induced Gab1 tyrosine-phosphorylation was not blocked by rottlerin pretreatment. Next, we used antisense PKC-α and PKC-ε oligonucleotides. CHO cells were stimulated with PDGF 72 hours after transfection. Expression of PKC-α and PKC-ε were effectively decreased (Fig. 5 B, C upper panels). PDGF induced Gab1 tyrosine-phosphorylation was significantly impaired in both antisense PKC-α and
PKC-ε transfected cells (Fig. 5 B, C middle and lower panels, p < 0.001 and p < 0.01 respectively). These results suggest that conventional PKC-α and novel PKC-ε are both involved in Gab1 tyrosine-phosphorylation by PDGF.

Protein tyrosine kinases (PTK) responsible for Gab1 tyrosine-phosphorylation – To study PTK responsible for Gab1 tyrosine-phosphorylation, we designed experiments using PTK inhibitors. VSMC were pretreated with general PTK inhibitors, herbimycin A and genistein, and stimulated by PDGF. As shown in Fig. 6A, Gab1 tyrosine-phosphorylation was significantly inhibited by genistein but not by herbimycin A. Herbimycin A is known to show inhibitory activity preferably to Src related kinases. Src family kinase inhibitor PP2 also failed to inhibit Gab1 tyrosine-phosphorylation by PDGF (Fig. 6B). These results show that Gab1 tyrosine-phosphorylation is regulated by genistein-sensitive kinases different from Src family kinases.

Effect of Gab1 overexpression on PDGF-induced ERK activation – To investigate the role of Gab1 in signaling pathways downstream of PDGFβ receptor, we prepared CHO cells expressing PDGFβR-WT that were co-transfected with a hemagglutinin (HA)-epitope tagged ERK2 and with a HA-tagged Gab1. PDGF stimulation of ERK2 phosphorylation was significantly increased (p < 0.001) in CHO cells that overexpress Gab1 as compared with control (vector) cells (Fig. 7A).

It has been suggested that tyrosine-phosphorylated Gab1 associates with other signaling molecules such as SHP2 and PI3-K, and subsequently regulates mitogen-activated protein kinase (MAPK) activity by IL6 (9,14), EGF (15-17) and HGF (18). Therefore, we next studied the effect of inhibiting the PLCγ/PKC pathway on the association between Gab1 and SHP2 or PI3-K
in PDGF-stimulated VSMC. As shown in Fig. 7B, the amount of SHP2 and PI3-K co-immunoprecipitated by Gab1 was increased by PDGF in VSMC. However, when we pretreated VSMC with U73122 (PLCγ inhibitor) or chelerythrin (PKC inhibitor), PDGF-stimulated association of Gab1 with SHP2 and PI3-K was significantly reduced. Because Gab1 tyrosine-phosphorylation is abrogated by the inhibitors (Fig. 3C and Fig.4B), the results suggest that Gab1 needs to be tyrosine-phosphorylated to interact with SHP2 and PI3-K. Furthermore, the PLCγ/PKC-dependent Gab1 tyrosine-phosphorylation appears to be critical for association with other signaling molecules, thereby regulating PDGF-mediated ERK activation in VSMC.
DISCUSSION

The major findings of this study are that Gab1 is tyrosine-phosphorylated by PDGF via a pathway that requires PLCγ, PKC-α and PKC-ε activity, and genistein-sensitive tyrosine kinases (Fig. 8). Gab1 is important for PDGF signaling since overexpression leads to enhanced ERK activation upon stimulation by PDGF (Fig. 7A).

It has been reported that Gab1 is tyrosine-phosphorylated downstream of EGF receptor (6,24,25). In this report, we demonstrated that Gab1 is also tyrosine-phosphorylated by PDGF in VSMC. Because there is accumulating evidence that EGFR is transactivated by PDGF (21,26), there was a possibility that Gab1 was tyrosine-phosphorylated downstream of EGFR that was transactivated by PDGF. In fact, Daub et al. showed that Gab1 is tyrosine-phosphorylated downstream of transactivated EGFR in COS-7 cells when stimulated with G-protein coupled receptor agonist (24). We successfully excluded this possibility because AG1478 (a specific EGFR kinase inhibitor) failed to inhibit PDGF-induced Gab1 tyrosine-phosphorylation. Moreover, a direct association between Gab1 and PDGF receptor was demonstrated because PDGF receptor was co-immunoprecipitated by anti-Gab1 (Fig. 1 and 2).

To characterize signaling molecules that are involved in Gab1 tyrosine-phosphorylation, we first investigated PLCγ which was already reported to be associated with Gab1 (11,12). Our study demonstrated PDGF-induced Gab1 tyrosine-phosphorylation was impaired in PDGFB receptor mutant (Y977/989F: lacking biding sites for PLCγ) expressing CHO cells as compared to PDGFB receptor wild type. The PLCγ inhibitor (U73122) dramatically reduced Gab1 tyrosine-phosphorylation induced by PDGF. These results strongly suggest that PLCγ and its downstream signaling cascade (but not PDGFB receptor tyrosine kinase activity) play an important role in Gab1 tyrosine-phosphorylation.
PKC isozymes are serine-threonine kinases activated by diacylglycerol (DG), which is a product generated by PLCγ. PKC consists of three subtypes, cPKC, nPKC and atypical PKC. Our data using PMA and PDBU suggested that phorbol ester-responsive PKC isoforms (cPKC and nPKC) may be responsible for Gab1 tyrosine-phosphorylation. In a previous report, we found PKC-α (cPKC), PKC-δ and PKC-ε (nPKC) are expressed predominantly in cultured VSMC (13). There is accumulating evidence that PKC-α and PKC-ε are involved in important VSMC functions. Haller et al observed these two PKC isoforms were translocated to focal adhesions during integrin-induced VSMC spreading, and inhibition of these PKC isoforms with antisense oligonucleotides significantly inhibited cell spreading (27). Giardina et al demonstrated that oxidized-LDL enhanced coronary vasoconstriction by increasing the activity of PKC-α and PKC-ε (28). In the present study, our results showed the involvement of PKC-α and PKC-ε in PDGF-induced Gab1 tyrosine-phosphorylation. PKC-δ was considered unlikely based on experiments using rottlerin (a PKC-δ inhibitor). However, the potential lack of specificity and cell-specific ineffectiveness of drug inhibitors such as rottlerin limits our ability to completely rule out PKC-δ or other PKC isozymes. Further study with other independent approaches will be required to define the precise role of PKC isozymes.

The ubiquitously expressed Src family kinase members seem to be important mediators of PDGFβ receptor signal events, regulating the mitogenic response to PDGF (29). We also previously reported that Src activity was important for ERK activation through transactivated EGF receptor by PDGF (22). However, the present study showed no evidence for Src family kinase involvement in Gab1 tyrosine-phosphorylation because herbimycin A and PP2 failed to inhibit this signaling event. Genistein successfully inhibited PDGF-induced Gab1 tyrosine-
phosphorylation. Further study is required to identify the particular tyrosine kinases that phosphorylate Gab1.

In this study, we observed that overexpression of Gab1 in CHO cells significantly enhanced ERK activation by PDGF, suggesting that Gab1 plays an important role for ERK activation downstream of PDGFβ receptor in VSMC. Although the mechanism by which Gab1 contributes to ERK activation is still not fully understood, it has been reported that Gab1 needs to be tyrosine-phosphorylated to interact with other signaling molecules. For example, SHP2 and PI3-K bind to Gab1 that is tyrosine-phosphorylated by insulin (6), IL-6 (9,14), EGF (15-17,25,30), erythropoietin (10,31), NGF (8,32) and HGF (4,7,18). The activation of the ERK members of the MAPK family are regulated by SHP2 and PI3-K bound to tyrosine-phosphorylated Gab1 (9,14-18). Moreover, SHP2 and PI3-K activation mediated by Gab1 tyrosine-phosphorylation has been suggested to promote cell survival (8), DNA synthesis, cell differentiation (32), and Gab1 localization to the plasma membrane (33-35). Itoh et al reported that Gab1-deficient mice are embryonic-lethal and display developmental defects in the heart, placenta, and skin, similar to phenotypes observed in mice lacking signals mediated by the HGF, PDGF and EGF pathways. Because ERK is activated at much lower levels in cells from Gab1-deficient embryos in response to these growth factors or cytokines, these findings suggest that Gab1 is a common player in a broad range of growth factor and cytokine signaling pathways linking ERK MAPK activation (14). In the present study, we demonstrated that the binding of SHP2 and PI3-K to Gab1 was increased in proportion to Gab1 tyrosine phosphorylation induced by PDGF in VSMC. A significant reduction in SHP2 and PI3-K associated with Gab1 was observed when Gab1 tyrosine-phosphorylation was inhibited by the blocking PLCγ/PKC activity. Considering that the expression of Gab1 protein is not affected by 5 minute-PDGF
incubation in VSMC, our results suggest that Gab1 tyrosine-phosphorylation is an important factor to regulate the PDGF-induced ERK activation, interacting with other molecules including SHP2 and PI3-K.

In summary, PDGF-mediated ERK activation is positively regulated by tyrosine-phosphorylated Gab1, which is also positively controlled by two different PKC isoforms, PKC-α and PKC-ε, when VSMC are stimulated with PDGF. We conclude that activation of the PLCγ/PKC pathway including PKC-α and PKC-ε is indispensable for Gab1 tyrosine-phosphorylation and Gab1 plays a pivotal role in ERK activation in VSMC by PDGF.
ACKNOWLEDGEMENTS

This study was supported by grants from the NIH-HLBI to BCB (HL49192 and 63462). Banyu Fellowship in Lipid Metabolism and Atherosclerosis to YH. We thank Dr. Harlan E. Ives for providing the wild type and mutant PDGF receptor expressing CHO cells. We thank Dr. Brett Monia (ISIS Pharmaceuticals) for providing the antisense and scramble oligonucleotides for mouse PKC-α and PKC-ε.
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FIGURE LEGENDS

Figure 1: Gab1 is tyrosine-phosphorylated by PDGF as well as EGF.
Serum-starved VSMC were stimulated with 10 ng/ml EGF or PDGF-BB for time as indicated. A, Cell lysates were immunoprecipitated (IP) with anti-Gab1 and immunoblotted (IB) with anti-phosphotyrosine (4G10) (upper panel), and reprobed with anti-Gab1 (lower panel). Gab1 was identified at 115 kDa. Co-immunoprecipitated EGF receptor (EGFR) and PDGFβ receptor (PDGFβR) were identified at 170 kDa and 180 kDa respectively.

Figure 2: PDGF-induced Gab1 tyrosine-phosphorylation after AG compound treatment.
Serum-starved VSMC were stimulated with 10 ng/ml EGF or PDGF-BB for 5 minutes after preincubation with 10 µM AG1295, AG 1296 or AG1478 for 30 minutes. Cell lysates were immunoprecipitated with anti-Gab1 followed by immunoblot with 4G10 (upper panel), and reprobed with anti-Gab1 (lower panel). Effects of AG compounds on receptor kinases can be observed by abrogation of tyrosine-phosphorylation in co-immunoprecipitated EGFR (170 kDa) or PDGFR (180 kDa).

Figure 3: Gab1 tyrosine-phosphorylation is impaired in CHO cells expressing Y977/989F mutant PDGF receptor.
CHO cells expressing mouse wild type PDGFβR -WT or the mutant PDGFβR -Y977/989F that lacks the binding site for PLCγ were used. Serum-starved CHO cells were stimulated with 10 ng/ml PDGF-BB for 5 minutes. A, Cell lysates were immunoprecipitated with anti-PDGFβR and analyzed by immunoblot probed with 4G10 (upper panel), and reprobed with anti-PDGFβR (lower panel). B, Cell lysates were immunoprecipitated with anti-Gab1 and analyzed by
immunoblot probed with 4G10 (upper panel), and reprobed with anti-Gab1 (lower panel). C, Serum-starved VSMC were pretreated with 10 µM U73122 for 10 minutes, followed by stimulation with 10 ng/ml PDGF-BB for 5 minutes. Cell lysates were immunoprecipitated with anti-Gab1 and analyzed by immunoblot probed with 4G10 (upper panel), and reprobed with anti-Gab1 (lower panel).

Figure 4: Role of PKC in PDGF-induced Gab1 tyrosine-phosphorylation and ERK activation.
A, Serum starved VSMC were stimulated with 200 nM phorbol-12-myristate-13-acetate (PMA) for 5 min (lanes 2). VSMC were pretreated with 1 µM phorbol-12,13-dibutyrate (PDBU) for 24 hr (lane 4) and stimulated with 10 ng/ml PDGF-BB for 5 min. Cell lysates were immunoprecipitated with anti-Gab1 and analyzed by immunoblot with 4G10 (upper panel), and reprobed with anti-Gab1 (lower panel). B, Serum starved VSMC were pretreated with 5 µM chelerythrine (Chel) for 10 min and stimulated with 10 ng/ml PDGF-BB for 5 min. Cell lysates were immunoprecipitated with anti-Gab1 and analyzed by immunoblot with 4G10 (upper panel), and reprobed with anti-Gab1 (lower panel).

Figure 5: Positive involvement of PKC-α and PKC-ε in PDGF-induced Gab1 tyrosine-phosphorylation.
A, Serum-starved VSMC were stimulated with 10 ng/ml PDGF-BB for 5 minutes after preincubation with 10 µM rottlerin for 30 minutes. Cell lysates were immunoprecipitated with anti-Gab1 followed by immunoblot with 4G10 (upper panel), and reprobed with anti-Gab1 (lower panel). B, C, Antisense oligonucleotides for PKC-α (αAS) and PKC-ε (εAS), and scramble oligonucleotides for PKC-α (αS) and PKC-ε (εS) were transfected into CHO cells as
indicated in Experimental Procedures. Cells were stimulated with 10 ng/ml PDGF-BB for 5 minutes and cell lysates were analyzed by immunoblot with anti-PKC-α (B) or PKC-ε (C) (upper panel), or immunoprecipitated with anti-Gab1 followed by immunoblot with 4G10 (middle panel) and reprobed with anti-Gab1 (middle panel). Lower panels show relative tyrosine-phosphorylation level of Gab1. Each antisense oligonucleotide significantly inhibited Gab1 tyrosine-phosphorylation induced by PDGF (*, P < 0.001; †, P < 0.01).

Figure 6: Effect of general tyrosine kinase inhibitors on PDGF-induced Gab1 tyrosine-phosphorylation.

Serum-starved VSMC were stimulated with 10 ng/ml PDGF-BB for 5 minutes after preincubation with 50 µM genistein (Gen) or 10 µM herbimycin A (Herb) (A), or 1 µM PP2 (B) for 30 min. Cell lysates were immunoprecipitated with anti-Gab1 followed by immunoblot with 4G10 (upper panel), and reprobed with anti-Gab1 (lower panel).

Figure 7: Effect of Gab1 overexpression and tyrosine-phosphorylation on ERK activity.

A, pcDNA3.1 vector (vector) or pcDNA3.1 Gab1 (HA-Gab1) was co-transfected with pcDNA3.1 hemagglutinin (HA)-epitope tagged ERK2 into PDGFβR-WT expressing CHO cells. Serum starved CHO cells were stimulated with 10 ng/ml PDGF-BB for 5 min. Cell lysates were immunoprecipitated with anti-HA and analyzed by immunoblot probed with anti-phospho-ERK antibody (upper panel), and reprobed with anti-HA (middle panel). Lower panels show relative tyrosine-phosphorylation level of ERK2. ERK2 tyrosine-phosphorylation was significantly enhanced in Gab1-overexpressed CHO cells by PDGF stimulation (*, P < 0.001). B, Serum starved VSMC were pretreated with 10 µM U73122 or 5µM chelerythrine (Chel) for 10 min and
stimulated with 10 ng/ml PDGF-BB for 5 min. Cell lysates were immunoprecipitated with anti-Gab1 and analyzed by immunoblot with anti-SHP2 (upper panel), and reprobed with anti-PI3-K p85 (middle panel) or anti-Gab1 (lower panel).

Figure 8: Signaling pathways for Gab1 tyrosine-phosphorylation downstream of PDGFβ receptor.

PDGF-induced Gab1 tyrosine-phosphorylation requires PKC-α and PKC-ε that are activated downstream of PLCγ. Genistein-sensitive tyrosine kinases are considered to be responsible for Gab1 tyrosine-phosphorylation. Gab1 is suggested to play a critical role in PDGF-induced ERK activation interacting with SHP2 and PI3-K in the present study.
Fig. 1
Fig. 2

**EGF**

- IP: Gab1
- IB: 4G10

**PDGF**

- IP: Gab1
- IB: Gab1

Treatment:

- AG 1295 1296 1478
- AG 1295 1296 1478

- PDGFβR (180 kDa)
- EGFR (170 kDa)
- Gab1 (115 kDa)
Fig. 3
C.

IP: Gab1  
IB: 4G10

IP: Gab1  
IB: Gab1

Gab1

115kDa

PDGF  -  +  +
U73122 -  -  +

Fig. 3
A.

IP: Gab1  
IB: 4G10  

IP: Gab1  
IB: Gab1  

PDGF  -  -  +  +  
Treatment  -  PMA 5min  -  PDBU 24hr

B.

IP: Gab1  
IB: 4G10  

IP: Gab1  
IB: Gab1  

PDGF  -  +  +  
Chel  -  -  +

Fig. 4
A.

| Treatment     | IP: Gab1 | IB: Gab1 | IB: 4G10 |
|---------------|----------|----------|----------|
| PDGF          | -        | +        | +        |
| Rottlerin     | -        | -        | +        |

Fig. 5
Fig. 5
**A.**

| Treatment | IB: 4G10 | IP: Gab1 | IB: Gab1 | 115 kDa |
|-----------|----------|----------|----------|---------|
| PDGF      | -        | +        | +        | +       |

**B.**

| Treatment | IB: 4G10 | IP: Gab1 | IB: Gab1 | 115 kDa |
|-----------|----------|----------|----------|---------|
| PDGF      | -        | +        | +        |         |

Fig. 6
A.

**Fig. 7**

**Vector**

IP: HA

IB: phosphoERK

**HA-Gab1**

IP: HA

IB: HA

PDGF: - + - +

ERK2 tyrosine-phosphorylation (Relative value)

- 5
- 10
- 15

Vector | HA-Gab1

PDGF: - + - +

Vector | HA-Gab1

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B.

| IP: Gab1 | IB: SHP2 | SHP2 (64 kDa) |
|---------|---------|---------------|
| IP: Gab1 | IB: PI3-K p85 | PI3-K (85 kDa) |
| IP: Gab1 | IB: Gab1 | Gab1 (115 kDa) |

PDGF: - + + +
Treatment: - - U73122 Chel

Fig. 7
PDGF-BB

PDGFβR

PLCγ

PKC-α and PKC-ε activation

Genistein-sensitive tyrosine kinases

Gab1

SHP2

PI3-K

ERK1/2 activation

Fig. 8
Protein kinase C-α and protein kinase C-<IMG SRC="/math/epsilon.gif" ALIGN="BASELINE" ALT="epsilon"> are required for Gab2-associated binder-1 tyrosine-phosphorylation in response to platelet-derived growth factor

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*J. Biol. Chem.* published online April 8, 2002

Access the most updated version of this article at doi: [10.1074/jbc.M200605200](http://10.1074/jbc.M200605200)

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