Association of Grb7 with Phosphoinositides and Its Role in the Regulation of Cell Migration*

Tang-Long Shen, Dong Cho Han‡, and Jun-Lin Guan§

From the Department of Molecular Medicine, Cornell University, Ithaca, New York 14853

Received for publication, April 1, 2002
Published, JBC Papers in Press, May 20, 2002, DOI 10.1074/jbc.M203085200

Grb7 is the prototype of a family of adaptor molecules that also include Grb10 and Grb14 that share a conserved molecular architecture including Src homology 2 (SH2) and pleckstrin homology (PH) domains. Grb7 has been implicated as a downstream mediator of integrin-FAK signal pathways in the regulation of cell migration, although the molecular mechanisms are still not well understood. In this paper, we investigated the potential role and mechanisms of PH domain in Grb7 in the regulation of cell migration. We found that the PH domain mediated Grb7 binding to phospholipids both in vitro and in intact cells. Furthermore, both Grb7 and its PH domain preferentially interacted with phosphatidylinositol phosphates showing strongest affinity to the D3- and D5-phosphoinositides. The PH domain interaction with phosphoinositides was shown to play a role in the stimulation of cell migration by Grb7. It was also shown to be necessary for Grb7 phosphorylation by FAK, although it was not required for Grb7 interaction with FAK or recruitment to the focal contacts. Last, we found that PI 3-kinase activity played a role in both Grb7 association with phosphoinositides and its stimulation of cell migration. In addition, both FAK binding to PI 3-kinase via its autophosphorylated Tyr397 and integrin-mediated cell adhesion increased Grb7 association with phosphoinositides. Together, these results identified the Grb7 PH domain interaction with phosphoinositides and suggested a potential mechanism by which several signaling molecules including Grb7, FAK, and PI 3-kinase and their interactions cooperate to mediate signal transduction pathways in integrin-mediated cell migration.

Cell migration plays essential roles in a variety of biological and disease processes such as embryonic development, inflammation, wound healing, and cancer metastasis. Cell migration is a multistep process that involves extension of lamellipodia in response to environmental cues, contraction of cell bodies regulated by the cytoskeleton, and detachment from adhesion sites at the rear of cells (1). Initiation of cell migration involves sensing of the gradients of soluble chemoattractants and/or immobilized extracellular matrix proteins or other cell surface molecules by cell surface receptors. In mammalian fibroblasts, various growth factor receptors and the integrin family of cell adhesion receptors have been shown to play essential roles in the initiation and regulation of cell migration. Recent studies have suggested that focal adhesion kinase (FAK) serves as a major mediator of integrin signaling in the regulation of cell migration (2–6). Furthermore, several different signaling pathways downstream of FAK have been implicated in cell migration. These include formation of signaling complexes between FAK and Src and their subsequent phosphorylation of p130cas (7–9) and interaction of FAK with the p85 subunit of PI 3-kinase (10, 11). Our recent studies also suggest that FAK binding and phosphorylation of the adaptor molecule Grb7 play an important role in the regulation of cell migration (12, 13).

Grb7 is a family member of Src homology 2 (SH2) domain-containing adaptor molecules that also include Grb10 and Grb14 (14–17). The Grb7 family members share similar structural organizations, including an amino-terminal proline-rich region; a central segment termed the GM region (for Grb and Mig), which includes a pleckstrin homology (PH) domain and shares sequence homology with the Caenorhabditis elegans protein, Mig-10, and a carboxyl-terminal SH2 domain. Like other adaptor molecules, Grb7 family members have been shown to interact with a variety of cell surface receptors and other signaling proteins (17). Most of these interactions are mediated by the SH2 domain of the Grb7 family members and a phosphotyrosine motif in the activated receptors/other signaling molecules. It is likely that these binding partners are upstream regulators for the Grb7 family molecules. In contrast, little is known about potential downstream effectors of the Grb7 family proteins. Given the role of Grb7 family proteins as adaptor molecules, it is likely that potential downstream effectors will interact with other conserved domains of Grb7 like the Pro-rich region or the GM region.

The high homology of Grb7 family proteins with the C. elegans protein Mig-10, which is involved in the regulation of neuronal cell migration during embryonic development (18, 19), suggests that Grb7 may play a role in the regulation of mammalian cell migration. This is supported by our recent data showing the role of Grb7 in mediating the FAK downstream pathways in cell migration (12, 13). We found that the SH2 domain of Grb7 can directly interact with autophosphorylated Tyr397 of FAK in a cell adhesion-dependent manner. Association of Grb7 with FAK promotes its recruitment to focal contacts and phosphorylation by FAK, which are both critical for Grb7 to mediate FAK-stimulated cell migration. These studies also suggested an important role for the central GM

* This work was supported by National Institutes of Health (NIH) Grant GM48050 and a subcontract from UCSD (NIH Grant HL-64382) (to J.-L. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Korea Research Institute of Bioscience and Biotechnology, Yusong, Taejon 305-600, Republic of Korea.
§ To whom correspondence should be addressed. Tel.: 607-253-3586; Fax: 607-253-3708; E-mail: jjg19@cornell.edu.

1 The abbreviations used are: FAK, focal adhesion kinase; PH, pleckstrin homology; SH2, Src homology 2; FN, fibronectin; HA, hemagglutinin; PI 3-kinase, phosphatidylinositol 3-kinase; GFP, green fluorescent protein; PBS, phosphate-buffered saline; GST, glutathione S-transferase; PtdIns, phosphatidylinositol; CHO, Chinese hamster ovary.
region of Grb7 in cell migration. Furthermore, we showed that the formation of the FAK-Grb7 complex was independent of the FAK complexes with Src or its binding to PI 3-kinase (13, 20). It is not clear, however, whether the FAK/Grb7 pathway cooperates with these other two pathways triggered by FAK in the regulation of cell migration.

PH domains are a module of about 100 amino acids that has been reported in a variety of signaling molecules including protein kinases, phospholipases, GTPases, adaptor proteins, and cytoskeletal proteins (21, 22). Besides interactions with other proteins, PH domains have been shown to interact with phospholipids (23, 24). Interestingly, a number of proteins showed specific binding to the PI 3-kinase product D3 phosphoinositides through their PH domains, which are consistent with their role as downstream mediators of PI 3-kinase (25). The presence of the PH domain in Grb7 suggested its possible interactions with phospholipids, which could potentially contribute to regulation of cell migration by Grb7. Furthermore, this interaction could also play a role in the cooperation between FAK/Grb7 and FAK/PI 3-kinase signaling pathways in cell migration. In this report, we investigated the potential role and mechanisms of the PH domain of Grb7 in the regulation of cell migration as well as the potential cooperation of FAK/Grb7 signaling pathway with the PI 3-kinase signaling pathway.

MATERIALS AND METHODS

Reagents—Protein A-Sepharose 4B, glutathione-agarose beads, human plasma fibronectin (FN), poly-L-lysine, and mouse mAb anti-vinculin were from Sigma. LipofectAMINE, CO2-independent medium, and Opti-MEM were from Life Technologies, Inc. 32Pi was from Amersham Biosciences. The presence of the PH domain in Grb7 suggested its possible interactions with phospholipids, which could potentially contribute to regulation of cell migration by Grb7. Furthermore, this interaction could also play a role in the cooperation between FAK/Grb7 and FAK/PI 3-kinase signaling pathways in cell migration. In this report, we investigated the potential role and mechanisms of the PH domain of Grb7 in the regulation of cell migration as well as the potential cooperation of FAK/Grb7 signaling pathway with the PI 3-kinase signaling pathway.

Grb7 Interaction with Phosphoinositides in Cell Migration

Grb7 Association of Phosphoinositides in Intact Cells—For detection of association of Grb7 or its segments with phosphoinositides in intact cells, 293 or CHO cells were transfected with pDHGST vectors encoding Grb7 or its segments. In some experiments, another expression vector was co-transfected. In other experiments, the cells were treated with 50 nM LY294002, 15 μM wortmannin, or Me3SO alone prior to cell lysis.

Protein-Lipid Overlay Assay—Membrane arrays (PIP-Stripes®) spotted with 100 pmoles of phospholipids were purchased from Echelon Research Laboratories (Salt Lake City, UT) and used for protein-lipid overlay assays by following the manufacturer’s guidelines. Briefly, membranes were blocked with 3% (w/v) fatty acid-free bovine serum albumin (Sigma) in TBST (10 mM Tris pH 8.0, 150 mM NaCl, and 0.1% (v/v) Tween 20) for 1 h at room temperature. Blocked membranes were then washed with PBS and incubated with 0.5 μg/ml GST fusion proteins overnight at 4°C with gentle agitation. They were then washed three times (10 min each) with TBST plus 3% fatty acid-free bovine serum albumin. After washing, membranes were subjected to Western blotting using anti-GST to detect the bound GST fusion proteins, as described below.

Cell Migration Assay—Cell migration assays for transiently transfected CHO cells were performed as described previously (13), except that constructs encoding GFP fusion proteins were used directly (instead of co-transfection with a vector encoding GFP) to identify positively transfected cells in some experiments. For experiments with the PI 3-kinase inhibitors wortmannin and LY294002, cells were pretreated for 30 min with the inhibitors prior to initiation of the cell migration assays in the presence of 50 nM LY294002, 15 μM wortmannin, or Me3SO alone.

Immunoprecipitation and Western Blotting—Subconfluent cells were washed twice with ice-cold PBS and then lysed with 1% Nonidet P-40 lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 1% Nonidet P-40, 10 mM glycerol, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 20 mg/ml leupeptin). Lysates were cleared by centrifugation, and total protein concentration was determined using the BioRad protein assay. Immunoprecipitations were carried out by incubating cell lysates with appropriate antibodies for 3 h at 4°C, followed by incubation for 1 h with protein A-Sepharose 4B (Sigma) or Protein G Plus (Santa Cruz Biotechnology) beads. After washing, immune complexes were resolved using SDS-PAGE. Western blotting was carried out using horseradish peroxidase-conjugated IgG as a secondary antibody and the Amersham ECL system as described above.
plasma fibronectin overnight at 4 °C. They were incubated in a 37 °C, 5% CO2 incubator to allow for cell attachment and spreading. Cells were then fixed with 3.7% formaldehyde for 15 min at room temperature, washed three times with PBS, and then permeabilized with 0.5% Triton X-100 for 10 min at room temperature. After washing three times with PBS, the cells were stained with polyclonal anti-GFP (1:300; Santa Cruz Biotechnology) and anti-vinculin monoclonal antibody (1:50; Sigma) in the presence of 10% goat serum for 1 hour at 37 °C. After washing three times with PBS, the bound primary antibody was detected using fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:150) and Texas Red-conjugated anti-mouse IgG (1:100) antibodies, respectively. Images of stained cells were captured using an immunofluorescence microscope under a 100× oil immersion objective and a CCD camera.

RESULTS

The PH Domain Mediates Grb7 Interaction with Phosphoinositides—To investigate potential functions of the PH domain in Grb7, we first examined whether it could bind phospholipids using both in vitro and in vivo binding assays. GST fusion proteins containing Grb7 or its segments were immobilized on glutathione-agarose beads and then incubated with lysates prepared from 32P-metabolically labeled 293 cells. After washing, the bound 32P-labeled phospholipids were separated on a TLC plate and quantified by a PhosphorImager. The relative binding was normalized to binding to GST alone (B). Duplicate samples were analyzed on SDS-PAGE followed by Coomassie Blue staining (C). D, 293 cells were transfected with plasmids pDHGST, pDHGST-Grb7, pDHGST-PH, or pDHGST-R239L, as indicated. The cells were then metabolically labeled with 32P, as described under “Materials and Methods.” Grb7 or its segments were pulled down from the lysates using glutathione-agarose beads. The associated 32P-labeled phospholipids were extracted and analyzed on a TLC plate and quantified by a PhosphorImager. The relative binding was normalized to binding to the wild type Grb7.

GST-SH2 did not. Duplicate samples were analyzed by Coomassie Blue staining to show similar amounts of the fusion proteins present in all samples (Fig. 1C). These results suggest that Grb7 can bind to phospholipids through its PH domain.

We also examined the association of Grb7 with phospholipids in intact cells. Mammalian expression vectors encoding GST-tagged Grb7 or its mutants (see Fig. 1A) were transiently transfected into 293 cells. The cells were then metabolically labeled with 32P, as described under “Materials and Methods.” Cell lysates were prepared, and GST-Grb7 or its mutants were precipitated with glutathione-agarose beads. The bound phospholipids were then extracted and analyzed by thin layer chromatography and quantified using a PhosphorImager. As shown in Fig. 1D, Grb7 as well as its PH domain alone associated with phospholipids in intact cells. Based on site-directed mutagenesis and x-ray crystal structures for PH domains in a number of proteins, several highly conserved residues within PH domains play critical roles for their association with phospholipids (24, 31, 32). To determine whether the Grb7 PH domain also requires these critical residues for phospholipid binding, we generated several point mutations at these sites. Mutation of Arg239 in the PH domain (R239L mutant) as well as other key residues (data not shown) abolished binding of Grb7 to phospholipids. These data are consistent with the in vitro binding results that the PH domain in Grb7 can mediate its binding to phospholipids. They also suggest that it inter-
acted with phospholipids in a similar manner as other PH domain because the same critical residues were necessary for the binding.

To evaluate the binding specificity of the Grb7 PH domain to various phospholipids, we employed a protein-lipid overlap assay using membranes that had been spotted with an equal amount (100 pmol) of different phospholipids (Fig. 2A). The purified GST fusion proteins containing Grb7 or its mutants were incubated with the membrane strips as described under “Materials and Methods.” After washing, the bound GST fusion proteins were detected by Western blotting with anti-GST antibody. As expected, the GST control did not bind to any of the phospholipids (Fig. 2B). On the other hand, GST-Grb7 showed binding to several phosphatidylinositol phosphates with various affinity (Fig. 2C). It bound to PtdIns-3-P and PtdIns-5-P with the greatest strength, followed by moderate binding to PtdIns-4-P and PtdIns-3,5-P_2, and a very weak binding to PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3. Similarly, the PH domain alone also bound to these phosphatidylinositol phosphates, although it exhibited a slightly stronger binding to PtdIns-3,4,5-P_3 than the full-length Grb7 (Fig. 2E). Mutation of the critical Arg^{239} residue (R239L mutant) abolished the binding of full-length Grb7 to all phosphatidylinositol phosphates (Fig. 2D), and the SH2 domain alone did not bind to any of the phospholipids (Fig. 2F). Together, these results indicated that Grb7 preferentially interacted with D3- and D5-phosphoinositides via its PH domain.

**Role of Grb7 Interaction with Phosphoinositides in Cell Migration**—To investigate the role of the Grb7 PH domain and its interaction with phosphatidylinositol phosphates in cell migration, we compared the effects of R239L mutant and wild type Grb7 on cell motility using a time-lapse imaging-based com-

---

**Fig. 2. The phospholipid binding specificity of the Grb7 PH domain.** Equal amounts of purified GST fusion proteins containing various Grb7 constructs (see Fig. 1A) were incubated with a nitrocellulose strip that had been spotted with various phospholipids (A), as indicated (B–F). The bound proteins were detected by Western blotting using anti-GST antibody.
puterized motility analysis method called OMAware, as described previously (13). CHO cells were transiently transfected with expression vectors encoding Grb7 or its mutants with GFP fused at their N termini. The expression of transfected Grb7 or its mutants was verified by Western blotting using anti-GFP (Fig. 3A). The effects of these constructs on cell motility were then evaluated using OMAware. Fig. 3B shows that GFP-Grb7 stimulated cell migration when compared with untransfected cells, which is consistent with our previous results using Grb7 alone (13). In contrast, the GFP-R239L mutant did not stimulate but actually inhibited cell migration. This inhibitory effect could be due to its functioning as a dominant negative mutant by competing with endogenous Grb7 for binding to FAK (via its still intact SH2 domain (see Ref. 12 and Fig. 4B)) yet failing to interact with phosphatidylinositol phosphates to trigger downstream events. The Grb7 PH domain alone also inhibited cell migration. This domain may function in a dominant negative manner by competing with endogenous Grb7 for binding phosphatidylinositol phosphates yet failing to associate with FAK due to lack of the SH2 domain. This possibility is supported by the lack of an effect on cell migration by the corresponding PH domain mutant with Arg239 mutated to Leu. The PH domain from Akt did not affect cell migration either, providing further support for a specific role of the Grb7 PH domain in the regulation of cell migration. Together, these data suggested that the Grb7 PH domain and its interaction with phosphatidylinositol phosphates play a critical role in the regulation of cell migration.

Interaction of various PH domains with phospholipids has been suggested to facilitate their localization to the plasma membranes (22, 33), and localization of Grb7 to the focal contacts is required for its stimulation of cell migration (13). Therefore, we examined the subcellular localization of the R239L mutant that unlike the wild type Grb7 does not bind to phosphatidylinositol phosphates. CHO cells were transfected with expression vectors encoding Grb7 or R239L mutant with GFP fused to their N termini and were plated on fibronectin-coated coverslips. Fig. 4A shows that both GFP-Grb7 and GFP-R239L mutant were localized in the cytoplasm and focal contacts in similar patterns (upper panels). Immunofluorescence staining of the same cells with anti-vinculin confirmed the focal contacts localization of both GFP-Grb7 and GFP-R239L (lower panels). These results suggest that the interaction of the Grb7 PH domain with phosphatidylinositol phosphates is not required for its focal contact localization and that the inability of
the R239L mutant to stimulate cell migration is not due to its mislocalization in these cells.

Since Grb7 phosphorylation by FAK has also been shown to be important in regulation of cell migration by Grb7 (13), we examined the association of R239L mutant with FAK and its phosphorylation by FAK. CHO cells were co-transfected with expression vectors encoding GFP-FAK and HA-tagged Grb7 or R239L mutant. The lysates were prepared and immunoprecipitated with anti-HA polyclonal antibody. Analysis of the immune complex by Western blotting with anti-phosphotyrosine antibody, PY20, showed that Grb7 is phosphorylated by FAK, but R239L mutant is not (Fig. 4, upper panel). However, both Grb7 and R239L mutant were associated with FAK (Fig. 4B, lower panel). The relative binding was normalized to the binding to Grb7 when transfected alone. The relative binding was normalized to the binding to Grb7 when transfected alone. Therefore, it was concluded that Grb7 interaction with phosphoinositides in cell migration is not due to its mislocalization in these cells.

Since Grb7 phosphorylation by FAK has also been shown to be important in regulation of cell migration by Grb7 (13), we examined the association of R239L mutant with FAK and its phosphorylation by FAK. CHO cells were co-transfected with expression vectors encoding GFP-FAK and HA-tagged Grb7 or R239L mutant. The lysates were prepared and immunoprecipitated with anti-HA polyclonal antibody. Analysis of the immune complex by Western blotting with anti-phosphotyrosine antibody, PY20, showed that Grb7 is phosphorylated by FAK, but R239L mutant is not (Fig. 4B, upper panel). However, both Grb7 and R239L mutant were associated with FAK (Fig. 4B, lower panel). The relative binding was normalized to the binding to Grb7 when transfected alone.

**Fig. 4.** Subcellular localization and tyrosine phosphorylation of Grb7 and R239L mutant. A, CHO cells that had been transfected with pEFGP-Grb7 or pEFGP-R239L were plated on fibronectin-coated coverslips. They were then fixed, permeabilized, and double-stained with rabbit polyclonal anti-GFP antibody (upper panels) and the mouse monoclonal anti-vinculin antibody (bottom panels). Representative focal contacts are marked by small arrows. B, CHO cells were co-transfected with pEFGP-FAK and pKH3, pKH3-Grb7, or pKH3-R239L, as indicated. They were lysed and immunoprecipitated (IP) by polyclonal anti-HA antibody Y-11. The immune complexes were analyzed by Western blotting (IB) with PY20 (upper panel), monoclonal anti-HA antibody (12CA5; middle panel), or monoclonal anti-FAK (bottom panel). The positions of phosphorylated Grb7 (top panel), total Grb7 (middle panel), and associated FAK (bottom panel) are marked on the left.

**Fig. 5.** Regulation of Grb7 binding to phospholipid by PI 3-kinase. A, 293 cells were transfected with pDHGST-Grb7 or pDHGST vector alone control and then treated with LY294002, wortmannin, or Me2SO solvent alone, as indicated. The binding of Grb7 with phospholipids in intact cells was then measured as described in the legend to Fig. 1D. The relative binding was normalized to the association in cells treated with the Me2SO solvent alone. B, 293 cells were transfected with pDHGST-Grb7 with or without the expression vector encoding M.p110*, as indicated. The binding of Grb7 with phospholipids in intact cells was then measured as described in the legend to Fig. 1D. The relative binding was normalized to the binding to Grb7 when transfected alone.

Because the PH domain alone showed greater binding to phosphoinositides than the full-length Grb7 (see Fig. 1D), Grb7 binding to FAK (via its SH2 domain) is unlikely to be necessary for its association with phosphoinositides. However, the above results (see Fig. 5) raised the interesting possibility that FAK might influence Grb7 association with phosphoinositides indirectly through its binding and activation of PI 3-kinase (35). To determine their effects on Grb7 association with phospholipids. Therefore, it was concluded that Grb7 interaction with phosphoinositides in cell migration is not due to its mislocalization in these cells.
test this possibility, CHO cells were co-transfected with expression vector encoding Grb7 and vectors encoding wild type FAK and its mutants. The in vivo association of Grb7 with phospholipids was then measured as described in the legend to Fig. 1D. The binding of Grb7 with phospholipids in intact cells was then measured as described in the legend to Fig. 1D for cells that had been suspended and replated on FN or poly-L-lysine (PLL) as indicated. The relative binding was normalized to the binding to cells replated on poly-L-lysine.

Fig. 6. Regulation of Grb7 binding to phospholipid by FAK and cell adhesion. A, CHO cells were co-transfected with pDHGST, pDHGST-Grb7, or pDHGST-Grb7 with pCDM8-FAK, pCDM8-Y397F, pCDM8-kd, or pCDM8-Y925F, as indicated. The binding of Grb7 with phospholipids in intact cells was then measured as described in the legend to Fig. 1D. The relative binding was normalized to the binding to Grb7 when transfected alone. B, CHO cells were transfected with pDHGST-Grb7. The binding of Grb7 with phospholipids were then measured as described in the legend to Fig. 1D for cells that had been suspended and replated on FN or poly-L-lysine (PLL) as indicated. The relative binding was normalized to the binding in cells replated on poly-L-lysine.

whether cell adhesion could regulate the association of Grb7 with phospholipids. CHO cells were transfected with expression vector encoding Grb7 and then metabolically labeled with 32P as described under “Materials and Methods.” The relative migration rate (normalized to untransfected cells as 1.0) from three independent experiments is shown. B, CHO cells were transfected with pEGFP-Grb7, the expression vector encoding M.p110* with pEGFP or pEGFP-R239L, as indicated. They were then subjected to the cell migration assays as described under “Materials and Methods.” The mean and S.D. of relative migration rate (normalized to untransfected cells as 1.0) from three independent experiments are shown.

Finally, we examined the effects of PI 3-kinase on Grb7-stimulated cell migration. As shown in Fig. 7A, treatment of cells with the solvent Me2SO did not affect stimulation of CHO cell migration upon transfection of vector encoding Grb7 (see Fig. 3B). However, treatment of the cells with the PI 3-kinase inhibitors LY294002 or wortmannin abolished the ability of Grb7 to stimulate cell migration. These results provided further evidence that the Grb7 association with D3-phosphoinositides is necessary for its function in promoting cell migration and also suggested that Grb7 might be at least one of the downstream mediators of PI 3-kinase in the regulation of cell

FIG. 6. Regulation of Grb7 binding to phospholipid by FAK and cell adhesion. A, CHO cells were co-transfected with pDHGST, pDHGST-Grb7, or pDHGST-Grb7 with pCDM8-FAK, pCDM8-Y397F, pCDM8-kd, or pCDM8-Y925F, as indicated. The binding of Grb7 with phospholipids in intact cells was then measured as described in the legend to Fig. 1D. The relative binding was normalized to the binding to Grb7 when transfected alone. B, CHO cells were transfected with pDHGST-Grb7. The binding of Grb7 with phospholipids were then measured as described in the legend to Fig. 1D for cells that had been suspended and replated on FN or poly-L-lysine (PLL) as indicated. The relative binding was normalized to the binding in cells replated on poly-L-lysine.

FIG. 7. Role of PI 3-kinase in Grb7-stimulated cell migration. A, CHO cells were transfected with pEGFP-Grb7 and then subjected to the cell migration assays in the presence of LY294002, wortmannin, or Me2SO solvent alone, as described under “Materials and Methods.” The mean and S.D. of relative migration rate (normalized to untransfected cells as 1.0) from three independent experiments are shown. B, CHO cells were transfected with pEGFP-Grb7, the expression vector encoding M.p110* with pEGFP or pEGFP-R239L, as indicated. They were then subjected to the cell migration assays as described under “Materials and Methods.” The mean and S.D. of relative migration rate (normalized to untransfected cells as 1.0) from three independent experiments are shown.
migration. Consistent with this latter possibility, we found that co-transfection of the R239L mutant (lacking binding for phospholipids) abolished the ability of the constitutively active p110 (M.p110*) to stimulate cell migration (Fig. 7B).

DISCUSSION

The PH domain is a conserved protein module in many signaling and cytoskeletal proteins, which has been shown to mediate interaction with various phospholipids and in some cases other proteins in signal transduction (23, 36–38). Although a conserved PH domain is present in all Grb7 family adaptor molecules (16–18), there have not been reports on whether this protein motif could bind to any specific phospholipids or whether such interactions play a role in any cellular functions mediated by Grb7 family proteins. Using both in vitro and in vivo binding assays, we showed here that Grb7 can interact with phosphatidylinositol phosphates through its PH domain. This interaction probably employs mechanisms similar to those of other PH domains, because mutation of Arg239 in Grb7 within the conserved arginine/lysine residue in the β1 strand (24, 32) abolishes its ability to bind to phospholipids both in vitro and in vivo. Furthermore, we show that the interaction of the Grb7 PH domain with phosphatidylinositol phosphates is crucial in its regulation of cell migration, which is consistent with our previous data suggesting a role for the central GM region of Grb7 in stimulation of cell migration (13).

The mechanism by which PH domain-mediated Grb7 interaction with phosphoinositides regulates cell migration is incompletely understood at present. Interaction of phospholipids with PH domain-containing proteins has been suggested to facilitate protein localization to the plasma membranes and/or induce conformational changes of the target proteins (22, 39). Localization of Grb7 to the focal contacts and its phosphorylation by FAK are both important for its promotion of cell migration (13). Therefore, binding of phosphatidylinositol phosphates to the Grb7 PH domain might affect its function in the regulation of cell migration by either or both of the above mechanisms. Data presented here support the latter possible mechanism. The R239L mutant deficient in phospholipid binding was localized to focal contacts as efficiently as the wild type Grb7 (Figs. 1D and 4A). This was consistent with our previous

---

**FIG. 8.** Working hypothesis of Grb7 interaction with FAK and phospholipids in cell migration.
finding that the SH2 domain of Grb7 was responsible for its subcellular localization (13). On the other hand, the R239L mutant showed a significant decrease in its phosphorylation by FAK (Fig. 4B). This suggested that phospholipids binding to the Grb7 PH domain might induce a conformational change in Grb7 to allow its phosphorylation by FAK, and disruption of this interaction prevented Grb7 phosphorylation by FAK. A similar mechanism was proposed for Akt phosphorylation at Thr<sup>308</sup> by PDK1, which was dependent on phospholipid binding to the Akt PH domain (39). Further support for a role of the Grb7 PH domain in inducing conformational change for FAK phosphorylation rather than targeting to focal contacts was provided by examination of chimeric molecules that replaced the SH2 domains of Grb7 or R239L mutant with the focal adhesion targeting sequence. Both chimeric molecules were targeted to focal contacts as expected. However, in contrast to the wild type Grb7, the R239L mutant chimera was unable to bind to phospholipids not phosphorylated by FAK or to stimulate cell migration (data not shown).

Besides Grb7, the activated FAK also recruits other downstream signaling molecules including Src family kinases and PI-3 kinase to focal contacts (7, 10, 11, 30). Our previous results suggested that the FAK-Grb7 signaling complex was independent of the FAK-Src complex that targets the p130<sup>cas</sup>/CrkII/DOCK/Rac signaling cascade in the regulation of cell migration (8, 9, 13). However, data presented here suggested that FAK-PI 3-kinase complex formation and PI 3-kinase activation could cooperate with FAK-Grb7 complex in the stimulation of cell migration. Analysis using protein-lipid overlay assays indicated binding of Grb7 and its PH domain to D3-phosphoinositides PtdIns-3-P, PtdIns-3,4-P<sub>2</sub>, and PtdIns-3,4,5-P<sub>3</sub> (Fig. 2). These results suggested that increased PI 3-kinase products upon its activation could enhance Grb7/phospholipid interaction, which in turn leads to Grb7 phosphorylation by FAK and stimulation of cell migration. This possibility is supported by a number of other observations. Co-expression of a constitutively active PI 3-kinase catalytic subunit M.p110<sup>+</sup> increased it. Consistent with this, treatment of cells with active M.p110* increased association of Grb7 with phospholipids, whereas treatment of cells inhibited by the R239L mutant that is deficient in phospholipid binding to Grb7 to allow its phosphorylation by FAK, and disruption of this interaction prevented Grb7 phosphorylation by FAK. A similar mechanism was proposed for Akt phosphorylation at Thr<sup>308</sup> by PDK1, which was dependent on phospholipid binding to the Akt PH domain (39). Further support for a role of the Grb7 PH domain in inducing conformational change for FAK phosphorylation rather than targeting to focal contacts was provided by examination of chimeric molecules that replaced the SH2 domains of Grb7 or R239L mutant with the focal adhesion targeting sequence. Both chimeric molecules were targeted to focal contacts as expected. However, in contrast to the wild type Grb7, the R239L mutant chimera was unable to bind to phospholipids not phosphorylated by FAK or to stimulate cell migration (data not shown).

Besides Grb7, the activated FAK also recruits other downstream signaling molecules including Src family kinases and PI-3 kinase to focal contacts (7, 10, 11, 30). Our previous results suggested that the FAK-Grb7 signaling complex was independent of the FAK-Src complex that targets the p130<sup>cas</sup>/CrkII/DOCK/Rac signaling cascade in the regulation of cell migration (8, 9, 13). However, data presented here suggested that FAK-PI 3-kinase complex formation and PI 3-kinase activation could cooperate with FAK-Grb7 complex in the stimulation of cell migration. Analysis using protein-lipid overlay assays indicated binding of Grb7 and its PH domain to D3-phosphoinositides PtdIns-3-P, PtdIns-3,4-P<sub>2</sub>, and PtdIns-3,4,5-P<sub>3</sub> (Fig. 2). These results suggested that increased PI 3-kinase products upon its activation could enhance Grb7/phospholipid interaction, which in turn leads to Grb7 phosphorylation by FAK and stimulation of cell migration. This possibility is supported by a number of other observations. Co-expression of a constitutively active PI 3-kinase catalytic subunit M.p110<sup>+</sup> increased association of Grb7 with phospholipids, whereas treatment of cells with active M.p110* increased association of Grb7 with phospholipids, whereas treatment of cells with active M.p110* increased association of Grb7 with phospholipids, whereas treatment of cells with active M.p110* increased...