Association of Focal Adhesion Kinase with Grb7 and Its Role in Cell Migration

(Received for publication, January 28, 1999, and in revised form, May 5, 1999)

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Focal adhesion kinase (FAK) has been implicated to play a key role in integrin-mediated signal transduction in cell migration. Grb7 is an Src homology (SH) 2-containing and pleckstrin homology domain-containing molecule, which shares significant homology with the Caenorhabditis elegans gene for Mig-10 involved in cell migration during embryogenesis. Here, we report that the SH2 domain of Grb7 can directly interact with FAK through Tyr-397, a major autophosphorylation site in vitro and in vivo. This interaction is cell adhesion-dependent, suggesting that the FAK-Grb7 complex is involved in integrin signaling. Using tetracycline-regulated expression system, we showed that overexpression of Grb7 enhanced cell migration toward fibronectin, whereas overexpression of its SH2 domain alone inhibited cell migration. In addition, we found that phosphorylation of FAK or p130Cas was not affected by the expression of either Grb7 or its SH2 domain alone, suggesting that Grb7 is downstream of FAK and does not compete with Src for binding to FAK in vivo. Taken together, these results suggest that the FAK-Grb7 complex plays a role in cell migration stimulated by integrin signaling through FAK.

Focal adhesion kinase (FAK)1 is a cytoplasmic tyrosine kinase that has been shown to play a critical role in integrin-mediated signal transduction (1–3). FAK is localized to focal contacts in many adherent cells, and it rapidly becomes activated and autophosphorylated following cell adhesion to extracellular matrix proteins or integrin clustering by antibodies (1, 4–6). The major tyrosine phosphorylation site of FAK has been mapped to Tyr-397, the phosphorylation of which creates binding sites for the Src homology (SH) 2 domains of other intracellular signaling molecules. Indeed, both Src family kinases (7–10) and phosphatidylinositol 3-kinase (PI3K) (11, 12) have been shown to form complexes with FAK at this site through their SH2 domains in cell adhesion-dependent manner. The formation of FAK-Src family kinase complexes have been proposed to allow phosphorylation of additional sites on FAK, which include Tyr-925 for binding the SH2 domain of Grb2 (13). Finally, FAK has been shown to interact with p130Cas through its proline-rich sequences at the COOH-terminal domain (14, 15) and cytoskeletal proteins Paxillin and talin by sequences also located in the COOH terminus (16–18). FAK interactions with these and potentially other proteins are believed to mediate the functions of FAK in integrin-dependent signal transduction.

Recent studies have suggested several roles for integrin signaling through FAK in regulation of cell survival (19, 20), proliferation (21, 22), spreading (23), and migration (21, 24, 25). Using Chinese hamster ovary (CHO) cells as a model system, we have previously shown that overexpression of FAK stimulated cell migration and mutation of Tyr-397 to Phe abolished its ability to promote cell migration (25). Furthermore, we have identified p130Cas as a downstream mediator of FAK-Src family kinases in the regulation of cell migration (26). Interestingly, tyrosine phosphorylation of Tyr-397 has also been found to be necessary for FAK to promote cell spreading (27), survival (19), and cell cycle progression (22). These studies implicate a key role for FAK complexes with Src family kinases and/or other signaling molecules at Tyr-397 in integrin-mediated signal transduction.

Grb7 is a member of an emerging family of signaling proteins that include Grb7, Grb10, and Grb14 (28–31). The family members have a highly conserved structure: the NH2-terminal region has proline-rich motifs, the central region has a pleckstrin homology (PH) domain, and the COOH-terminal region has an SH2 domain. Consistent with their domain structures, Grb7 family proteins have been shown to interact with a variety of other proteins, including tyrosine kinase receptors and proto-oncogenes (32–39). These interactions are believed to play a role in the regulation of mitogenic signaling pathways. In addition, the central domain of Grb7 family proteins contains a region of >300 amino acids that shows a high homology to a Caenorhabditis elegans gene, mig-10 (29, 32, 40). The Mig-10 protein has been shown to be involved in long range migration of neuronal cells in embryonic development (41). This suggests a possible role for Grb7 in the regulation of migration of mammalian cells.

In this paper, we present results showing a direct interaction of Grb7 with FAK at Tyr-397 and a potential role for FAK-Grb7 complex in cell migration stimulated by integrin signaling through FAK.

EXPERIMENTAL PROCEDURES

Materials—Protein A-Sepharose 4B, glutathione-agarose beads, and human plasma fibronectin (FN) were from Sigma. G418 and LipofectAMINE were from Life Technologies, Inc. The phosphopeptide Tyr(P)-397 surrounding Tyr-397 of FAK (SETDpYAEIIDE) was from Chiron Mimotopes (Victoria, Australia). The control peptide corresponding to the carboxyl-terminal 15 residues of FAK (IDQKLRKMISOSSRPH) was synthesized by the Biotecinology Program of Cornell University. The mouse mAb 12CA5, the mouse mAb KT3, the mouse monoclonal α-Src antibody 2–17, and the rabbit polyclonal α-FAK serum have been described previously (11, 25). The mouse α-phosphotyrosine mAb PY-20 was from Transduction Laboratories (Lexington, Kentucky).
KY), and the rabbit polyclonal α-Grb7 and α-p130<sup>SH2</sup> antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Preparation of Glutathione S-transferase (GST) Fusion Proteins

The cDNA encoding human Grb7 was as described previously (42). The sequences encoding the Grb7 SH2 domain was amplified by PCR using primers showing forward and reverse primers: 5′-CCGGGATCCAC

CAACACTGTTTTCACAG and 5′-CCGATTCCGGCTGAGGACATGACG

CAG. The PCR product was digested with BamHI and EcoRI and then cloned into pGEX2T to generate pGEXT-Grb7.SH2. pGEX-Src.SH2 was described previously (7). GST fusion proteins were produced and purified as described previously (7).

In Vivo and Peptide Competition Assays—Cell lysates were prepared in 1% Nonidet P-40 lysis buffer as described previously (43). GST fusion proteins (5 μg) were immobilized on glutathione-agarose beads and then incubated with lysates (200 μg) prepared from attached or suspended CHO cells for 90 min at 4 °C. The complexes were washed 4 times with 1% Nonidet P-40 lysis buffer, resolved by SDS-polyacrylamide gel electrophoresis, and analyzed by Western blotting with anti-FAK (1:3000) or anti-c-Src (1:5000) using the Amersham Pharmacia Biotech enhanced chemiluminescence (ECL) system, as described previously (18). For peptide competition assays, immobilized GST fusion proteins were preincubated with various concentrations of oligopeptides in 1% Nonidet P-40 lysis buffer for 15 min at 4 °C and then incubated with 100 μg of lysates from attached CHO cells in the presence of the oligopeptides. The bound proteins were analyzed as described above.

Transient Transfections of 293 Cells—To generate a vector for expression of GST fusion proteins in mammalian cells, the plasmid pKH3 (12) was linearized by digestion with Sall, filled in with T4 polymerase, and digested with BamHI. The coding sequence for GST was amplified by PCR using pGEX-2T as a template and the following primers: 5′-CTGTCGCAGGAGGAAATACG and 5′-CTCAGCTCATCCGAC

AAACGC. The PCR product was digested with BamHI and inserted into the linearized pKH3 described above. The resulting vector was designated pDHGST.

The full-length Grb7 was amplified from hGrb7 cDNA by PCR using the sense (5′-GGAACTCATAGGAGCCTGATCTGTCAC) and antisense (5′-CCGATTCCGGCTGAGGACATGACG) oligonucleotides as primers. The PCR product was digested with BamHI and EcoRI and inserted into pDHGST at the corresponding cloning site to generate pDHGST-Grb7. Expression vectors encoding SV40 T antigen epitope-tagged FAK (pCDM8-FAK), Y397F mutant (pCDM8-Y397F), and Y925F mutant (pCDM8-Y925F) were described previously (25, 26).

293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and were plated on poly-L-lysine or fibronectin (FN) substrates. For some experiments, cell lysates were prepared from suspended cells or cells that had been re-plated on poly-L-lysine or FN using ice-cold modified radiolabeled precipitation buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 30 mM Na<sub>2</sub>PO<sub>4</sub>, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotonin, and 20 μg/ml leupeptin), as described previously (4, 43). Lysates were cleared by centrifugation and total protein concentration was determined using the Bio-Rad protein assay. For “pull-down” assays using GST fusion proteins, 30–50 μl of glutathione-coupled beads were added to lysates and rotated for 30 min at 4 °C. After washing five times with lysis buffer, the samples were resolved using SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membrane (Schleicher & Schuell). Immunoprecipitations were carried out by incubating cell lysates with α-FAK or α-p130<sup>SH2</sup> antibody for 2 h at 4 °C, followed by incubation for 1.5 h with protein A-Sepharose. After washing, immune complexes were resolved using SDS-polyacrylamide gel electrophoresis. Western blotting was carried out using horseradish peroxidase-conjugated IgG as a secondary antibody and the Amersham Pharmacia Biotech ECL system for detection.

Cell Migration Assay—Cell migration assays were carried out using a Neuro Probe (Cabin John, MD) 48-well chemotaxis Boyden chamber as described previously (25) with slight modification as indicated. Prior to experiments, cells were incubated for 18 h in medium with (uninduced) or without (induced) 0.5 μg/ml tetracycline. 7.5 × 10<sup>3</sup> cells were added in each well, and the cells were allowed to migrate for 4 h in a 37 °C humidified incubator. At the end of experiment, cells were fixed with methanol for 8 min and stained with modified Giemsa stain (Sigma). The migrated cells were counted under light microscope at 100 × magnification using Image-Pro Plus software, version 3.0 (Media Cybernetics, Silver Spring, MD).

RESULTS

Previous studies have shown that upon cell adhesion FAK is autophosphorylated and associated with a number of SH2 domain-containing molecules, such as Src, Grb2, and PI3K (7–13). To further understand the mechanisms of signaling pathways initiated by integrin-activated-FAK, we investigated potential FAK interactions with additional SH2 domain-containing proteins. Cell lysates were prepared from either suspended or attached CHO cells and then incubated with various GST fusion proteins immobilized on glutathione beads. The bound proteins were then analyzed by Western blotting with anti-FAK. As shown in Fig. 1A, FAK from attached cells bound GST fusion protein containing the Grb7 SH2 domain (GST-Grb7.SH2) but not GST alone. In contrast, FAK from suspended cells did not bind to either GST-Grb7.SH2 or GST. Similar experiments demonstrated that the src-binding domain did not bind to another tyrosine-phosphorylated protein, c-Src, from either suspended or attached cells (Fig. 1B). These results demonstrated a specific FAK interaction with the Grb7 SH2 domain in vitro in a cell adhesion-dependent manner.

Tyroid-397 has been identified as the major autophosphorylation site of FAK in cell adhesion, suggesting that Grb7 SH2 domain may bind to FAK at Tyroid-397. Alternatively, adhesion-dependent Src binding to Tyroid-397 of FAK could lead to phos-
Comparison of the IC50 also suggested an apparently stronger phosphorylation of FAK at other tyrosine residues, creating binding sites for the Grb7 SH2 domain (13, 44, 45). To distinguish these two possibilities, a synthetic phosphopeptide Tyr(P)-397 (12-mer containing phosphorylated Tyr-397 and its flanking sequences) was tested for its ability to inhibit FAK binding to GST-Grb7.SH2 in vitro. Fig. 2A shows that the phosphopeptide Tyr(P)-397 decreased FAK binding to GST-Grb7.SH2 in a dose-dependent manner. As expected, it also inhibited FAK binding to GST-Src.SH2 (Fig. 2B). The IC50 values to inhibit FAK-GST-Grb7.SH2 and FAK-GST-Src.SH2 associations were approximately 30 and 3 μM, respectively. A control peptide corresponding to the carboxyl 15 residues of FAK did not inhibit FAK binding to either GST-Grb7.SH2 or GST-Src.SH2 even at 560 μM. These results indicated that the Grb7 SH2 domain bound to phosphorylated Tyr-397 of FAK rather than other sites.

To examine whether Grb7 also binds to FAK via phosphorylated Tyr-397 in intact cells, 293 cells were transiently co-transfected with plasmid pDHGST (−) or pDHGST-Grb7 (+) and pCDM8-FAK, pCDM8-Y397F, or pCDM8-Y925F, as indicated. Grb7 was pulled down from the lysates using glutathione-coupled agarose beads. They were then analyzed by Western blotting with KT3 (A) or PY-20 (B). Aliquots of the lysates (whole cell lysates (WCL)) were also analyzed to verify similar expression levels of FAK (C). D, lysates from NIH 3T3 cell were immunoprecipitated (IP) by anti-Grb7 or the control rabbit anti-mouse IgG (RAM). The immune complexes or an aliquot of the lysate (WCL) were analyzed by Western blotting with anti-FAK. The arrow indicates the position of FAK. Molecular mass positions (in kDa) are shown on the left.

Grb7 by Western blotting using 12CA5, a mAb against the hemagglutinin epitope tag fused to the NH2 terminus of exogenous Grb7. Fig. 4A shows expression of the exogenous Grb7 in several clones upon induction by removal of tetracycline in the media (compare lanes 1 with lanes U).

FAK has been shown to play an important role in cell migration (21, 24, 25) with phosphorylation of Tyr-397 being crucial for its activities (25). Therefore, NIH 3T3 cells with inducible overexpression of Grb7 were evaluated for their migration toward FN using modified Boyden chamber assays (Fig. 4B), as described previously (25). Little migration was observed under both induced and uninduced conditions in the absence of FN. At 1 μg/ml FN, however, induction of exogenous Grb7 expression (Fig. 4B, open columns) caused an approximately 2-fold increase in cell migration compared with the uninduced cells (filled columns). Smaller but significant increases were also observed upon induction of exogenous Grb7 expression at higher FN concentrations. Control experiments with Mock cells transfected with pTet-Splice vector alone showed similar migration rates under both induced and uninduced conditions (Fig. 4C). These results suggested that Grb7 and possibly its binding to FAK play a role in stimulation of cell migration.

To test directly the possibility that Grb7 binding to Tyr-397 of FAK is crucial for FAK-stimulated cell migration, we generated NIH 3T3 cells with inducible expression of the SH2 domain of Grb7 using the tetracycline-regulated expression system. Because Grb7 binds to FAK by its SH2 domain, overexpression of the SH2 domain alone is expected to compete with endogenous Grb7 for binding to FAK, thus acting in a dominant-negative manner. Fig. 5A shows expression of the
SH2 domain of Grb7 in several clones after induction by removal of tetracycline in the media, but not under uninduced conditions. Fig. 5B shows that induction of the Grb7 SH2 domain expression reduced cell migration by approximately one-half as compared with uninduced cells at several FN concentrations. These results provided further support that Grb7 association with FAK plays a crucial role in stimulation of cell migration, although we cannot exclude the possibility that the Grb7 SH2 domain inhibited cell migration by interfering with Grb7 interactions with other proteins.

The results presented so far are consistent with the idea that Grb7 is an important downstream mediator of FAK-promoted cell migration. However, it is also possible that Grb7 functions upstream of FAK to stimulate migration of NIH 3T3 cells as observed above. To investigate this possibility, we examined the effects of induced overexpression of Grb7 or its SH2 domain on tyrosine phosphorylation of FAK in these clones. Grb7 or Grb7.SH2 cells under either uninduced or induced conditions were removed from the plates and replated on FN or poly-L-lysine (PLL), as indicated. Lysates were immunoprecipitated with anti-FAK followed by Western blotting with PY-20 (A) or anti-FAK (B). The position of FAK is marked by arrows on the left.

FIG. 5. Expression of the Grb7 SH2 domain inhibits cell migration. A, lysates from several NIH 3T3 clones with inducible Grb7.SH2 expression under uninduced (U) and induced (I) conditions were analyzed by Western blotting with 12CA5. The arrow marks the position of the exogenous Grb7. Molecular mass positions (in kDa) are shown on the left. B, one representative clone with inducible Grb7.SH2 expression under uninduced (filled columns) or induced (open columns) conditions were subjected to cell migration assays as described under “Experimental Procedures.” Mean cells counts from at least 12 fields and three experiments are shown. Error bars represent S.D.

FIG. 6. Tyrosine phosphorylation of FAK in cells with inducible expression of Grb7 or Grb7.SH2. Grb7 or Grb7.SH2 cells under uninduced (U) or induced (I) conditions were detached by trypsin and replated on FN or poly-L-lysine (PLL), as indicated. Lysates were immunoprecipitated with anti-FAK followed by Western blotting with FY-20 (A) or anti-FAK (B). The position of FAK is marked by arrows on the left.
FAK has been implicated to play an important role in signal transduction by integrins (1–3). Consistent with this, FAK has been shown to interact with several cytoskeletal proteins as well as other signaling molecules. Analysis of FAK mutants lacking binding sites for these proteins has implicated specific cellular functions for some of these interactions (19, 22, 25–27). Here, we have identified association of FAK with another signaling molecule Grb7. Mutational analysis and peptide competition experiments have indicated a direct binding of FAK to Grb7 through its SH2 domain. Furthermore, this interaction was dependent on cell adhesion and required the major autophosphorylation site of FAK at Tyr-397.

The functional significance of the FAK-Grb7 interaction is not well understood at present. However, integrin signaling through FAK has been shown to stimulate cell migration, and autophosphorylation of FAK at Tyr-397 is critical for triggering downstream events leading to cell migration. Therefore, identification of FAK binding to Grb7 at this site raised the possibility that FAK-Grb7 interaction plays a role in FAK-stimulated cell migration. Interestingly, Grb7 shares extensive sequence homology with mig-10, a C. elegans gene implicated in cell migration in embryonic development (41). In addition, Tanaka et al. (48) have recently reported that inhibition of Grb7 by antisense RNA suppressed invasion of esophageal carcinoma cells using matrigel assays. Using an inducible expression system in NIH 3T3 cells, we show here that overexpression of Grb7 stimulated cell migration, whereas expression of the Grb7 SH2 domain decreased cell migration, possibly due to its disruption of Grb7 complex with FAK. Even though we cannot exclude the possibility that the Grb7 SH2 domain inhibited cell migration by interfering with Grb7 interactions with other proteins, these results together suggested a possible role for Grb7 and its association with FAK in the regulation of migration of mammalian cells.

Previous results from our laboratory showed that the auto-phosphorylation site Tyr-397 is essential for the binding of Src and P13K to FAK and the function of FAK in cell migration (12, 25). This raised the possibility that Grb7 influenced cell migration indirectly by competing with Src or P13K for FAK binding. However, several lines of data exclude this possibility. First, if Grb7 exerted its effects on cell migration by competing with binding of Src or P13K to Tyr-397 of FAK, we would have expected that both Grb7 and its SH2 domain would have inhibited cell migration. However, our data indicated that Grb7 stimulated cell migration, whereas the Grb7 SH2 domain inhibited it (Figs. 4 and 5). Second, tyrosine phosphorylation of p130

![Fig. 7. Tyrosine phosphorylation of p130 in cells with inducible expression of Grb7 or Grb7.SH2. Grb7 or Grb7.SH2 cells under uninduced (U) or induced (I) conditions were detached by trypsin and replated on FN or poly-L-lysine (PLL), as indicated. Lysates were immunoprecipitated by anti-p130 followed by Western blotting with PY-20 (A) or anti-p130 (B).](image)

DISCUSSION

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FAK at Tyr-397. We did not observe any changes in p130 phosphorylation in NIH 3T3 cells expressing either wild-type Grb7 or Grb7 SH2 domain alone (Fig. 7), suggesting that FAK-Src complex formation was not affected. Finally, similar amounts of FAK-associated P13K activities were detected in NIH 3T3 cells with induced expression of Grb7, its SH2 domains, or the control cells (data not shown). Together, these results indicated that FAK-Grb7 association regulates cell migration independent of FAK-Src or FAK-P13K complex formations.

These results also suggest that FAK may bind to Grb7, Src, and P13K through its phosphorylated Tyr-397 in an independent manner. It is possible that only a fraction of FAK is actually bound to Src, P13K, and Grb7. There may be sufficient unbound FAK in the cellular pool so that binding of one protein to FAK at Tyr-397 will not affect the association of FAK with other molecules. It is also possible that FAK binding to these molecules is spatially separated in cells due to different subcellular distributions. Finally, these interactions may be temporarily separated during cell migration. Further study will be necessary to test these possibilities and to determine the mechanisms by which FAK interactions with these molecules cooperate to regulate cell migration.

Grb7 has been shown to bind several other tyrosine-phosphorylated proteins, including the adapter molecule Shc (32) and tyrosine kinase receptors PDGFR (34), erbB2 (33), and erbB3 (35). Examination of the sequences surrounding the phosphorylated tyrosines revealed that the Grb7 SH2 domain recognized the Tyr(P)-X-Asn motif in these proteins (35). Although Tyr-925 of FAK is present in such a motif and is phosphorylated in a cell adhesion-dependent manner, mutation of Tyr-925 to Phe did not block FAK binding to Grb7 in in vivo (Fig. 3), which excluded its involvement in FAK binding to Grb7. In contrast, the Grb7 binding site in FAK (Tyr(P)-397) is not followed by an Asn at the +2 position, suggesting a possible exception to the binding motif for Grb7 observed in other proteins. Interestingly, Tyr-397 in FAK is preceded by two acidic residues, and one or two such residues are also present in the −3 to −1 positions of Grb7 binding site in these other proteins (33–35). It is possible that the residues amino-terminal to the phosphotyrosine also contribute to the specific binding of the phosphotyrosine to Grb7 SH2 domain, as this has been demonstrated for other SH2 domains (49). The specificity of FAK binding to Grb7 is also illustrated by its lack of binding to the closely related adapter molecule Grb10 (37). Nevertheless, the mechanisms and specificity of FAK-Grb7 interactions await further investigation.

The downstream pathway of Grb7 binding to FAK in cell migration is unknown at present. Both serine and tyrosine phosphorylations of Grb7 family proteins have been reported upon growth factor stimulation and their association with the tyrosine kinase receptors (29–32, 48). However, the functional consequences of phosphorylation of Grb7 family proteins have not been characterized yet, and additional studies are necessary to determine whether FAK association with Grb7 and/or cell adhesion can induce phosphorylation of Grb7. Another clue for potential mechanisms of Grb7 in cell migration comes from sequence comparison of Grb7 with Mig-10, a protein known to be involved in cell migration in C. elegans (29, 32, 40). Such a comparison reveals that the SH2 domain of Grb7 involved in interaction with FAK is not present in Mig-10. In contrast, the central regions of these two molecules containing a PH domain are highly homologous. The PH domain is involved in protein-protein interactions or protein-lipid metabolites interactions (50). This suggests the interesting possibility that the SH2 domain of Grb7 is responsible for its interaction with the up-
stream regulator FAK and that its Pro-rich domain or PH domain is involved in triggering downstream pathways in cell migration. It will be interesting to identify cellular proteins that interact with these regions of Grb7.

Cell migration is important in biological processes such as embryogenesis, wound healing, and tumor invasion and metastasis (51, 52). Increased expression of FAK and Grb7 has been found in certain cancers, which has been proposed to correlate with the invasive potential of tumors (53–55). Understanding the mechanisms by which FAK-Grb7 complex formation and their increased expression contribute to tumor progression will be an important area for future investigation.

Acknowledgments—We are grateful to Dr. H. Sasaki of the National Cancer Center Research Institute, Japan, for the generous gift of the cDNA clone pRe-Grb7 encoding human Grb7. We thank Leslie Cary, Molly Ingersoll, Ji He Zhao, and Renee Christopher for critical reading of the manuscript and helpful comments.

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