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The cell migration protein Grb7 associates with transcriptional regulator FHL2 in a Grb7 phosphorylation-dependent manner

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Abstract

Grb7 is an adaptor molecule that can mediate signal transduction from multiple cell surface receptors to various downstream signaling pathways. Grb7, along with Grb10 and Grb14, make up the Grb7 protein family. This protein family has been shown to be overexpressed in certain cancers and cancer cell lines. Grb7 and a receptor tyrosine kinase (RTK), erbB2, are overexpressed in 20–30% of breast cancers. Grb7 overexpression has been linked to enhanced cell migration and metastasis, though the participants in these pathways have not been determined. In this study, we report that Grb7 interacts with four and half lim domains isoform 2 (FHL2), a transcription regulator with an important role in oncogenesis, including breast cancer. Additionally, in yeast 2-hybrid (Y2H) assays, we show that the interaction is specific to the Grb7 RA and PH domains. We have also demonstrated that full-length (FL) Grb7 and FHL2 interact in mammalian cells and that Grb7 must be tyrosine phosphorylated for this interaction to occur. Immunofluorescent microscopy demonstrates possible co-localization of Grb7 and FHL2. A model with supporting NMR evidence of Grb7 autoinhibition is proposed.

Keywords

Grb7; FHL2; adaptor molecules; cell migration; cancer; NMR; protein recognition and binding; autoinhibition

Introduction

Intracellular signaling pathways regulate many critical processes in the cell. Errors in these pathways can lead to organismal pathology, such as cancer, diabetes, and a multitude of developmental diseases. It is clear that proteins in these pathways do not always function in a modular sense, that is, acting through linked, but autonomous, domains. In particular, autoinhibitory mechanisms, where one element of a protein masks or modulates the function of another element, have been reported in many proteins (for a review see Pufall and Graves, 2002).

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The Grb7 protein contains an N-terminal pro-rich region, a Ras associating-like (RA-like) domain (Wojcik *et al.*, 1999), a pleckstrin homology (PH) domain, “a between pleckstrin and Src” (BPS) domain, and a C-terminal SH2 domain (Figure 1). Research from our laboratory (Ivancic *et al.*, 2005) and others (Han and Guan, 1999; Han *et al.*, 2000; Tsai *et al.*, 2007) suggests that the central region of the cell migration protein Grb7 (the Grb7-RA and -PH domains) may operate, or be regulated, as a single functional unit. Guan *et al.* showed that this region of Grb7 is essential for Grb7-mediated cell migration of cancer cells (Han and Guan, 1999; Han *et al.*, 2000). Studies clarifying the functional role and regulation of this region in the Grb7 protein are an important step in understanding the mechanisms of cancer progression involving Grb7, with the desired goal of exploiting vulnerabilities for treatment options.

Grb7 protein family cellular signaling is an active research area (Stein *et al.*, 1994; Daly, 1998; Han and Guan, 1999; Jones *et al.*, 1999; Han *et al.*, 2000; Morrione *et al.*, 2000; Cariou *et al.*, 2002; Lim *et al.*, 2004; Shen and Guan, 2004; Tsai *et al.*, 2007). Members of this protein family have been shown to be important for normal and abnormal (cancer) signaling (Stein *et al.*, 1994; Tanaka *et al.*, 1998; Han and Guan, 1999; Jones *et al.*, 1999; Joyce *et al.*, 1999; Han *et al.*, 2000; Morrione *et al.*, 2000; Tanaka *et al.*, 2000) and for receptor tyrosine kinase (RTK) regulation (Bereziat *et al.*, 2002; Cariou *et al.*, 2002; Wick *et al.*, 2003). Grb7 has been shown to bind to erbB2 (epidermal growth factor receptor 2) and focal adhesion kinase (FAK) through its SH2 domain, and has been observed localized to focal adhesions (Han *et al.*, 2000; Lee *et al.*, 2000; Shen *et al.*, 2002).

ErbB2 (a.k.a. HER2) and Grb7 are both overexpressed in 20–30% of breast cancer patients (Slamon, 1987; Seshadri *et al.*, 1993; Stein *et al.*, 1994). Grb7 is phosphorylated on serine and threonine residues in both non-EGF, and EGF stimulated cells, and growth factor stimulation does not result in differential Ser/Thr phosphorylation patterns (Stein *et al.*, 1994; Fiddes *et al.*, 1998).

In contrast, FAK/Grb7 interaction results in phosphorylation of Grb7 on tyrosine residues (Jones *et al.*, 1999; Han *et al.*, 2000; Tanaka *et al.*, 2000). The Grb7-SH2 domain, FAK interaction is essential for FAK-mediated cell migration, with FAK phosphorylating Grb7 at tyrosine residues after initial Grb7 binding (Han *et al.*, 2000). Overexpression of Grb7 has been significantly correlated with the presence of lymph node metastases (Tanaka *et al.*, 1998), and the addition of exogenous Grb7-SH2 domain into an esophageal carcinoma cell line inhibits the association of Grb7 with endogenous FAK (Han and Guan, 1999), resulting in complete elimination of cell migration. The downstream signaling partners in FAK/Grb7 mediated cell migration are currently unknown.

The protein four and half lim domains isoform 2 (FHL2) binds to cytoskeletal structural proteins such as α -actin ACTA1 and titin (Lange *et al.*, 2002; Coghill *et al.*, 2003), has been observed localized to focal adhesions in several studies (Li *et al.*, 2001; Samson *et al.*, 2004; Lai *et al.*, 2006), and can interact directly with FAK (Gabriel *et al.*, 2004). Cell migration is severely impaired in FHL2-deficient cells in wound healing (Wixler *et al.*, 2007), thus highlighting the importance of FHL2 in these pathways.

The current studies seek to identify interaction partner(s) of Grb7 involved in FAK/Grb7-mediated cell migration, while clarifying the role of the Grb7-RA and -PH domains in this pathway. We explore the phosphorylation state of Grb7 necessary for (or during) interactions with these partner(s), and finally, propose a Grb7 autoinhibitory mechanism based upon these results.

Material and Methods

Yeast 2-hybrid screen

As an initial method to screen for other proteins involved in Grb7 signaling, a yeast 2-hybrid assay (Y2H) was performed using CLONTECH's Matchmaker GAL4 Two Hybrid System 3 according to the manufacturer's instruction (Clontech). The Grb7-RA-PH, Grb7-RA, or Grb7-PH domains were amplified from a pCMV-Grb7 full-length (FL) template by PCR. Primer sequences are available in the Supplementary Data Section. The “bait” Grb7-RA-PH, Grb7-RA, or Grb7-PH was directionally subcloned into the pGBKT7 vector (providing the DNA-binding domain (BD): bait). Initially, the yeast *Saccharomyces cerevisiae* strain AH109 was transformed with a pGBKT7 vector carrying the Grb7-RA-PH domain according to the manufacturer's instructions (Clontech). The Clontech pretransformed Human HeLa Matchmaker cDNA Library in *S. cerevisiae* strain Y187 containing the pGADT7-Rec vector (providing the DNA-activation domain (AD): prey) was used for the screen (Clontech). The results of the mating of Y187/pGADT7-Rec with the AH109/pGBKT7-Grb7-RA-PH were subjected to additional selection screening using appropriate media (medium stringency quadruple dropout media synthetic minimal medium (SD)/-Ade/-His/-Leu/-Trp, and high stringency SD/-Ade/-His/-Leu/-Trp/X- α -Gal media) to verify the interaction between the transcriptional factor BD and AD. The *MEL1* gene encodes the secreted enzyme α -galactosidase that hydrolyzes colorless X- α -Gal into a blue end product; thus, positive two-hybrid interaction colonies are blue (Supplemental Data, Figure 1A). Plasmids from positive clones were extracted using Zymoprep™ II spin columns (Zymo Research Corporation). The plasmids were transformed into *Escherichia coli* TOP 10 cells (Invitrogen), and grown on LB/ampicillin plates. Colonies were selected for plasmid extraction, followed by sequencing. The sequencing results were used for homology searching using BlastX (<http://www.ncbi.nlm.nih.gov/blast/>). Protein candidates with links to cell migration and proliferation (e.g., FHL2), and/or proteins that have been linked to cancer development and progression were given highest priority for analysis.

To determine which Grb7-RA-PH domain(s) were necessary for the protein–protein binding in the Y2H assay (i.e., with FHL2; Supplemental Data, Figure 1B), the isolated pGADT7-Rec plasmids carrying high-priority sequences were co-transformed with pGBKT7-Grb7-RA or pGBKT7-Grb7-PH into AH109 competent cells using the Lithium acetate method according to the manufacturer's instruction (Clontech). The cells were initially plated on double dropout media SD/-Leu/-Trp. AH109 cells growing on SD/-Leu/-Trp confirmed the presence of both pGBKT7 and pGADT7-Rec plasmids. Colonies grown on the SD/-Leu/-Trp were subcloned using quadruple dropout media (SD/-Ade/-His/-Leu/-Trp/X- α -Gal). The positive control for the Grb7 single domain (i.e., Grb7-RA or Grb7-PH) Y2H screens consisted of the pGBKT7-Grb7-RA-PH and pGAT-T7-FHL2 plasmids co-transfected into AH109 competent cells. The negative control consisted of empty pGBKT7 (i.e., no Grb7 domain insert) and pGADT-T7-FHL2 plasmids co-transfected into AH109 competent cells.

Construction of mammalian expression vectors

The pCMV-HA and pCMV-cMyc mammalian expression vectors were used to subclone the sequences of the FL-Grb7 and FHL2 obtained through the initial Y2H assay. These expression vectors express a fusion of proteins of interest with either the cMyc, or hemagglutinin (HA) epitope tag (Clontech). The amplified FL-Grb7 gene was directionally subcloned into pCMV-cMyc using the *SfiI* and *Sall* restriction sites. The FL FHL2 gene was subcloned into the pCMV-HA vector using the *Sall* and *NotI* restriction sites. Primer sequences are available in the Supplementary Data Section. Positive control vectors were also constructed by subcloning the P53 insert in the pCMV-cMyc and the large T antigen sequence in the pCMV-HA vector (data not shown).

Construction of Grb7-RA-PH domain protein expression vector for NMR analysis

The ligation-independent cloning method (Aslanidis and de Jong, 1990), using the pET46 EK/LIC vector according to the manufacturer's protocol (Novagen), was used to subclone the Grb7-RA-PH domains (residues 103–341 of the hGrb7 protein). Primer sequences are available in the Supplementary Data Section. The Grb7-SH2 domain expression vector has been described previously (Brescia *et al.*, 2002).

Antibody immobilization

The Grb7 (N20) antibody (Santa Cruz Biotechnology) was immobilized using the ProFound™ co-immunoprecipitation method according to the manufacturer's suggestions (Pierce Biotechnology). Samples of the immobilized Ab-bead slurry were boiled at 95°C for 5 min and were analyzed on SDS-PAGE gels with Coomassie or silver staining to confirm the presence of the immobilized antibody.

Site directed mutagenesis

Site-directed mutagenesis of selected Tyr residues was performed by polymerase chain reaction according to the manufacturer's guidelines, using the GeneTailor site-directed mutagenesis system (Invitrogen). Primer sequences are available in the Supplementary Data Section. Mutations were verified by DNA sequencing. All Grb7 mutants were expressed as FL-Grb7 proteins: FL-Grb7(Y262F), FL-Grb7(Y263F), FL-Grb7(Y262F/Y263F), FL-Grb7(Y287F), FL-Grb7(Y483F), FL-Grb7(Y495F), and FL-Grb7(Y483F/Y495F). Tyrosine residues were selected for mutation based upon their prediction as possible phosphorylation sites using the software NetPhos 2.0 (www.cbs.dtu.dk/zservices/netphos/).

Cell culture and transfections

HeLa cells were transfected with the indicated constructs using the transfection reagent Lipofectamine™ LTX (Invitrogen). Cells were maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% sodium bicarbonate, 0.5% sodium pyruvate, and 1% antibiotic-antimycotic. Cells were co-transfected with pCMV-HA-FHL2 and pCMV-cMyc-Grb7 vectors (wild type or mutants, as described previously), and were processed for pull-down assays or staining 48 h post-transfection. As the goal of this study was to determine Grb7 interaction partners in FAK-mediated signaling pathways (as opposed to erbB2-mediated pathways), cells for analysis were fully adherent and not stimulated with growth factors such as EGF.

Co-immunoprecipitation and western blotting

After transfection and incubation, adherent, non-growth-factor stimulated HeLa cells were lysed using the mammalian protein extraction reagent (M-PER) according to the manufacturer's recommendation (Pierce Biotechnology Inc.), and cellular debris removed by centrifugation at 15 000g. Lysates were incubated with immobilized Grb7 antibody beads (4°C) overnight, washed, eluted, and loaded onto SDS-PAGE gels. Proteins were transferred to PVDF membrane, and western blotting was carried out using FHL2 or Grb7 (H70) as the primary antibody, with horseradish peroxidase-conjugated IgG as the secondary antibody. The SuperSignal West Femto substrate was used for visualization (Pierce Biotechnology Inc.) for FHL2 and Grb7 primary antibody blots. The signal for the PY20 (phosphotyrosine) primary antibody blots was so intense a more insensitive (Amersham ECL—Enhanced ChemiLuminescence, GE Biosciences) visualization reagent was used to decrease the signal to levels that avoided overly intense (i.e., white) bands.

Immunofluorescence

Cells were fixed and processed for immunofluorescence staining using the Cytoskelfix II solution according to the manufacturer's instructions (Cytoskeleton Inc). Cells were incubated with primary antibodies against Grb7 (H70), and FHL2 (Santa Cruz Biotechnology). The cells were then washed and incubated with DAPI and secondary antibodies Alexa 488, and Alexa 546. Images were acquired using a 63× Planapo objective (NA = 1.4) mounted on a Zeiss Axiovert 200M inverted microscope equipped with epifluorescence optics and an Apotome structured illumination module to generate optical sections (Carl Zeiss, Thornwood, NY). Twelve bit images were acquired using an Axiocam MrM CCD camera driven by Axiovision 4.5 software. Acquired images were exported into eight-bit tiff files, and figures were prepared using the software Adobe Photoshop®.

Antibodies

The following antibodies were purchased from Santa Cruz Biotechnology: Grb7 (H-70) rabbit polyclonal, Grb7 (N-20) rabbit polyclonal, *p*-Tyr (PY20) mouse monoclonal, FHL2 mouse monoclonal, *p*-Tyr (PY350) rabbit polyclonal. The HA-Tag rabbit polyclonal and the cMyc mouse monoclonal antibodies were purchased from Clontech. The Alexa Fluor® 488 goat anti-rabbit and Alexa Fluor® 546 goat anti-mouse secondary antibodies were purchased from Invitrogen. The ImmunoPure® peroxidase conjugated goat anti-rabbit, and ImmunoPure® peroxidase conjugated goat anti-mouse antibodies were obtained from Pierce Biotechnology Inc.

Grb7 protein domain(s): NMR sample preparation and NMR spectroscopy

Sample Preparation: expression and purification of the human Grb7-SH2 domain has been described previously (Brescia *et al.*, 2002). Uniformly ¹⁵N-labeled Grb7-SH2 domain was produced in minimal media containing 1 g/L (¹⁵NH₄)₂SO₄ (Cambridge Isotope Labs, Woburn, MA, USA). A pET expression plasmid containing the hGrb7-RA-PH gene insert was transformed into *E. coli* BL21 (Rosetta, Novagen) cells and expressed at 37°C (310 K). The RA-PH domains, consisting of residues 103–341 of the hGrb7 protein (numbering according to Tsai *et al.*, 2007), were expressed as a hexa-histidine fusion protein and purified by affinity chromatography utilizing NiNTA resin (Invitrogen). Typical yields of pure hGrb7-RA-PH domain were 2–4 mg/L of culture. For both the free ¹⁵N-Grb7-SH2 domain alone and ¹⁵N-Grb7-SH2 domain in the presence of ¹⁴N-Grb7-RA-PH, sample conditions for NMR spectroscopy were: 0.05–0.1 mM ¹⁵N-Grb7-SH2 domain, 0.015–0.11 mM ¹⁴N-Grb7-RA-PH domain, 250 mM imidazole, 50 mM NaH₂PO₄, 0.5 M NaCl, pH 8.

NMR spectroscopy: all ¹H-¹⁵N correlation HSQC (Nhsqc) spectra of both the ¹⁵N-Grb7-SH2 domain alone, and ¹⁵N-Grb7-SH2 domain in the presence of ¹⁴N-Grb7-RA-PH, were recorded at a temperature of 25°C on a Varian Unity Plus 500 MHz NMR spectrometer equipped with a ¹H/¹³C/¹⁵N probe and Z-axis pulsed field gradient capabilities. Spectra were recorded in the States-TPPI mode for quadrature detection with carrier frequencies for ¹H and ¹⁵N at 4.73 and 120.0 ppm, respectively. In the direct dimension, 1024 real data points were collected, and in the indirect dimension 128 real data points were collected, with 64 scans per increment. Spectral widths of 2200 and 8000 Hz were employed in F1 and F2, respectively. Nhsqc spectra were acquired for the following sample ratios of SH2 domain to RA-PH domain: 1:0, 1:0.3, 1:0.6, 1:1.1. Each sample ratio combination of SH2 and RA-PH domain was prepared individually and concentrated to a total NMR sample volume of 0.6 ml.

All ¹H-¹⁵N correlation spectra were processed identically using instrument-supplied Varian VNMR software with sinebell squared (direct dimension), or sinebell (indirect dimension) apodization functions and zero filling to the next power of 2 in both dimensions.

Results

Grb7 interacts with FHL2 (four and a half lim domains, isoform 2) via its RA and PH domains

The initial Y2H screen with Grb7-RA-PH revealed a protein potentially involved in Grb7-mediated cellular signaling: FHL2 (1 essentially FL clone out of 35 positive clones, the remaining positive clones will be reported elsewhere). Specifically, FHL2 was identified as binding positively to the Grb7-RA-PH region (Supplemental Data, Figure 1A). Y2H assays performed using only the Grb7-RA domain or the Grb7-PH domain (Supplemental Data, Figure 1B) as bait indicated each of the Grb7-RA or Grb7-PH domains alone may be sufficient for binding to FHL2.

FHL2 interacts with full-length Grb7 in mammalian cells

For all the mammalian cell studies described, cells were adherent and non-growth-factor stimulated. HeLa cells were co-transfected with vectors expressing HA-tagged FHL2 and cMyc-tagged FL-Grb7, and tested for interaction between the two proteins by co-immunoprecipitation assay. The calculated molecular weight of cMyc-tagged Grb7 is 64 315 Daltons, while that of HA-tagged FHL2 is 33 193 Daltons. Figure 2, Panel A shows the results of lanes probed with FHL2 antibody. Figure 2, panel B, depicts stripping and re-probing with Grb7 antibody. In the final panel (Figure 2, panel C), the membrane is stripped and re-probed with phosphotyrosine antibody. Appropriate positive and negative controls appear in lanes 1–3 as described in the figure legend. The FL-Grb7 protein migrates at an apparent molecular weight of approximately 70–72 kD, in accordance with H70 antibody probed Grb7 from A-431 cell lysates (Santa Cruz Biotechnology, Grb7-Ab H70 datasheet, <http://datasheets.scbt.com/sc-13954.pdf>). The apparent molecular weight of FHL2 is approximately 48–50 kD, and is a doublet. It has been shown that FHL2 can be post-translationally modified, resulting in multiple forms and abnormal electrophoretic mobility (El Mourabit *et al.*, 2003). Figure 2 verifies that FL-Grb7 protein co-immunoprecipitates with the FHL2 protein in a mammalian cell environment.

Full-length Grb7 is phosphorylated on tyrosine residues

As described above, the FL-Grb7/FHL2 co-immunoprecipitation assay results were also probed with a phosphotyrosine antibody (Figure 2, panel C). The bands verified as Grb7 in lanes 4 and 5 in panel B (molecular weight 70–72 kD) react positively with the phosphotyrosine antibody (lanes 4 and 5, panel C), while there is no phosphotyrosine antibody signal observed at the molecular weight corresponding to FHL2 (approximately 48–50 kD, as determined by reaction with FHL2 antibody). This result demonstrates that the FL-Grb7 protein is phosphorylated on one or more tyrosine residues, and that FHL2 is not tyrosine phosphorylated.

Grb7 and FHL2 co-localize in HeLa Cells

In Figure 3, Grb7 and FHL2 show apparent co-localization in HeLa cells. Grb7 is visualized in panels B, F, D, and H; FHL2 is visualized in panels A, E, D, and H; and the cell nucleus is visualized in panels C, G, D, and H. Areas of Grb7/FHL2 overlap in panels D and H are shown as yellow. Panels E–H provide a magnification of the region denoted by a square in panel D, inset box. Arrows in panels E, F, and H indicate particular regions of Grb7/FHL2 co-localization on the inner cell membrane.

The phosphorylation state of the Grb7 PH and SH2 domains affects Grb7 function

All of the Grb7 tyrosine mutations resulted in negation of FHL2 binding to Grb7, as can be seen in lanes 2–8 of Figure 4. In lane 1 of Figure 4, wild-type FL-Grb7 co-

immunoprecipitates with FHL2 readily. However, all co-immunoprecipitation lanes involving the FL-Grb7 Tyr→Phe mutants, give virtually no signal (panel A). Panel B demonstrates, by probing with Grb7 Ab after coimmunoprecipitation, that all the Grb7 protein mutants are binding to the Grb7 antibody immobilized beads.

Panel C provides transfected cell lysate loading controls for wild-type FL-Grb7, and each of the studied mutants. The loading controls demonstrate approximately the same amount of Grb7 protein is contained in each cell lysate prior to exposure to Grb-7 antibody immobilized beads.

Grb7(Tyr→Phe) mutants lack phosphorylation on tyrosine residues

The same co-immunoprecipitation results in Figure 4 were probed with phosphotyrosine antibody, as in Figure 2, to discover the tyrosine phosphorylation state of wild-type FL-Grb7 and the FL-Grb7(Tyr→Phe) mutants. As can be seen in panel C, only the wild-type FL-Grb7 reacts appreciably with the phosphotyrosine Ab, implying the Grb7(Tyr→Phe) mutants lack phosphorylation on tyrosine residues. A very weak phosphotyrosine signal is observed for the FL-Grb7(Y263F) mutant.

Grb7-SH2 domain NMR chemical shifts are perturbed by the presence of the Grb7-RA-PH domains

Based on evidence that members of the Grb7 family may exist in a multimeric state (Dong *et al.*, 1998; Stein *et al.*, 2003; Ivancic *et al.*, 2005; Porter *et al.*, 2005; Porter *et al.*, 2007), different combinations of Grb7 protein domains were expressed in a bacterial system, isotopically labeled with magnetically sensitive nuclei, and studied by NMR spectroscopy. Specifically, the uniformly ^{15}N -labeled Grb7-SH2 domain was expressed and purified as described previously (Brescia *et al.*, 2002). A Grb7-RA-PH construct was expressed and purified, without ^{15}N -labeling (see Materials and Methods). ^1H - ^{15}N correlation HSQC spectra (Nhsqc) of the ^{15}N -labeled Grb7-SH2 domain and the unlabeled Grb7-RA-PH domain were acquired in the following ratios: 1:0, 1:0.3, 1:0.6, 1:1.1. In all spectra the sample buffer conditions, pH, total volume, and temperature were the same. In the upper right quadrant of Figure 5 is the ^1H - ^{15}N correlation HSQC spectrum of the Grb7-SH2 domain (red contours, ratio 1:0) overlaid on the ^1H - ^{15}N correlation HSQC spectrum of the Grb7-SH2 domain in the presence of the Grb7-RA-PH domain (black contours, ratio 1:1.1). Several labeled regions of the Nhsqc spectra are enlarged and shown in the other quadrants, with small labeled boxes representing the same areas in the original spectra.

The amplified regions of the overlaid Nhsqc spectra demonstrate that the Grb7-SH2 domain undergoes many chemical shift changes in the presence of the Grb7-RA-PH domains (indicated by arrows). The imidazole buffer in which the Grb7-RA-PH domain has the best solubility characteristics is a buffer in which the Grb7-SH2 domain is only transiently soluble, and results in precipitation of the sample within a 1–3 days. Despite this, direct comparison of the Nhsqc spectrum of the Grb7-SH2 domain in its published buffer conditions (Brescia *et al.*, 2002; Ivancic *et al.*, 2003), versus the same domain in imidazole, allowed sequence specific assignment of several residues undergoing chemical shift change, specifically, residues 45V, 46R, 48S, 64K, and 101Q. Boxes D and E demonstrate fast exchange chemical shift values between the free and bound resonances of 101Q and 64K for the different ratios of SH2 domain and RA-PH domains (as labeled), while boxes A–C show the chemical shift changes observed in the amide resonances of 45V, 46R, and 48S in the presence of a slight excess of unlabeled RA-PH (ratio 1:1.1).

Discussion

This study has shown that the cell migration protein Grb7 and transcriptional regulator FHL2 interact in a mammalian cell environment. Further, these studies demonstrate that FL-Grb7 is phosphorylated on tyrosine residues in cell lysate pull-downs of FHL2. As shown by Y2H analysis, the Grb7 interaction with FHL2 can take place through either the Grb7-RA or Grb7-PH domains alone. That Grb7 can exist in a tyrosine phosphorylated form has been reported previously (Jones *et al.*, 1999; Han *et al.*, 2000; Tanaka *et al.*, 2000), however, the existence of a Grb7/FHL2 complex, and co-localization of Grb7 and FHL2 in HeLa cells, is a new finding. In addition, many chemical shift resonances in the Nhsqc spectrum of the Grb7-SH2 domain are affected by the presence of the Grb7-RA-PH domains, implying a binding event may be occurring.

Evidence for a Grb7 multimeric/autoinhibited form

There is evidence that members of the Grb7 protein family (consisting of Grb7, Grb10, and Grb14) can exist in a multimeric form, and that this form could offer an autoinhibitory regulation mechanism.

The Grb10-SH2 domain crystal structure (PDB ID # 1NRV) solved by Stein *et al.* indicates the SH2 domain exists as a dimer (Stein *et al.*, 2003), implying possible tail-to-tail oligomerization. This is in direct contrast to the finding of Dong *et al.*, which shows that Grb10 exists as a head-to-tail dimer or tetramer (Dong *et al.*, 1998). Dong *et al.* proposed a mechanism for Grb10 regulation (Dong *et al.*, 1998), theorizing Grb10 exists in multimeric form in cells, based upon Y2H assays and mutagenic analysis.

Another Grb7 protein family member, Grb14, has emerged as an inhibitory regulator of insulin signaling (Kasus-Jacobi *et al.*, 1998; Hemming *et al.*, 2001; Bereziat *et al.*, 2002; Cariou *et al.*, 2002; Cariou *et al.*, 2004; Cooney *et al.*, 2004; King and Newton, 2004; Depetris *et al.*, 2005; Rajala and Chan, 2005; Rajala *et al.*, 2005; Nouaille *et al.*, 2006; Park *et al.*, 2006). Depetris *et al.* showed that, “high-affinity binding of the Grb14 protein to the insulin receptor requires the BPS domain and a dimerized SH2 domain” (Depetris *et al.*, 2005). This conclusion was based upon the decreased inhibitory action of an SH2-dimerization defective Grb14 mutant.

Our previous work also shows the Grb7-SH2 domain undergoes a competing dimerization reaction in solution (PDB ID # 1MW4, Ivancic *et al.*, 2005). It is possible that in the truncated form SH2-domain dimerization in the Grb7 protein family may provide only a partial binding interface. Porter *et al.* showed that FL-Grb7 dimerizes with a dissociation constant of 11 μ M, while the SH2 domain alone dimerizes with a dissociation constant of 21.8 μ M (Porter *et al.*, 2007). This factor of two decrease in dimerization affinity could result from loss of critical contacts with the RA and/or PH domain regions in the truncated Grb7-SH2 domain form. How much significance to attribute to these affinity differences remains a question, since the authors do not report a root-mean-square deviation value for their NONLIN fit of the ultracentrifugation data, and there is considerable scatter in the residuals of the fit.

Recently, Tsai *et al.* showed that Grb7 acts as a translational repressor by binding to the mRNA 5'UTR of the kappa opioid receptor (KOR) through its N-terminal pro-rich region (Tsai *et al.*, 2007). Phosphorylation of Grb7 in its carboxy terminus, specifically in the Grb7-SH2 domain, relieves Grb7 repression of KOR mRNA translation by preventing Grb7 association with the KOR mRNA 5'UTR. This work for the first time suggests Grb7 may play a broader regulatory role than originally envisioned, a role that may be regulated by the phosphorylation state of Grb7.

Grb7 and FHL2

The FHL2 transcriptional regulator is composed of multiple LIM domains. LIM domains are common protein–protein interaction motifs found in several types of proteins, including transcription factors, kinases, and scaffolding proteins. The presence of LIM domains in a protein is often associated with both transcriptional and cytoskeletal rearrangement functions (Johannessen *et al.*, 2006). The predicted FHL2 protein is 279 amino acid residues in length and has a calculated mass of 32 kD. LIM domains themselves are Zn metal binding cysteine-rich motifs of about 50 amino acids in length. Since FHL2 has been shown to localize with FAK at focal adhesions, and this study implies Grb7 and FHL2 co-localize, it is logical to extrapolate that Grb7 and FHL2 are co-localizing at focal adhesions, as seen in Figure 3. However, although Grb7 has been observed localized to focal adhesions in prior studies (Han *et al.*, 2000; Lee *et al.*, 2000; Shen *et al.*, 2002), our study shows Grb7 localization typically *proximal* to areas of vinculin antibody fluorescence activity representing focal adhesions (Supplemental Data, Figure 2). This could suggest a Grb7 functional connection between the advancing lamellipodial leading edge and lagging focal adhesion formation.

Y2H screens using only the Grb7-RA and Grb7-PH domains alone yielded instructive results, and indicated either domain was sufficient for binding to FHL2. The repeating LIM domain structure of FHL2 can allow selective binding to only certain LIM domains, and may explain the finding that both the Grb7-RA and Grb7-PH domains alone are capable of binding to FL-FHL2. Future efforts are focused on investigating the validity of this model.

Previous Y2H screens employing FL-Grb7 yielded no identifiable binding partners (Leavey *et al.*, 1998). Our Y2H studies focused on the Grb7-RA-PH domains alone as bait, investigating whether the FL-Grb7 molecule somehow prevents access to these domains in the non-mammalian environment. This hypothesis was borne out by isolating the FHL2 protein as a Grb7 interacting partner. The resulting Grb7/FHL2 interaction was verified using FL-Grb7 in a mammalian cell environment, suggesting some posttranslational modification needed for Grb7 activation was lacking in the yeast environment.

Model for Grb7 autoinhibition

To explain our results, and the observations noted in Dong *et al.* (1998) and Tsai *et al.* (2007), we present a model in which Grb7 is autoinhibited through a binding interaction between the N-terminal domains of the protein and the C-terminal SH2 domain region (Figure 6). In this model, FAK recruits Grb7 via its SH2 domain (Han and Guan, 1999), and subsequently phosphorylates Grb7 at tyrosine residues in the PH and/or SH2 domains (Han *et al.*, 2000; Tsai *et al.*, 2007). Whether the N- to C-terminal autoinhibition mechanism is intra- or inter-molecular is equally supported by the available data, and both scenarios are pictorially represented in Figure 6 (panel A vs. panel B). However, only panel A in Figure 6 satisfies the proposed requirement that Grb7 dimerization is mediated via its SH2 domain (Stein *et al.*, 2003; Porter *et al.*, 2005; Porter *et al.*, 2007). In both mechanisms, we theorize steric and/or ionic clash produced by Grb7 phosphorylation could force the Grb7 domains apart, providing access to the RA-PH region for communication with signaling proteins such as FHL2.

Experimental support of the models in Figure 6 is seen in ^{15}N - ^1H resonances of the Grb7-SH2 domain, which are perturbed in the presence of the Grb7-RA-PH domains, and display many instances of substantial chemical shift change (Figure 5). Such chemical shift changes are conventionally interpreted as evidence for a binding interaction (Shuker *et al.*, 1996), and suggest that Grb7 molecule can indeed interact in a head-to-tail fashion. Based upon the

resonances affected by the presence of the RA-PH domains, the β B strand of the Grb7-SH2 domain may form at least part of the binding interface with the RA-PH domains.

One would expect mutation of potential tyrosine phosphorylation sites in Grb7 to affect Grb7's ability to bind FHL2, if phosphorylation is required for the Grb7 central domains to be available for binding to signaling proteins such as FHL2. Indeed, we have shown this to be the case. However, the *differential* effect of mutating various tyrosine residues to phenylalanine is not highly pronounced. All mutations resulted in abrogation of Grb7 binding to FHL2, with the exception of a slight phosphotyrosine signal for FL-Grb7(Y263F) (Figure 4).

There are several scenarios that potentially explain why each Grb7(Tyr→Phe) mutant remains non-phosphorylated (presumably non-phosphorylated by FAK (Jones *et al.*, 1999; Han *et al.*, 2000; Tanaka *et al.*, 2000)). It is possible that all the PH- and SH2-domain tyrosines may be required for proper recognition of Grb7 as a FAK substrate, or that a Grb7/FHL2 complex supplies the FAK substrate recognition. Perhaps loss of even a single hydroxyl group changes the recognition site sufficiently such that FAK will no longer phosphorylate Grb7. Another explanation for Grb7(Tyr→Phe) mutant non-phosphorylation is that FAK is somehow kinase inactive. It has been demonstrated FAK itself exists in an autoinhibited form, with its N-terminal FERM domain obstructing both access to the Y397 required for Grb7 binding, and the activation loop required for FAK kinase activity (Lietha *et al.*, 2007). Grb7 could be involved in FAK activation, and mutation of tyrosine residues interferes with this process. Finally, perhaps every Tyr→Phe mutation performed in this study results in a denatured Grb7 protein, incapable of binding to FAK, and therefore not phosphorylated by FAK. This is unlikely, considering that the Grb7 sister protein Grb14 naturally contains Phe residues at the Grb7 Y263, Y483, and Y495 equivalent positions.

Conclusions

The adaptor protein Grb7 interacts with FHL2 in a mammalian cell environment, and Grb7 is phosphorylated on tyrosine residues in cell lysate pull-downs of FHL2. The presence of the RA and PH domains of Grb7 alone is sufficient for binding between FHL2 and Grb7. Mutation of any of the five predicted tyrosine phosphorylation sites of Grb7 results in loss of binding to FHL2 and an apparent global loss of tyrosine phosphorylation on Grb7. This loss of tyrosine phosphorylation on Grb7 could perhaps be explained by a Grb7(Y→F) mutant substrate deficiency for FAK phosphorylation. NMR evidence suggests the Grb7-SH2 and Grb7-RA-PH domains may be involved in a head-to-tail binding event. Two potential autoinhibition models have been proposed to explain the combined results of Grb7-RA-PH domain signaling availability and the observed NMR chemical shift changes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

BPS	between Pleckstrin and Src
CORT	cloning of receptor targets
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
erbB2 (a.k.a. HER2)	Erythroblastosis B
FAK	focal adhesion kinase
FHL2	four and a half LIM domains isoform 2
Grb	growth factor receptor bound

HSQC	heteronuclear single quantum coherence
LB	luria broth
LIM	Lin-11 Isl-1 Mec-3
Nhsqc	nitrogen heteronuclear single quantum coherence
NMR	nuclear magnetic resonance
PH	pleckstrin homology
RA	Ras associating
RTK	receptor tyrosine kinase
SH2	Src homology 2
Src	Sarcoma
Y2H	yeast 2-hybrid

Figure 1.

Domain topology of the Grb7 protein. The approximate locations of the Grb7 tyrosine to phenylalanine mutation sites as described in the text are identified by arrows in the primary amino acid sequence.

Figure 2.

Co-immunoprecipitation results for co-transfected HeLa cells with the vectors full-length FHL2/HA-pCMV and full-length Grb7/cMyc-pCMV. The same membrane is stripped and probed with antibodies a total of three times (A-C). Lane 1: co-transfected HeLa cell lysate. Lane 2: 40 μ l co-transfected HeLa cell lysate, pull down with beads, no Ab. Lane 3: 40 μ l native HeLa cell lysate, pull down with Grb7-Ab-immobilized beads. Lane 4: pull down of co-transfected HeLa cell lysate with Grb7-Ab-immobilized beads, 10 μ l. Lane 5: pull down of co-transfected HeLa cell lysate with Grb7-Ab-immobilized beads, 40 μ l. Panel A: all lanes probed with FHL2-Ab. Panel B: all lanes probed with Grb7-Ab. Panel C: all lanes probed with phospho-Tyr-Ab.

Figure 3.

For co-localization of FHL2 and Grb7 in HeLa cells, the cells were co-transfected with plasmids carrying epitope-tagged FHL2 and Grb7, and after 48 h were fixed and processed for immunolocalization. FHL2 localized to what may be focal adhesion plaques on the ventral plasma membrane (Panels A and E), whereas Grb7 (Panels B and F) was predominantly cytoplasmic. However, co-localization could be detected at discrete cellular protrusions (denoted by arrows). Panels E–H represent a magnification of the region denoted by a square shown in Panel D. Bars, 10 μ m.

Figure 4.

The effect of the loss of possible tyrosine phosphorylation sites in Grb7 was measured in terms of the ability of FL-Grb7 to interact with FHL2. Lanes 1–8 demonstrate the ability of FL-Grb7 and FL-Grb7 mutants to interact with FHL2 by immunoprecipitation assay as described in the materials and methods section. Panel A: all lanes probed with FHL2 Ab. Panel B: all lanes probed with Grb7 Ab. Panel C: all lanes probed with phospho-Tyr Ab. Panel D: transfected cell lysate loading control, probed with Grb7 Ab. Lane 1: wild type FL-Grb7, Lane 2: FL-Grb7(Y262F), Lane 3: FL-Grb7(Y263F), Lane 4: FL-Grb7(Y262F/Y263F), Lane 5: FL-Grb7(Y287F), Lane 6: FL-Grb7(Y483F), Lane 7: FL-Grb7(Y495F), and Lane 8: FL-Grb7(Y483F/Y495F). This figure is available in color online at www.interscience.wiley.com/journal/jmr

Figure 5.

Nhsqc NMR spectrum of the ^{15}N -labeled Grb7-SH2 domain alone (red contours, ratio 1:0), and in the presence of non-labeled Grb7-RA-PH domain (black contours, ratio 1:1.1). The x -frequency represents the amide proton chemical shift, while the y -frequency represents the attached amide nitrogen chemical shift. The entire overlaid Nhsqc spectra for the domains is shown in the upper right panel, and blowups of the indicated boxed regions are shown in the remaining panels. Boxes D and E demonstrate the titration effect of adding different sub-equivalent amounts of the Grb7-RA-PH domains to the ^{15}N -labeled SH2 domain. Purple contours represent the 1:0.3, and blue contours represent the 1:0.6 ratio, of SH2 domain to RA-PH domains.

Figure 6.

Proposed autoinhibition mechanisms of the Grb7 protein. In both scenarios (Panels A and B) activated focal adhesion kinase (FAK) recruits Grb7 via its SH2 domain (Han and Guan, 1999). FAK subsequently phosphorylates Grb7 at tyrosine residues in the PH and/or SH2 domain (Han *et al.*, 2000; Tsai *et al.*, 2007). Steric/ionic clash produced by tyrosine phosphorylation forces the Grb7-SH2 and Grb7-RA-PH domain regions apart, providing access to the RA-PH region for interaction with downstream signaling proteins such as FHL2.