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Deletion of the calmodulin-binding domain of Grb7 impairs cell attachment to the extracellular matrix and migration

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ABSTRACT

The adaptor Grb7 is a calmodulin (CaM)-binding protein that participates in signaling pathways involved in cell migration, proliferation and the control of angiogenesis, and plays a significant role in tumor growth, its metastatic spread and tumor-associated neo-vasculature formation. In this report we show that deletion of the CaM-binding site of Grb7, located in the proximal region of its pleckstrin homology (PH) domain, impairs cell migration, cell attachment to the extracellular matrix, and the reorganization of the actin cytoskeleton occurring during this process. Moreover, we show that the cell-permeable CaM antagonists *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and *N*-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide (W-13) both retard the migration of cells expressing wild type Grb7, but not the migration of cells expressing the mutant protein lacking the CaM-binding site (Grb7Δ), underscoring the proactive role of CaM binding to Grb7 during this process.

1. Introduction

Grb7 is a mammalian adaptor protein that plays important roles in mediating the transmission of signals from tyrosine kinase receptors and cytoplasmic tyrosine kinases by coupling protein complexes. Grb7, Grb10 and Grb14 form a protein family [1–6] that is phylogenetically related to the *Caenorhabditis elegans* Mig10 protein, which is involved in the regulation of embryonic neural cell migration [7,8]. These proteins share significant sequence homology and a well conserved modular structure divided in several domains. Human Grb7 is a 532 amino acid protein harboring an amino-terminal proline-rich region; a central GM region (for Grb and Mig10) that includes three domains: RA, PH and BPS; and a C-terminal region formed by a SH2 domain [1–6].

Grb7 participates in integrin-mediated signaling pathways by interacting with FAK [9] and also binds to the EphB1 receptor [10], triggering in both cases cell migration. Upon FAK autophosphorylation at Tyr397, Grb7 interacts with the kinase through its SH2 domain resulting in its phosphorylation at Tyr188 and Tyr338 [11,12]. The interaction of the PH domain of Grb7 with phosphoinositides at the plasma membrane seems to be necessary for proper FAK-mediated Grb7 phosphorylation; however, the subsequent downstream signaling events are largely unknown [11].

Our laboratory described the presence of a CaM-BD in the proximal region of the PH domain of Grb7 [13], and demonstrated the relevance of this site in the translocation of Grb7 into the nucleus [14], and its role in tumor growth and tumor-associated angiogenesis *in vivo* [15]. We have shown that a Grb7 mutant lacking the CaM-BD (Grb7Δ) lost the ability to bind to membranes and different phosphoinositides, although partial binding to phosphatidyl-3-phosphate and phosphatidyl-3,5-bisphosphate remained [13]. In this report we show that deletion of the CaM-BD of Grb7 results in impaired cell migration, adhesion and reorganization of the actin cytoskeleton, suggesting that CaM is involved in these processes via a Grb7-mediated mechanism.

Abbreviations: AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; CaM, calmodulin; CaM-BD, calmodulin-binding domain; BPS, between PH and SH2; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; E-64, *N*-[*N*-(L-3-trans-carboxyirane-2-carbonyl)-L-leucyl]-agmatine; ECL, enhanced chemiluminescence; EDTA, ethylene-diamine-tetraacetic acid; EGTA, ethylene-glycol-tetraacetic acid; EphB1, ephrin type-B receptor 1; ERK1/2, extracellular regulated kinases 1 and 2; FAK, focal adhesion kinase; FBS, fetal bovine serum; Grb7/10/14, growth factor receptor bound proteins 7, 10 and 14; NP-40, nonylphenoxypolyethoxylethanol; PH, pleckstrin homology; PBS, phosphate buffer saline; PMSF, phenylmethylsulfonyl fluoride; PVDF, poly(vinylidenedifluoride); RA, Ras-associating; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; SH2, Src homology 2; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; W-13, *N*-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide.

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2. Materials and methods

2.1. Reagents

Anti-Grb7 (N-20), anti-FAK and anti-phospho-FAK (Tyr397) rabbit polyclonal antibodies, and anti-phospho-ERK1/2 (Thr202/Tyr204) mouse monoclonal antibody were from Santa Cruz Biotechnology. Anti-ERK1/2 and anti-GAPDH rabbit monoclonal antibodies were from Cell Signaling Technology. Anti-rabbit IgG (goat) polyclonal antibody coupled to horseradish peroxidase was from Zymed Laboratories or Invitrogen. The anti-mouse Fc specific IgG polyclonal (goat) antibody coupled to horseradish peroxidase, EGTA and fibronectin (from bovine plasma) were from Sigma–Aldrich. PVDF membranes were from Pall Corporation, and DMEM, FBS, G418 (Geneticin), ProLong® Gold Antifade reagent, DAPI and Alexa Fluor® 488 phalloidin were from Invitrogen. The ECL kit was from GE Healthcare-Amersham, and the X-ray films from Konika Minolta. Glass-bottom culture dishes (35 mm diameter) coated with collagen or poly-D-lysine were from MatTek, the 6-wells Transwell® clusters equipped with 24 mm polycarbonate membranes (8 μm pore size) were from Corning Incorporated, andW-13 and W-7 were from Calbiochem.

2.2. Cell culture and transfection

Authenticated human embryonic kidney (HEK) 293 cells (ATCC® number CRL-1573™) were grown in DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 40 μg/ml gentamicin (plus 1 mg/ml G418 for stable transfectants) at 37 °C in an humidified air atmosphere containing 5% CO_2. The cells (≈80% confluent) were treated with 25 μM chloroquine and transfected with pcDNA3.1 (empty vector), pcDNA3/FLAG-Grb7, pcDNA3/FLAG-Grb7Δ, pEYFP-Grb7 or pEYFP-Grb7Δin a medium containing 120 mM $CaCl_2$, 25 mM Na_2HPO_4, 150 mM KCl, 250 mM NaCl, 50 mM glucose and 25 mM Hepes-NaOH (pH 7.0). Stable transfectants were selected with 1 mg/ml G418 for 15–20 days and isolated using cloning rings and trypsin/EDTA. Grb7 and Grb7Δ expression was tested by Western blot using the anti-Grb7 (N-20) antibody. Control experiments were performed with cells transfected with the empty vector or non-transfected cells.

2.3. Preparation of cell extracts

Cells were washed twice with cold PBS and lysed with a buffer containing 50 mM Tris–HCl (pH 8), 150 mM NaCl, 1% (w/v) deoxycholic acid, 1% (v/v) NP-40, phosphatase inhibitors (100 mM NaF and 1 mM Na_3VO_4) and protease inhibitors (5 μM pepstatin A, 10 μM leupeptin, 0.5 mM AEBSF, 0.4 μM aprotinin, 7.5 μM E-64, 25 μM bestatin, and freshly prepared 1 mM PMSF). Lysates were cleared by centrifugation at 15,000*g* for 30 min at 4 °C.

2.4. Electrophoresis and Western blot

Proteins (30–50 μg) were separated by SDS–PAGE using a 5–20% (w/v) linear gradient gel, electrotransferred to a PVDF membrane, fixed with 0.2% (v/v) glutaraldehyde for 10 min, and transiently stained with 0.1% (w/v) Fast Green FCF using standard procedures. The PVDF membranes were blocked with 5% (w/v) bovine serum albumin or 5% (w/v) fat-free powdered milk according to the instructions of the antibodies' manufacturers, and incubated overnight at 4 °C with the primary antibody (1/2000 dilution) and for 1 h at room temperature with the secondary antibody coupled to horseradish peroxidase (1/5000 dilution). The bands were visualized with the ECL reagents following instructions from the manufacturer.

2.5. Artificial wound assays

Artificial wounds were done scratching a monolayer of confluent cells with a pipette tip. The plates were washed twice with fresh medium to remove non-adherent cells before photographs were taken at different times using a Nikon Eclipse TS100 microscope at low magnification to follow the repopulation of the wounds. When required, 15 μM W-7 or 15 μM W-13 were added to the medium replacing it daily during the course of the experiment.

2.6. Cell motility assays

Cells expressing EYFP-Grb7 or EYFP-Grb7Δ were seeded 24 h post-transfection on 35 mm diameter glass-bottom plates coated with collagen or poly-D-lysine, and their migration was visualized with a Leica TCS SP5 confocal microscope using the 514 nm or 488 nm lasers and a 63× oil-immersion objective. Images were acquired every 10 min for 14 h to generate a time-lapse video using the Leica Microsystem computer software. The extent of migration of individual cells was measured frame-by-frame to determine the average migration rate. Alternatively, cell migration assays across a porous membrane were performed using the Transwell® system.

2.7. Cell detachment experiments

Cells were seeded 24 h post-transfection and the rate of detachment determined upon trypsin/EDTA treatment or calcium chelation with 1 mM EGTA counting the number of cells in aliquots of the medium at different times using a Neubauer chamber.

2.8. Cytoskeleton analysis

Serum-deprived cells were seeded on fibronectin-coated coverslips and fixed at the times indicated in the legend of the figure with 4% (w/v) paraformaldehyde. Cells were thereafter permeabilized with 0.1% (v/v) Triton X-100, blocked with 2% (w/v) bovine serum albumin overnight at 4 °C, and probed with Alexa Fluor® 488 phalloidin (1:40 dilution) for 1 h at room temperature to detect the actin cytoskeleton. The cells were also stained with DAPI to visualize the nuclei. Coverslips were placed faced-down on microscope slides using ProLong® Gold Antifade mounting solution. Images were acquired with a Leica TCS SP5 confocal microscope using the 488 nm and 358 nm lasers to visualize the actin cytoskeleton and the nuclei, respectively, using a 63× oil-immersion objective and focusing the planes at 1 μm intervals. The images were processed using the Leica Microsystems software.

2.9. Statistics

The two-way analysis of variance (ANOVA) and the Student's *t* tests were performed using the GraphPad Prism software program. Data were expressed as the mean ± SEM. Differences were considered significant at $p \leqslant 0.05$ as indicated in the legends of the figures.

3. Results

3.1. Grb7Δ expression impairs cell motility

As a first approach, we transiently transfected HEK293 cells with pcDNA3.1 (empty vector), pcDNA3/FLAG-Grb7 and pcDNA3/FLAG-Grb7Δ and performed artificial wound assays to follow the closing of the wounds along the time. Fig. 1A shows that