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The adaptors Grb10 and Grb14 are calmodulin-binding proteins

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We identified the Grb7 family members, Grb10 and Grb14, as Ca^{2+}-dependent CaM-binding proteins using Ca^{2+}-dependent CaM-affinity chromatography as we previously did with Grb7. The potential CaM-binding sites were identified and experimentally tested using fluorescent-labeled peptides corresponding to these sites. The apparent affinity constant of these peptides for CaM, and the minimum number of calcium ions bound to CaM that are required for effective binding to these peptides were also determined. We prepared deletion mutants of the three adaptor proteins lacking the identified sites and determined that they lost or strongly diminished their CaM-binding capacity following the sequence Grb7 > > Grb14 > Grb10. More than one CaM-binding site and/or accessory CaM-binding sites appear to exist in Grb10 and Grb14, as compared to a single one present in Grb7.

Keywords: adaptor proteins; calmodulin; calmodulin-binding sites; Grb10; Grb14; Grb7

The growth factor receptor bound proteins 7, 10 and 14 (Grb7, Grb10 and Grb14) form a family of mammalian adaptor proteins that play important roles mediating the transmission of signals from tyrosine kinase receptors and nonreceptor tyrosine kinases by coupling protein complexes to attain cellular responses [1–6]. These proteins are phylogenetically related to the *Caenorhabditis elegans* Mig10 protein, which is involved in the regulation of embryonic neural cell migration [7,8]. They all share significant sequence homology and a well-conserved modular structure divided into several domains: a proline-rich (PR) domain in the N-terminal, a central GM region (for Grb and Mig10) comprising a Ras-associating (RA) domain, a pleckstrin homology (PH) domain and a BPS domain (for between PH and SH2); and finally a Src homology 2 (SH2) domain located in the C-terminal [1–3,5].

Grb7 is implicated in cell migration processes by interacting with focal adhesion kinase by an integrin-mediated mechanism [9] and with the ephrin type-B receptor 1

Abbreviations
BPS, between PH and SH2; CaM, calmodulin; CaM-BD, CaM-binding domain; CaM-BP, CaM-binding protein; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; EGFR, epidermal growth factor receptor; EGTA, ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; Grb7/10/14, growth factor receptor bound proteins 7, 10, 14; IGF-1R, insulin-like growth factor 1 receptor; IgG, immunoglobulin G; ORF, open reading frame; PCR, polymerase chain reaction; PH, pleckstrin homology; PMSF, phenylmethylsulfonyl fluoride; PR, proline-rich; PVDF, polyvinylidene difluoride; RA, Ras-associating; SDS/PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; SH2, Src homology 2; TAMRA, tetramethylrhodamine; Tris, tris(hydroxymethyl)aminomethane.

[10]. In tumor cells, coexpression of Grb7 with the tyrosine kinase receptor ErbB2, and its natural occurring variant Grb7V lacking the SH2 domain, contribute to tumor cells invasiveness and metastasis formation [11–13]. In addition, Grb7 also plays important roles in cell proliferation [14,15], cell adhesion [16] and tumor-associated angiogenesis [15,17].

Several isoforms of the human Grb10 generated by alternative splicing have been described, including hGrb10α, hGrb10β and hGrb10γ [2], the hGrb10γ isoform being the longest [18,19]. The hGrb10α isoform is shorter and lacks a 46 amino acids long section corresponding to the PH domain [19,20]. The hGrb10β isoform differs from the hGrb10α isoform in the 5′-UTR and in the 5′ coding region, and although has an intact PH domain as the hGrb10γ isoform, it has a shorter N-terminal region [21]. These isoforms differ in their expression pattern, which seems to be tissue-specific [22,23]. Although Grb10 has been found to interact with different tyrosine-kinase receptors including the epidermal growth factor receptor (EGFR) [1], EphB1/ELK [24], platelet-derived growth factor receptor [21] and vascular endothelial growth factor receptor [25], and nonreceptor tyrosine kinases including Tec [26] and Bcr-Abl [27], its most important described role is to bind with high affinity to the insulin receptor [19,28] and the insulin-like growth factor 1 receptor (IGF-1R) [20,29–33] causing the inhibition of these receptors upon ligand stimulation. Besides, Grb10 has been found to interact with the ubiquitin ligase Nedd4 [34] triggering the internalization and degradation of the IGF-1R [35].

Grb14 was first identified in human breast epithelial cells [36]. This adaptor protein was also found to inhibit the insulin receptor and IGF-1R after insulin stimulation mediating the coordinated downregulation of the receptors [37]. This interaction was mediated by the BPS domain of Grb14 inhibiting the catalytic activity of the receptor, process that was enhanced by the SH2 domain of the adaptor protein [38,39]. The *GRB14* gene is overexpressed in breast and prostate tumors [36,40], and several mutations occurring with high frequency in human colorectal cancers have been described [41].

Calmodulin (CaM) is a ubiquitous Ca^{2+}-receptor protein in all eukaryotic cells that transduces intracellular Ca^{2+} signals generated by a variety of biological effectors (e.g. growth factors, hormones, neurotransmitters) or physical stimulus (e.g. mechanical, light, temperature) regulating hundreds of enzymes and nonenzymatic proteins implicated in the control of multiple cellular functions [42–46]. In addition, Ca^{2+}-free CaM (apo-CaM) is also able to regulate a variety of target proteins [47]. We have previously demonstrated *in vitro* and in living cells Ca^{2+}-dependent binding of CaM to human Grb7 and identified its CaM-binding domain (CaM-BD) located at the proximal region of the PH domain comprising the sequence ^{243}RKLWKRFFCFLRRS^{256} [17]. The deletion of this site affects Grb7-mediated cellular functions including its nuclear translocation, cell attachment, cell migration, tumor growth and tumor-associated angiogenesis [15–17,48]. In this report, we demonstrate that Grb10 and Grb14 are also Ca^{2+}-dependent CaM-binding proteins and identify the location of their CaM-BDs using deletion mutants.

Materials and Methods

Reagents

Anti-FLAG®-M2 (clone M2, isotype IgG_1) mouse monoclonal antibody was purchased from Sigma-Aldrich Co (St. Louis, MO, USA). Anti-HA tag rabbit monoclonal antibody was from Upstate-Millipore (Billerica, MA, USA). Anti-Grb7 (N-20) rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-Grb10 mouse monoclonal and anti-Grb14 rabbit polyclonal antibodies were obtained from ABCAM (Cambridge, UK). Anti-rabbit immunoglobulin G (IgG) goat polyclonal antibody coupled to horseradish peroxidase was purchased from Zymed Laboratories Inc. (San Francisco, CA, USA), Invitrogen (Carlsbad, CA, USA) or GE Healthcare-Amersham (Little Chalfont, UK), and the anti-mouse Fc-specific IgG polyclonal (goat) secondary antibody coupled to horseradish peroxidase was obtained from Sigma-Aldrich Co. or GE Healthcare-Amersham. Polyvinylidene difluoride (PVDF) membranes were obtained from Pall Co. (New York, NY, USA), and Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin/EDTA solution and L-glutamine were purchased from Invitrogen. The enhanced chemiluminescence (ECL) kit and CaM-Sepharose 4B were obtained from GE Healthcare-Amersham, and the X-ray films were from Konika Minolta (Tokyo, Japan). Triton X-100, ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), trichloroacetic acid and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich Co. The protease inhibitors cocktail (set III) was from Merck-Calbiochem (Darmstadt, Germany), and Tween-20, acrylamide/bis-acrylamide solution and precision Plus Protein Standards were purchased from Bio-Rad (Hercules, CA, USA). The ExSite™ mutagenesis kit was obtained from Stratagene (San Diego, CA, USA), and the Genopure plasmid maxi kit was obtained from Roche Life Science (Basel, Switzerland). Lipofectamine® 2000 was purchased from Life Technologies (Carlsbad, CA, USA). The three synthetic peptides labeled with tetramethylrhodamine (TAMRA) corresponding

to the CaM-BD of hGrb7 (RKLWKRFFCFLRRS), hGrb10γ (KKSWKKLYVCLRRS) and hGrb14 (KKSWKKIYFFLRRS) were synthesized by Schafer-N (Copenhagen, Denmark). Other reagents used in this work were of analytical grade.

Vectors preparation

The pCR3/FLAG-Grb7 vector was kindly donated by S. Tanaka (Kyushu University, Japan), and its open reading frame (ORF) was amplified by polymerase chain reaction (PCR) as previously described [17] using the following oligonucleotides: forward primer 5′-GAT GAC GAT CAT ATG GAG CCG GAT CTG TCT CCA CCT CAT C-3′ containing a *Nde*I restriction site (underlined) and reverse primer 5′-GCC AGT CCA CGC TCG AGT CAG AGG GCC ACC CGC GTG CAG C-3′, containing a *Xho*I restriction site (underlined). The PCR product was subcloned into the bacterial expression vector pET-14b to obtain pET-Grb7. Additionally, the CaM-BD (residues 243–256) deletion mutant denoted Grb7Δ was generated by mutagenesis using the ExSite™ mutagenesis kit, the vectors pCR3/FLAG-Grb7 and pET-Grb7 as templates and the following oligonucleotides: forward 5′-TCC TGA ACC CCG CAG CTG CAG AAA GCC CTG-3′ and reverse 5′-GGC GTC TAT TAC TCC ACC AAG GGC ACC TCT AAG-3′. Alternatively, *Eco*RI and *Xho*I restriction enzymes were used to subclone FLAG-Grb7 and FLAG-Grb7Δ into pcDNA3.1, yielding the pcDNA3/FLAG-Grb7 and pcDNA3/FLAG-Grb7Δ vectors.

The pCDNA3/Grb10γ-HA vector was kindly provided by S. Gioretti-Peraldi (INSERM, Faculty of Medicine, Nice, France) and was used as a template to obtain pcDNA3/Grb10Δ-HA with the ExSite™ mutagenesis kit and the following oligonucleotides: forward 5′-GCA TGT GAA AGA GCT GGG-3′ and reverse 5′-CTT TGA AGT TCC CTT GGT G-3′. Three different deletion mutants (termed Grb10Δ1, Grb10Δ2 and Grb10Δ3) were prepared by removing the nucleotides corresponding to the residues ^{246}RKNYAKYEFFKN^{257}, ^{304}KKSWKKLYVCLRRS^{317} or ^{387}RTCWMTAFRLLK^{398}, respectively. In the case of Grb10Δ2, the sequence was chosen on the basis to its similarity to the CaM-binding domain of Grb7 (^{243}RKLWKRFFCFLRRS^{256}) [17], and in the case of Grb10Δ1 and Grb10Δ3 using a software for predicting CaM-BDs (freely available at http://calcium.uhnres.utoronto.ca). Each single mutant was constructed using two PCR reactions, and directional ligation (subcloning) of resulting amplicons to pcDNA3. PCR reactions were done using Vent (New England Biolabs, Ipswich, MA, USA) polymerase to generate high fidelity blunt-ended amplicons, and pcDNA3-Grb10γ-HA as a template, to allow subcloning in the same plasmid backbone. Primers for the first PCR were designed to generate a 5′-most gene fragment, beginning at the unique *Hin*dIII restriction site of pcDNA3 (forward primer: 5′-ATA CGA CTC ACT ATA GGG AGA C-3′) and ending just before the Grb10γ region to be deleted (reverse primer: 5′-GAA TAG AAA TTT ACTCTC ACT G-3′ for Grb10Δ1, and 5′-GGT TTG CTC GTC CTC TGC-3′ for Grb10Δ3). Primers for the second PCR were designed to generate a 3′-most gene fragment, beginning just after the Grb10γ region to be deleted (forward primer: 5′-CCC ATG AAT TTC TTC CCA GAA-3′ for Grb10Δ1, and 5′-TAT GGA ATG CTC CTT TAC CAG -3 for Grb10Δ3) and ending at the unique *Apa*I restriction site of pcDNA3 (reverse primer: 5′-TAG CAT TTA GGT GAC TCT ATA G-3′). Directional subcloning was achieved by mixing in the same ligation reaction (containing T4 ligase provided by New England Biolabs) three DNA substrates: pcDNA3 (*Hin*dII and *Apa*I restriction digested), 5′-most amplicon (*Hin*dII restriction digested), and 3′-most amplicon (*Apa*I restriction digested). Note that the 5′-most and 3′-most amplicons ligate to pcDNA3 through their different cohesive ends, and that the 5′-most and 3′-most amplicons ligated to each other through their blunt ends. The double mutants Grb10Δ1Δ2 and Grb10Δ2Δ3 were constructed following the same strategy but using a precedent deletion mutant backbone as a template for second rounds of PCR and ligation.

The pRc/CMV/Grb14-FLAG vector was kindly donated by R. J. Daly (Monash University, Vic., Australia) and the pRc/CMV/Grb14Δ-FLAG vector was prepared amplifying the ORF of Grb14 using pRc/CMV/Grb14-FLAG as a template and the forward primer 5′-ATG ACC ACT TCC CTG CAA GAT G-3′. The PCR product was cloned into pMOSBlue plasmid and used as a template for PCR-aided mutagenesis using the forward primer 5′-GGT TTA TAT TTT TCT ACT AAA GGA AC-3′ and the reverse primer 5′-TCC CTG TTC TTT CGC ATG TAA GA-3′. The PCR product was purified and religated in the pMOSBlue vector. Finally, the Grb14Δ insert was subcloned into the pRC/CMV/FLAG vector using the *NdeI* and *Eco*RI restriction enzymes. Vectors replication was performed in transformed *Escherichia coli* DH5α grown in Luria-Bertani broth with 0.1 mg·mL^{-1} ampicillin. The isolation of the vectors was performed following the low-copy number procedure of the Genopure plasmid maxi kit.

Cells culture

Authenticated human embryonic kidney (HEK) 293 cells (ATCC® number CRL-1573™) were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 40 μg·mL^{-1} gentamicin at 37 °C in a humidified atmosphere of air containing 5% CO_2.

Cells transfection

The HEK293 cells were transfected with the following plasmids: pcDNA3.1 (empty vector), pcDNA3/FLAG-Grb7,

pcDNA3/FLAG-Grb7Δ, pCDNA3/Grb10γ-HA, pCDNA3/Grb10γΔ1-HA, pCDNA3/Grb10γΔ2-HA, pCDNA3/Grb10γΔ3-HA, pCDNA3/Grb10γΔ1Δ2-HA, pCDNA3/Grb10γΔ2Δ3-HA, pRc/CMV/Grb14-FLAG and pRc/CMV/Grb14Δ-FLAG with the calcium phosphate method as described [17] or the Lipofectamine® 2000 method following the instructions of the manufacturer.

Electrophoresis and western blot

Proteins were electrophoretically separated in 5–20% (w/v) polyacrylamide gradient slab gels in the presence of 0.1% (w/v) SDS at pH 8.3 [49] during 16–17 h at 6 mA. Proteins were electrotransferred from the gels to PVDF membranes for 2 h at 400 mA in a buffer containing 48 mM Tris-base, 36.6 mM L-glycine, 0.04% (w/v) SDS and 20% (v/v) methanol (TGSM buffer). Proteins were fixed with 0.2% (v/v) glutaraldehyde in 10 mM Tris-HCl (pH 8) and 150 mM NaCl (TBS buffer) for 10 min; and transiently stained with a 0.1% (w/v) Fast Green FCF solution in 50% (v/v) methanol and 10% (v/v) acetic acid to verify the regularity of the transfer procedure. The membranes were blocked with 5% (w/v) BSA or 5% (w/v) fat-free powdered milk according to the instructions of the antibodies' manufacturers in TBS containing 0.1% (v/v) Tween-20 (TBS-T buffer) and incubated overnight at 4 °C using a 1/2000 dilution of the corresponding primary antibody. Membranes were incubated thereafter for 1 h at room temperature using a 1/5000 dilution of the appropriate secondary antibody coupled to horseradish peroxidase. The positive bands were developed using the ECL reagents following instructions from the manufacturer.

Calmodulin-affinity chromatography

Ca^{2+}-dependent CaM-affinity chromatography was performed as previously described [17]. Briefly, cells were washed twice with cold PBS (130 mM NaCl, 2.7 mM KCl, 11.5 mM sodium/potassium phosphate pH 7.4) and resuspended in a buffer containing 20 mM Hepes-NaOH (pH 7.5), 150 mM NaCl, and the protease inhibitor cocktail supplemented with freshly prepared 1 mM PMSF. The cells were sonicated on ice for 5 min with 15 s on/off pulses and centrifuged at 100 000 ***g*** for 30 min at 4 °C. An aliquot of the supernatant was kept and processed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS/PAGE) and western blot to have a positive control for input in the column. $CaCl_2$ was added to the rest of the supernatant at a final concentration of 100 μM and the sample was passed through a 1–2 mL bed volume CaM-Sepharose 4B column previously equilibrated with 25 mM Hepes-NaOH (pH 7.5), 135 mM NaCl, 100 μM $CaCl_2$, and the protease inhibitor cocktail supplemented with freshly prepared 1 mM PMSF (Ca^{2+}-buffer). After extensively washing with the same buffer, 0.5 mL fractions were collected before and after elution with a buffer containing 25 mM Hepes-NaOH (pH 7.5), 135 mM NaCl, 2 mM EGTA, and the protease inhibitor cocktail supplemented with freshly prepared 1 mM PMSF (EGTA-buffer). The proteins in the fractions were precipitated with 10% (w/v) TCA and processed by SDS/PAGE and western blot as previously described.

Calmodulin binding to peptides upon addition of different calcium concentrations

Peptides were tagged with the fluorescent dye TAMRA. The binding of the tagged peptides to CaM was followed by fluorescence anisotropy in a medium of known changing free Ca^{2+} concentrations. Exhaustive description of the methodology used was previously described [50].

Results

Our laboratory previously described the presence of a CaM-BD in hGrb7 comprising the sequence ^{243}RKLWKRFFCFLRRS^{256} located in the proximal region of the PH domain, and found that this adaptor protein was able to bind CaM in a Ca^{2+}-dependent manner both *in vitro* and in living cells. To study the functionality of the CaM-BD of Grb7, we prepared a deletion mutant named Grb7Δ lacking the CaM-BD, and diverse techniques were used to ascertain its inability to bind CaM [17].

Sequence analysis of Grb10 and Grb14 in several species showed that they have significant sequence homology and a well-conserved molecular structure with that of Grb7. When we compared the CaM-BD of hGrb7 with the sequences of hGrb10β/hGrb10γ and hGrb14, we observed significant homology with the segments ^{246}KKSWKKLYVCLRRS^{259} in hGrb10γ, ^{304}KKSWKKLYVCLRRS^{317} in hGrb10β and ^{248}KKSWKKIYFFLRRS^{261} in hGrb14 (Table 1).

To study whether Grb10 and Grb14 are also CaM-BPs as Grb7, we generated deletion mutants lacking the indicated segments, respectively, denoted by Grb7Δ, Grb10Δ and Grb14Δ. The expression of the wild-type proteins and their respective deletion mutants in transfected HEK293 cells show the expected increase in electrophoretic mobility of the mutants (Fig. 1A). The cytosolic fractions of HEK293 cells transiently transfected with pcDNA3/Grb10γ-HA or pcDNA3/FLAG-Grb14 were applied to a CaM-Sepharose 4B column equilibrated with a buffer containing Ca^{2+}. After extensive washing with the Ca^{2+} buffer, the bound proteins were eluted with an EGTA-containing buffer. We also processed an aliquot of the cytosolic fraction as a positive control. The different fractions were separated by SDS/PAGE, transferred to

Table 1. Sequence homology of the canonical CaM-BD of Grb7 family members.

Protein	CaM-BD residues	CaM-BD sequence	CaM-BD identity (%)	Whole sequence identity (%)	Reference
Grb7 (human)	243–256	RKLWKRFFCFLRRS	–	–	[58]
Grb7V (human)	243–256	·····-·····	100	97	[12]
Grb7 (rat)	246–259	·····-·····	100	92	[37]
Grb7 (mouse)	246–259	·····-·····	100	90	[59]
Grb10γ (human)	304–317	K·s··KlYvc····	58	55	[20]
Grb10β (human)	246–259	K·s··KlYvc····	58	55	[21]
Grb10α (human)	N/A	N/A	N/A	49	[60]
Grb10α (mouse)	332–345	··s··KlYvc····	60	56	[61]
Grb10δ (mouse)	307–320	··s··KlYvc····	60	49	[31]
Grb14 (human)	248–261	K·s··KiYf·····	67	52	[36]
Grb14 (rat)	246–259	K·s··KaYf·····	67	50	[37]
Grb14 (mouse)	246–259	K·s··KaYf·····	67	51	[62]
Mig10 (*C. elegans*)	358–371	··s··KhYfv··p·	53	60	[7]

Identical amino acids are indicated as dots, conserved substitutions as capital letters and nonconserved substitutions as lower case letters. CaM-BD, calmodulin-binding domain. The sequence identity of the CaM-BD and the whole protein are compared to human Grb7. The homologous CaM-BD sequence of the Mig10 protein from *C. elegans* is also included. The human Grb10α isoform lacks the region of the pleckstrin homology domain where the CaM-BD is located due to alternative splicing (N/A).

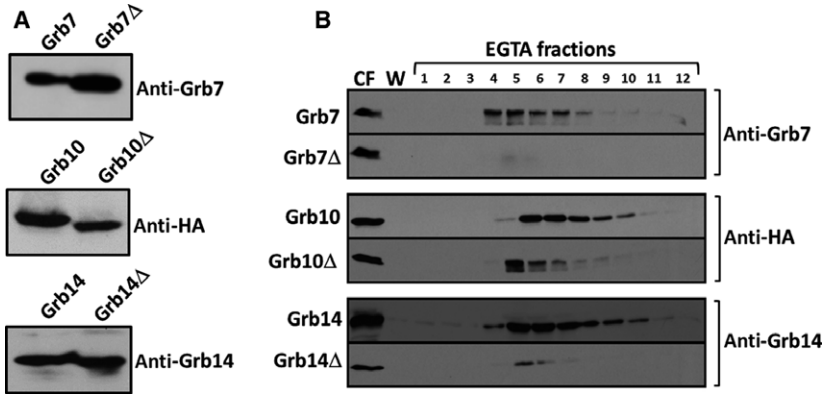

Fig. 1. Isolation of the three Grb7 family members by Ca^{2+}-dependent CaM-affinity chromatography and effect of deleting the proposed CaM-BDs. (A) FLAG-tagged Grb7, HA-tagged Grb10 and FLAG-tagged Grb14 were transfected in HEK293 cells and their expression patterns determined by western blot using anti-Grb7, anti-HA and anti-Grb14 antibodies to detect FLAG-tagged Grb7 and Grb7Δ, HA-tagged Grb10 and Grb10Δ, and FLAG-tagged Grb14 and Grb14Δ, respectively. (B) FLAG-tagged Grb7, FLAG-tagged Grb14, HA-tagged Grb10, and their corresponding CaM-BD deletion mutants were transfected in HEK293 cells and their cytosolic fractions were processed by Ca^{2+}-dependent CaM-affinity chromatography. After loading, the columns were extensively washed (W) with the Ca^{2+}-buffer to ascertain the absence of unattached proteins. The EGTA-eluted fractions were processed by SDS/PAGE and western blot using anti-Grb7, anti-HA and anti-Grb14 antibodies to detect FLAG-tagged Grb7 and Grb7Δ, HA-tagged Grb10 and Grb10Δ, and FLAG-tagged Grb14 and Grb14Δ, respectively. Samples of the cytosolic fractions (CF) were included to ascertain the positive detection of the proteins.

PVDF and probed with specific antibodies. Figure 1B shows that both wild-type Grb10 and Grb14 were detected in the EGTA-eluted fractions, as was the case for wild-type Grb7, suggesting Ca^{2+}-dependent bind to CaM. Once we identified the three Grb7 family members as CaM-BPs (Ref. [17] and Fig. 1B), and as the sequence homology of the putative CaM-BDs of the three proteins were notorious (Table 1), we tested whether the mutants lost their ability to bind CaM. We prepared cytosolic fractions of cells transiently transfected with pcDNA3/FLAG-Grb7Δ, pcDNA3/Grb10Δ-HA or pcDNA3/FLAG-Grb14Δ and subjected them to Ca^{2+}-dependent CaM-affinity chromatography as previously described. We determined the comparative behavior of the three deletion mutants in the column. Western blot analysis demonstrated that although Grb10Δ and Grb14Δ lost in part the ability to bind CaM when compared with their wild-type counterparts, they still had, particularly Grb10, significant CaM-binding capacity. In contrast, the deletion mutant Grb7Δ almost completely lost this ability (Fig. 1B).

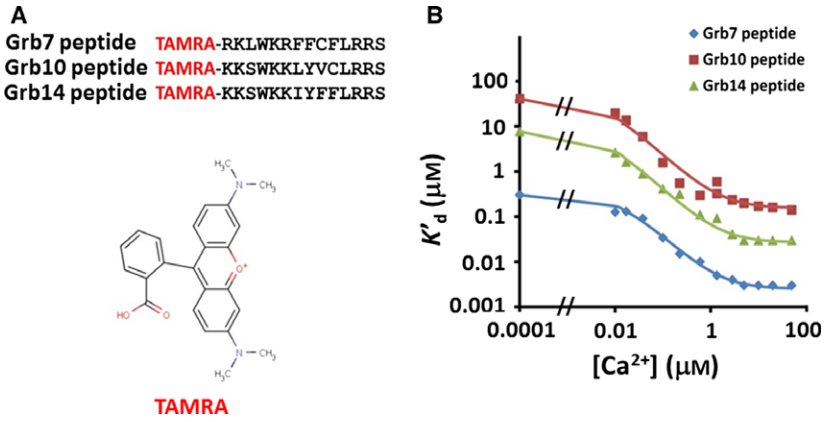

Fig. 2. Binding of CaM to TAMRA-labeled peptides corresponding to the CaM-BDs of Grb7, Grb10 and Grb14 at different Ca^{2+} concentrations. (A) Sequence of the TAMRA-labeled peptides corresponding to the CaM-BDs of Grb7, Grb10 and Grb14. The chemical structure of TAMRA is also shown. (B) The plot presents the apparent dissociation constant (K'_d) of the Grb7, Grb10 and Grb14 peptides bound to CaM at different concentrations of free Ca^{2+}.

To exclude that the adaptor proteins were piggy-back bound to other CaM-BPs present in the preparations, we decided to estimate the binding and affinity of peptides corresponding to the identified CaM-BDs of Grb7, Grb10 and Grb14 to CaM. We used a new technique based on fluorescence polarization to describe the binding affinity of CaM for its target, as well as the minimum number of Ca^{2+} bound to CaM to achieve efficient binding [50,51]. Peptides corresponding to the CaM-BD of Grb7, and the homologous sequences of Grb10 and Grb14 tagged with the fluorescent probe TAMRA (Fig. 2A) were assayed by fluorescent polarization of the CaM/peptide target interaction. We used a titration matrix in which both Ca^{2+} and CaM concentrations were changed to determine the minimum number of Ca^{2+} bound to CaM that was required for the interaction to occur with efficiency, as well as the apparent association and dissociation constants (K'_a and K'_d) [50,51]. Figure 2B shows the apparent dissociation constants (K'_d) of the fluorescent target peptides corresponding to the CaM-BD of Grb7, Grb10 and Grb14 when they bind to CaM as a function of the Ca^{2+} concentration. The peptide corresponding to the CaM-BD of Grb7 presented higher affinity for CaM (as given by a lower K'_d) than the Grb14 peptide, while the affinity for CaM of the peptide corresponding to the potential CaM-BD of Grb10 was even lower than the latter.

CaM is able to bind up to four Ca^{2+}, and the association constants of Ca^{2+} to the different binding sites were used to obtain the apparent association constant (K'_a) of the peptides when bound to CaM in the absence of Ca^{2+} and at different Ca^{2+} saturations, as described in Table 2 for apo-CaM (A), CaM-Ca^{2+} (B), CaM-2Ca^{2+} (C) CaM-3Ca^{2+} (D) and CaM-4Ca^{2+} (E). With this information, it was determined the minimum number of Ca^{2+} required to generate a conformational change in CaM to trigger effective binding to the peptides. In all cases, we found that this change occurred when a single Ca^{2+} was bound to CaM, but in the case of the Grb7 peptide the affinity further increased upon additional Ca^{2+} binding sites were saturated. Overall, these experiments confirmed that all the Grb7 family members are CaM-BPs and that the identified CaM-BD sequences are essential or contributed to Ca^{2+}-dependent CaM binding. However, their relative affinities for CaM were different, resulting in the following sequence: Grb7 > > Grb14 > Grb10.

Table 2. Association constants of CaM with different degrees of Ca^{2+} saturation for CaM-BD peptides of the three Grb7 family members.

CaM-BD peptide	A (μM^{-1})	B (μM^{-1})	C (μM^{-1})	D (μM^{-1})	E (μM^{-1})
Grb7	3.3	293.9	391.9	391.9	391.9
Grb10	0.02	6.4	6.4	6.4	6.4
Grb14	0.13	36.5	36.5	36.5	36.5

Association constants of CaM for peptides corresponding to the CaM-BDs of Grb7, Grb10 and Grb14 at different degrees of Ca^{2+} saturation. A, B, C, D and E are the association constant for apo-CaM, CaM-1Ca^{2+}, CaM-2Ca^{2+}, CaM-3Ca^{2+} and CaM-4Ca^{2+}, respectively.

Since Grb10Δ showed significant residual capacity to bind CaM (Fig. 1A), we searched for additional site(s) that could contribute to CaM binding. Although with low scores, we identified two additional sites with the sequences ^{246}RKNYAKYEFFKN^{257} and ^{387}RTCWMTAFRLLK^{398}. We prepared additional Grb10 deletion mutants lacking these new sites, denoted, respectively, Grb10Δ1 and Grb10Δ3; and assigning to the original mutant lacking the site ^{304}KKSWKKLYVCLRRS^{317} used in Fig. 1, that has higher homology to the CaM-BD of Grb7, the new name Grb10Δ2. In addition, we prepared the double deletion mutants Grb10Δ1Δ2 and Grb10Δ2Δ3.

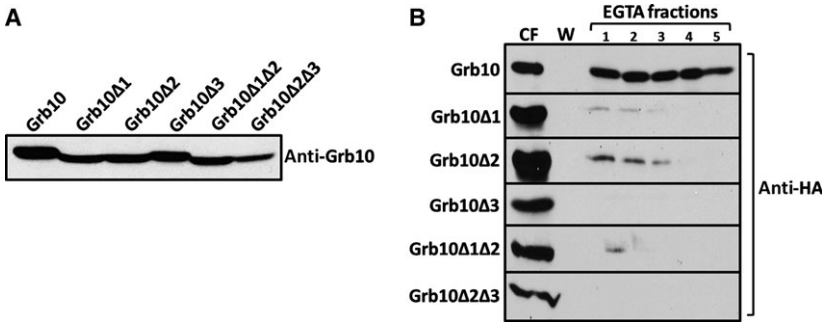

Fig. 3. Isolation of Grb10 by Ca^{2+}-dependent CaM-affinity chromatography and effect of deleting several sequence segments. (A) HA-tagged Grb10 and the deletion mutants Grb10Δ1, Grb10Δ2, Grb10Δ3, Grb10Δ1Δ2 and Grb10Δ2Δ3 were transfected in HEK293 cells and their expression patterns determined by western blot using an anti-Grb10 antibody. (B) HA-tagged Grb10, and the indicated deletion mutants were transfected in HEK293 cells and their cytosolic fractions were processed by Ca^{2+}-dependent CaM-affinity chromatography. After loading, the columns were extensively washed (W) with the Ca^{2+}-buffer to ascertain the absence of unattached proteins. The EGTA-eluted fractions were processed by SDS/PAGE and western blot using an anti-HA antibody. Samples of the cytosolic fractions (CF) were included to ascertain the positive detection of the proteins.

Figure 3A shows the expression pattern of this battery of Grb10 deletion mutants in transfected HEK293 cells, where a slight increase in electrophoretic mobility was apparent as expected, with the lowest mobility change corresponding to Grb10Δ3. We then determined the ability of all these mutants to interact with CaM using Ca^{2+}-dependent CaM-affinity chromatography. Figure 3B shows that Grb10Δ1, and particularly Grb10Δ3, strongly decreased or lost, respectively, the capacity to bind CaM, as compared to Grb10Δ2. Similar results were found using the double deletion mutants Grb10Δ1Δ2 and Grb10Δ2Δ3 as expected.

Discussion

The mammalian Grb7 protein family formed by Grb7, Grb10 and Grb14 shares a conserved structure with high sequence homology [1–6]. We have previously described human Grb7 and its variant Grb7V as CaM-BPs that bind CaM in a Ca^{2+}-dependent manner both *in vitro* and in living cells [17]. In this work, we aimed to test whether the other members of the Grb7 family, Grb10 and Grb14, were also able to interact with CaM. As shown in Table 1, sequence alignment showed that Grb10 and Grb14 might contain CaM-BDs homologues to the one in Grb7, comprising the sequences ^{246}KKSWKKLYVCLRRS^{259} in hGrb10β, ^{304}KKSWKKLYVCLRRS^{317} in hGrb10γ and ^{248}KKSWKKIYFFLRRS^{261} in hGrb14. We demonstrated using CaM-affinity chromatography that both hGrb10γ and hGrb14 were able to bind CaM in a Ca^{2+}-dependent manner. The fact that the Grb10 and Grb14 deletion mutants in these domains did not completely lose their ability to bind CaM suggested the possibility of alternative or additional CaM-BDs present in these proteins.

We have run *in silico* CaM-BD prediction and no other amphiphilic α-helix domain or any potential IQ motif were found in these proteins. In contrast to Grb7, where direct interaction with CaM was detected *in vitro* by CaM-affinity chromatography, CaM-overlay experiments and in living cells by FRET [17]; at first, we could not discard that Grb10 and/or Grb14 could be piggy-back bound to unidentified CaM-BPs during the chromatographic process. To determine whether the proposed sequences had the capacity to bind CaM, we determined the binding and affinity of fluorescent-tagged peptides corresponding to the CaM-BDs of hGrb7, hGrb10γ and hGrb14 to CaM in the presence of different concentrations of Ca^{2+}. Despite the high homology sequence shown between the three Grb7 protein family members, the affinity of these peptides for CaM was different, being the CaM-BD of Grb7 the one presenting the highest affinity while the one corresponding to the CaM-BD of Grb10 showed the lowest one, suggesting that structural differences might be found in these domains. As it has been previously described, several isoforms of hGrb10, generated by alternative splicing, have been identified, including hGrb10α, hGrb10β and hGrb10γ [2]. They basically differ in their PH domain, where the CaM-BD is located. The hGrb10β and hGrb10γ isoforms show high homology sequence between their putative CaM-BDs and the one of hGrb7 (Table 1), whereas, the hGrb10α isoform lacks the segment harboring the sequence corresponding to the CaM-BD present in the other isoforms.

Typical CaM-BDs form basic amphiphilic α-helixes with basic and hydrophobic residues exposed at

opposite sides of the helix [52]. Although in the predicted helical projections of the CaM-BDs of hGrb7, hGrb10β/hGrb10γ and hGrb14, there is a noticeable segregation of basic and hydrophobic amino acids at opposite sides of all the helixes, the helix corresponding to the CaM-BD of Grb7 is the one showing a more uniform distribution, followed by the one corresponding to the CaM-BD of Grb14 and in lesser extent the one corresponding to the CaM-BD of hGrb10β/hGrb10γ. This suggests that the CaM-BD of Grb7 would be the one presenting higher affinity for CaM, followed by the CaM-BDs of Grb14 and Grb10 as our results show. Crystallographic studies of the resolved PH domain of hGrb10β, where the CaM-BD is located, have shown that it is structured as a β-strand within a β-sheet [53] instead of an α-helix. This could be the reason why the affinity of the peptide corresponding to the CaM-BD of Grb10 shows the lowest affinity for CaM, as compare to the other Grb7 family members. The lack of crystallographic structures of the whole proteins, however, prevents us to establish whether or not the identified CaM-BDs form α-helixes or not in its native forms. Nevertheless, in the case of Grb10, our results show that two additional sites, upstream and downstream of the canonical CaM-BD with sequence similarity to the CaM-BD of Grb7, play an essential role in CaM binding, as its absence drastically impairs Ca^{2+}-dependent CaM binding. We could not identify in Grb14 additional sites with high score to be potential CaM-BD, although it cannot be discarded its existence due to the intrinsic uncertainty of the predicting software. Yet, we cannot exclude the existence in this protein of other accessory site(s) contributing to CaM binding. It is likely that conformational changes in the structure of these mutant proteins could contribute to the loss of their CaM-binding capacity.

As we have previously described, the minimum number of Ca^{2+} bound to CaM required to induce an effect depends on the target protein and might vary from protein to protein, offering extra regulatory mechanisms [43,47,51,54]. We found specific recognition of CaM for the CaM-BD peptides of Grb7, Grb10 and Grb14, when CaM had a single Ca^{2+} bound, suggesting that although the affinity for CaM is different, the recognition mechanism appears to be very similar. Nevertheless, in the case of Grb7, the affinity for CaM increased upon binding of the second Ca^{2+} (Table 2). In contrast, using an analogous approach, it was found that a peptide corresponding to the CaM-BD of the EGFR, also previously described by our laboratory as a CaM-BP [55–57], presented the highest affinity for the Ca^{2+}-CaM complex when 4 Ca^{2+} were bound to CaM [51].

Future work should be performed to determine the functional roles of the identified CaM-BDs of Grb10 and Grb14. As the different Grb10 isoforms differ in their expression pattern in a tissue-specific manner [23], it would be interesting to study whether there is connection between this differential expression pattern and the effect that CaM could play regulating the isoforms that contain a CaM-BD (hGrb10β, hGrb10γ) in contrast to the hGrb10α isoform lacking this domain.

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Author contributions

IG-P, NP-V, EV, and SG designed and performed the experiments; JH and AV designed and supervised the study; IG-P and AV wrote the paper. All authors discussed the results.

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