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Grb7 Protein RA Domain Oligomerization

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Abstract

The Grb7 protein is an adaptor protein that is often co-amplified with the ErbB2 receptor in 20–30% of breast cancer patients. Grb7 overexpression has been linked to increased cell migration and cancer metastasis. The RA and PH domain region of Grb7 has been reported to interact with various other downstream signaling proteins such as four and half LIM domains isoform 2 (FHL2) and filamin α. These interactions are believed to play a role in regulating Grb7 mediated cell migration function. The full-length Grb7 protein has been shown to dimerize, and the oligomeric state of the Grb7SH2 domain has been extensively studied; however, the oligomerization state of the RA and PH domains, and the importance of this oligomerization in Grb7 function is yet to be fully known. In this study, we characterize the oligomeric state of the Grb7RA domain using size exclusion chromatography, NMR nuclear relaxation studies, glutaraldehyde cross linking and dynamic light scattering. We report the Grb7RA domain can exist in transient multimeric forms, and, based upon modeling results, postulate the potential role of Grb7RA domain oligomerization in Grb7 function.

Keywords

cancer; cell migration; Grb7; oligomerization; RAPH domains

Introduction

The Grb7, Grb10 and Grb14 proteins compose the Grb7 family of adapter proteins. Each member shares similar topology consisting of an N-terminal pro-rich region followed by an RA (ras associating), PH (pleckstrin homology), BPS (between pleckstrin and src) region and a C-terminal SH2 (src homology 2) domain. In addition to the conserved domain topology, the three proteins also share considerable sequence homology and identity.

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Despite this topological similarity and homology, Grb7 protein family members are involved in distinct signaling pathways. Grb10 and Grb14 have both been found to function in insulin receptor signaling (for reviews see Cariou et al., 2004; Holt & Siddle, 2005). Grb10 has come under increased interest as researchers seek to understand the effects of Grb10 gene imprinting (Dent & Isles, 2014; Mroue, et al., 2015; and others).

Many studies have elaborated the link between Grb7 expression and the development of early metastases in cancers (for a review see Han et al., 2001). Besides its accepted roles as a cancer marker (Lacle et al., 2015; Saito et al., 2012) and potential therapeutic target (Pero et al., 2007; Pradip et al., 2013; Spuches et al., 2007; Tanaka et al., 2006; Villalobo et al, 2013) Grb7 has been shown to function in the formation of stress granules (Krisenko et al., 2015; Tsai et al., 2008) and in rheumatoid arthritis signaling (Kim et al., 2006; Xiao et al., 2016).

Members of the Grb7 protein family have been shown to exist in higher oligomeric forms. Grb10 can undergo tetramerization in mammalian cells (Dong et al., 1998). Stein et al. (2003) showed, using gel filtration and sedimentation equilibrium studies, that both the SH2 domain and the full length Grb10 protein exist in multimeric forms. The Grb7 full-length protein, and its SH2 domain alone, is also capable of forming a dimer (Ivancic et al., 2005; Porter et al., 2005). Previous work in our laboratory has additionally demonstrated the Grb7RAPH domains engage in an intramoleculer interaction with the C-terminal SH2 domain (Siamakpour-Reihani et al., 2011). Based upon this observation we hypothesized such an intramolecular association could constitute a form of Grb7 regulation.

The RAPH domain region is important in protein-protein interactions and can act as a discreet functional unit (Depetris et al., 2009; Paudyal et al., 2013; Qamra & Hubbard, 2013; Siamakpour-Reihani et al., 2009; Siamakpour-Reihani et al., 2011). There is evidence this region is also capable of oligomerization. Dong et al. (1998) provided a model for tetramerization of the Grb10 protein. According to that model, oligomerization occurs through the BPS, SH2 and PH domains. Also, the Grb10 and Grb14 RAPH domains may form integrated dimeric structural units of their own (Depetris et al., 2009). In the available structures (PDB #s 3HK0 and 4K81) the basis for dimerization appears to occur through a C-terminal helical extension of the PH domain (the Grb10RAPH structure, 3HK0), or through an N-terminally located helical region of the RA domain (the Grb14RAPH structure, 4K81). A structure of the Grb7RAPH domain is not available; therefore the potential role of Grb7RA domain oligomerization in Grb7 function remains undescribed.

A mechanism for Grb7 regulation has been suggested (Siamakpour-Reihani et al., 2009; Siamakpour-Reihani et al., 2011) whereby tyrosine phosphorylation of the RAPH/SH2 domains relieves the RAPH/SH2 intramolecular interaction. In the model, loss of interaction with the SH2 domain consequently increases access to the RA and PH domains for further downstream signaling. Although this mechanism elucidates a potential functional role for regulation of the Grb7RAPH region, the oligomerization state of these domains, and the importance of this oligomerization in their function, is yet to be understood. In this study, we characterize the physical state of the Grb7RA domain, and interpret these results with a view towards understanding the contribution of the RA domain to overall Grb7 oligomerization and function.

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Methods

Protein expression and purification

A full length Grb7/pCMV vector was used as a template to create Grb7RA domain (residues 102 through 187 in the intact Grb7 protein) bacterial expression constructs. The Grb7RA domain constructs amplified by PCR were ligated into pGEX-2T (for GST tagging) and pET 303 (for 6His tagging) vectors.

Plasmid constructs were transformed into BL21 Rosetta competent Escherichia coli (E. coli) cells (Millipore, Billerica, MA). The cells were grown in Luria-Bertani (LB) media containing 34 μg/ml Chloramphenicol and 50μg/ml Ampicillin at 37 °C with shaking until the OD_{600} reached approximately 0.5–0.7. For ^{15}N labeled protein expression, the cells were grown in 1X 3-(N-Morpholino)Propane Sulfonic acid (40 mM MOPS, 50 mM NaCl, pH 7) (Fisher Scientific) minimal media containing 1 g/L ^{15}N labeled $(NH_4)_2SO_4$ (Cambridge Isotope Laboratories, Andover, MA) and unlabeled glucose (Fisher Scientific). Protein expression was induced using 1 mM concentration IPTG (isopropyl-thiogalactoside) and the cells were allowed to grow at 17 °C with shaking for 16 hours. The cells were pelleted by centrifuging at 5,500 × g for 10 minutes at 4 °C and stored at −20 °C.

Purification of the 6His-Grb7RA domain was performed using Ni-NTA resin (Qiagen, Valencia, CA). The pellet from a 1 L bacterial cell culture was resuspended in 30 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol, pH 8.0 buffer containing 0.5 μg/ml pepstatin, 0.5 μg/ml leupeptin, and 50 μM phenylmethylsulfonyl fluoride). The cell suspension was sonicated on ice 5 times for 12 seconds each at 60% amplitude with a one-minute rest interval between each sonication. Triton X-100 (900 μl) was added to the solution and rocked for 30 minutes at 4 °C. The cell suspension was then centrifuged at 15,000 × g at 4 °C for 10 minutes to separate cell debris from the solution. Cleared lysate was then incubated with 3 ml of Ni-NTA resin pre-equilibrated with lysis buffer at 4 °C for 3 hours. The solution was poured into a gravity column and the post bead flow through collected. Next the beads were washed with 20 ml of wash buffer (50mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 10 mM β-mercaptoethanol, pH 8.0) and the protein was eluted with 12 ml of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 200 mM imidazole, 10 mM β-mercaptoethanol, pH 8.0) in four separate elutions. The eluted fractions were concentrated and further purified by size exclusion chromatography (SEC).

Purification of the GST cleaved Grb7RA domain was performed using Glutathione Sepharose 4B (GE Healthcare Life Sciences, Piscataway, NJ) beads. The lysis buffer for the Grb7RA domain purification was 1X PBS (phosphate buffered saline: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) pH 7.4 with 10 mM DTT (dithiothreitol) containing appropriate protease inhibitors as described above. The cleared lysate was prepared as described earlier and incubated with 2 ml of Glutathione Sepharose beads for 3 hours at 4 °C. The lysate was removed by centrifugation at 600 × g for 5 minutes at 4 °C and the beads were washed 4 times with 50 mM Tris, 100 mM NaCl pH 8.0 buffer. Twenty-five units of bovine thrombin were added to the solution containing beads and stirred overnight at room temperature. The supernatant was collected and thrombin was removed by rocking

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with 300 µl of p-aminobenzamidine agarose (Sigma Aldrich) for 30 min at 4 °C. Eluted proteins were further purified by size exclusion chromatography.

Size exclusion chromatography

A sephacryl S-200 HR (Amersham Biosciences, Pittsburgh, PA, USA) column was used for size-exclusion chromatography. The column was pre-equilibrated with 20 mM Tris, 100 mM NaCl, 1 mM DTT, pH 8.0 buffer at 4 °C. The protein sample (0.5 ml, 0.3–0.4 mM) was injected onto the column and eluted with the same buffer at 0.5 ml/min flow rate. The absorbance of the eluent was monitored at 280 nm. The molecular weight of the respective Grb7RA domain was estimated by comparing the elution volumes with those of gel filtration standards (Bio-Rad, CA, USA) (Figures 2 and 3, top panel).

Backbone nuclear relaxation study of the Grb7RA domain

The Grb7RA sample conditions for NMR (nuclear magnetic resonance) spectroscopy were 0.41–0.45 mM protein, 20 mM Tris, 100 mM NaCl, 1 mM DTT and 0.9 mM NaN_3 at pH 7.0. All NMR spectra were obtained at 298 K on a Varian INOVA 500 MHz spectrometer. ^{15}N T_1 values were measured from spectra recorded with eight different durations of the T_1 delay: 10, 70, 110, 280, 440, 610, 880, 1330 msec. ^{15}N T_2 values were measured from spectra recorded with seven different durations of the T_2 delay: 10, 30, 50, 70, 90 110 and 130 msec. All recycle delays were set to 1 sec for the T_1 and T_2 measurements.

Data were processed using Sparky 3.114 NMR Assignment and Integration Software (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco). All spectra were processed identically and the T_1 and T_2 values were determined by fitting measured peak heights to a decaying exponential, h = A*exp(-R*t) where h is height, R is the rate constant (R=1/T) and t is the spectrum time parameter (Figure 4).

Glutaraldehyde cross-linking experiments

GST cleaved Grb7RA domain and 6His tagged Grb7RA domain were cross-linked using glutaraldehyde as described previously (Fadouloglou et. al, 2008). Briefly, 40 µl of 25% (v/v) glutaraldehyde solution acidified with 0.8 µl of 6 M HCl was added into a micro well of a 24 well plate. Fifteen microliters of 1 mg/ml protein solution was placed on a cover slip, which was used to seal the well. The micro well plate was incubated at 30 °C for 5 minutes. Samples were collected and the reaction was quenched by adding 1 µl of 1 M Tris-HCl buffer at pH 8.0. The samples were then analyzed by SDS-PAGE. Horse heart myoglobin (Sigma), a known monomeric protein, was used as the negative control.

Dynamic Light Scattering

Protein molecular hydrodynamic diameters were measured by dynamic light scattering using a Zetasizer Nano-S (Malvern Instruments, Worcestershire, UK) instrument. Fifteen measurements were taken for each Grb7RA domain construct and/or condition and the average hydrodynamic diameter was calculated. All measurements were taken on protein samples at a concentration of 1 mg/mL and at 20 °C.

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Homology Modeling and Protein Docking

The Protein Data Bank (PDB, http://www.rcsb.org/pdb/home/home.do) provided the molecular structures used in this study. The structure used as the source structure for homology modeling was the Grb10RAPH domain (PDB# 3HK0). The structure used for protein-protein docking was the Grb7RA domain (PDB# 1WGR). Swiss-pdb viewer (www.genebee.msu.su/spdbv/) is software capable of basic model building, as well as molecular visualization. Swiss-pdb viewer was used to develop a homology model of the Grb7RAPH domain based upon the Grb10-RAPH domain structure (Figures 6 and 7). The Swiss-pdb viewer loop building, breaking and ligating protein backbone linkages, and molecular energy minimization tools were utilized in production of the Grb7RAPH model. The interactive protein docking and molecular program Hex (http://hex.loria.fr/) was used for calculating and displaying feasible docking modes for the Grb7RA domain (Figures 6 and 7).

Results

Size exclusion chromatography

Size exclusion chromatography was carried out using two different Grb7RA domain constructs. The first RA domain construct was expressed with a 6His tag at the C-terminus (Figure 1-C). The second construct was expressed as an N-terminal GST-fusion protein that, upon thrombin cleavage released the tagless Grb7RA domain (Figure 1-B), but with two additional non-native GS residues remaining at the N-terminus. The GST cleaved construct elutes at an approximate molecular weight of 20 kDa compared to the molecular weight standards (Figure 2). This suggests the GST cleaved construct may be a dimer, since the calculated molecular weight for the untagged Grb7RA domain is 9.4 kDa. The 6His tagged construct has two elutions; one peak corresponding to an approximate molecular weight of 15 kDa (second peak) and the other is at 30 kDa (first peak) (Figure 2). The calculated molecular weight for this construct is 10.2 kDa. Therefore, the second peak elutes at a size in between a monomer and a dimer. This lower molecular weight species, 15 kDa, could potentially be monomeric Grb7RA that exists in a more open, non-compact, globular form. The first eluted peak could be a trimeric form of the Grb7RA domain.

Figure 3 demonstrates the change in oligomerization of the 6His tagged construct with time. The results of this experiment imply the presence of the putative Grb7RA trimer decreases with time and is converted to the smaller species, as depicted by the increase in the “monomer” peak height.

Backbone Nuclear Relaxation study

A backbone nuclear relaxation study of the Grb7RA domain was carried out to further confirm the oligomeric state. ^{15}N longitudinal T_1 relaxation values were calculated from spectra recorded with eight different durations of the T_1 relaxation delay: 10, 70, 110, 280, 440, 610, 880, and 1330 msec. ^{15}N transverse T_2 relaxation values were calculated from spectra recorded with seven different durations of the T_2 relaxation delay: 10, 30, 50, 70, 90 110 and 130 msec. The longitudinal (T_1) and transverse (T_2) ^{15}N relaxation values were determined by fitting measured peak heights to the exponential function,

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$$h = A^* e^{(-Rt)}$$

Here h is height of the peak, R is the rate constant and t is the spectrum time parameter. The rotational correlation time, τ_c, was calculated using the following relationship (Kay et al., 1995).

$$\tau_c \approx \frac{\left(\sqrt{\frac{6T_1}{T_2} - 7}\right)}{4\pi\nu_N}$$

Here, ν_N represents the nuclear frequency (50,648,020 s^{-1} for Nitrogen). The T_1/T_2 ratios were calculated for each amino acid and the average value was used to estimate the molecular weight of the Grb7RA domain using the available standard curve at http://technology.sbkb.org/portal/page/199/. The average rotational correlation time, (τ_c) value for the 6His-Grb7 domain is 7.46 ns while it is 8.13 ns for the GST cleaved construct. Based upon these values, the estimated molecular weights are approximately 12 kDa and 13 kDa for the respective Grb7RA domain constructs (Figure 4, and supplemental data Figure S-1).

Cross-linking experiments

Cross-linking experiments were carried out in an attempt to stabilize and observe oligomeric forms of the Grb7 protein RA domain. GST cleaved Grb7RA domain and 6His tagged Grb7RA domain were cross-linked using glutaraldehyde as described previously (Fadouloglou et al., 2008). Both constructs form what appear to be a dimer and a trimer while myoglobin, which is a known monomeric protein, does not show any oligomerization (Figure 5) under the same conditions.

Dynamic light scattering

Dynamic light scattering experiments were performed using non-cross-linked and cross-linked Grb7RA domains, in an effort to preserve transient oligomeric Grb7RA domain species (Table 1, Table 2, and supplemental data Figures S-2 through S-5). The average diameter of the 6His-Grb7RA domain was determined to be 4.25 nm. When cross-linked, the average species diameter is 8.97 nm (Table 2 and supplemental data Figures S-2 and S-3). The GST cleaved Grb7RA domain has an average diameter of 4.18 nm and the same sample cross-linked has an average diameter of 7.94 nm (Table 2 and supplemental data Figures S-4 and S-5). All of these constructs show additional species of larger diameter, however, according to the size distribution by volume, their presence contributes little to the overall species (supplemental data Figures S-2 through S-5, panels “B”).

As shown in Table 2, based upon the assumption the protein is globular and approximately spherical in shape, the estimated molecular weight for the 6His-Grb7RA domain is 19.6 kDa, which is very close to the molecular weight for a dimer. When cross-linked, the estimated molecular weight becomes 112.8 kDa, implying the formation of a larger multimeric complex (possibly a multimer of 5–6 dimer units). Non-cross-linked, GST

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cleaved Grb7RA domain has an estimated molecular weight of 18.9 kDa. Again, this suggests the GST cleaved Grb7RA domain construct exists as a dimer. The cross-linked GST cleaved Grb7RA domain construct has an estimated molecular weight of 84.8 kDa, possibly suggesting a multimer of 4–5 dimer units. Table 1 shows the estimated values for the Perrin factor of each construct. According to these values, non-cross-linked Grb7RA domains are globular in shape while the cross-linked constructs are highly elongated.

Discussion

In this study we have shown the Grb7 protein RA domain can exist as a higher molecular weight oligomer. According to our size exclusion chromatography data, the GST cleaved Grb7 protein RA domain construct has an approximate molecular weight of 20 kDa. This observation suggests a dimer, considering the theoretical calculated molecular weight of the construct is 9.4 kDa. The 6His-Grb7 protein RA construct behaves in a different manner. It has two different elution peaks upon size exclusion chromatography. According to our analysis, one peak corresponds to an approximate molecular weight of 15 kDa and the other to approximately 30 kDa. The calculated theoretical molecular weight for this construct is 10.2 kDa. Therefore, the second peak elutes at an apparent size in-between a monomer and a dimer, and could represent a more conformationally open form of the monomeric Grb7RA domain. The first peak could possibly be a trimer oligomeric form, if one bases that assumption upon the theoretical molecular weight of 10.2 kDa.

A backbone nuclear relaxation study of the Grb7RA domain was carried out to further clarify the oligomeric state of this protein domain. From this study the correlation time (τ_c) for the 6His-Grb7RA domain was determined to be 7.46 ns, while the GST cleaved Grb7RA domain gave a τ_c value of 8.13 ns. The calculated molecular weights based upon these molecular correlation times are 12 kDa and 13 kDa for the 6His-Grb7RA and GST cleaved Grb7RA domains, respectively (for the reference curve, see supplemental data Figure S-1). These molecular weight values are 17% and 38% larger, respectively, than the calculated theoretical values of 10.2 kDa (6His-Grb7RA) and 9.4 kDa (GST cleaved Grb7RA) for the monomers. It is noted by definition, these Grb7RA domain samples are “old”. In other words, the NMR samples are at least several days old by the time the nuclear relaxation series of experiments are complete. Therefore, it may be difficult to observe transient oligomeric species observed in the size exclusion experiments such as seen in Figure 3.

Glutaraldehyde cross-linking is a simple method that can be used to create covalent bonds between protein molecules, when a binding interaction may be low affinity or transient in nature (Kluger & Alagic, 2004). Glutaraldehyde forms Schiff bases between its two carboxyl groups and any positively charged amino groups of a protein (Salem et al., 2010). Mixing of Glutaraldehyde directly into a protein solution can lead to non-specific cross-linking (Fadouloglou et al., 2008). Therefore, a hanging drop method was used to minimize these non-specific interactions (Figure 5). Both of the Grb7RA domain constructs (6His tagged and GST cleaved) form what appear to be a dimer and a trimer, based upon comparison to molecular weight standards. Myoglobin, a known monomeric protein, was used as a negative control (Figure 5). Under the same reaction conditions used for the Grb7RA domain constructs, myoglobin does not reveal any oligomerization products.

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Based upon the calculated molecular weights from our dynamic light scattering experiments, both of the Grb7 protein RA domain constructs are dimeric in nature, while cross-linked samples demonstrate much larger molecular weights. The cross-linked 6His RA domain construct has an estimated molecular weight of 112.8kDa. This could be a 6His RA pentamer (or hexamer) of dimers, considering the calculated molecular weight for a pentameric-dimer is 102 kDa. The apparent molecular weight of the cross-linked GST cleaved RA domain is 84.8 kDa. This potentially could be a GST cleaved RA domain tetramer of dimers, considering the calculated molecular weight for a tetrameric-dimer is 75.2 kDa.

Dynamic light scattering data allows the determination of the shape of a protein using the Perrin factor and axial ratios. The radius of a hypothetical sphere of homogeneous mass (R_{sph}) can be calculated using the following equation where M_W is the molecular weight, $\tilde{V}$ is the partial specific volume, and N_A is Avogadro's number (http://www.nanosafe.org/home/liblocal/docs/Nanosafe%202010/2010_oral%20presentations/O7-5_Malvern.pdf).

$$R_{sph} = \left(\frac{3M_W\bar{V}}{4\pi N_A}\right)^{1/3}$$

The frictional coefficient of the sphere (f_M) can then be calculated using the equation below, where η is the viscosity.

$$f_M = 6\pi\eta R_{sph}$$

The same equation can be modified for a non-spherical system by using the hydrodynamic radius (R_H) instead of the R_{sph}.

$$f_H = 6\pi\eta(R_H - \text{Solvent layer})$$

The ratio of the measured frictional coefficient to the frictional coefficient for a hypothetical sphere of the same radius is defined as the Perrin factor (F); $F = f_H/f_M$. According to the values we obtained for each construct, non-cross-linked Grb7RA domains are spherical while the cross-linked constructs appear highly elongated (Table 1). It has been suggested the Perrin factor may not be ideal for determining the shape of a protein (Erickson, 2009), however, it can be used to obtain relative general trends in protein shape.

Our size exclusion chromatography data suggests the GST cleaved Grb7RA domain exists as a dimer while the 6His-Grb7 domain may transiently form a trimer, that over time converts into a more conformationally open monomer. Molecular weights estimated from NMR relaxation data are larger than the molecular weights calculated for each monomer construct, again suggesting possible oligomerization may transiently occur. Glutaraldehyde cross-linking of the Grb7RA domains revealed the presence of a possible dimer and a trimer.

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This is also confirmed by dynamic light scattering where the calculated molecular weight for both RA domain constructs indicates dimers.

In total, these experiments hint that the Grb7RA domain has an ability to associate into at least dimers and trimers, and potentially even higher oligomeric forms. However, these multimeric Grb7RA forms appear to be highly transient, and only partially specific in their formation.

In terms of the functional significance of Grb7RA oligomerization, there are at least two (or more) possible mechanistic scenarios. It could be that RA oligomerization can contribute to the proposed dimerization of the Grb7 family RAPH domains (Depetris et al., 2009). A structure of the Grb7RAPH domain is not currently available, hence the model we have proposed here based upon the available Grb10RAPH structure (PDB# 3HK0). If the Grb7 family RAPH domains indeed dimerize, the Grb10RAPH domain structure suggests the dimerization interface is composed of C-terminal residues from the PH domain, while the Grb14RAPH domain structure (PDB# 4K81) suggests only an N-terminally located helical region of the RA domain may be involved. Regardless, the residues at the binding interface in the Grb7RA trimer model (Figures 6 and 7) are located distant from the RAPH dimer interface suggested by the available Grb10RAPH and Grb14RAPH domain structures. This observation sheds doubt upon the possibility that RA domain oligomerization can contribute to Grb7 dimerization overall.

A more likely explanation could be that Grb7RA oligomerization is simply a consequence of expressing the protein domain in isolation when it is normally part of a functional unit, the Grb7RAPH. Tandem RAPH domains are seen in several proteins and are believed to form integrated structural units (Chang et al. 2013; Depetris et al., 2009; Qamra et al., 2013; Zhang et al., 2014). It is especially noteworthy that the RA/PH domain interface in the Grb7RAPH model (Figures 6 and 7) involves two of the same residues that form the trimer interface in the Grb7RA docking model. Specifically, L54 (L62 in the trimer model) of the RA domain packs against Y184 of the PH domain (Figure 7, left panel). R56 (R64 in the trimer model) of the RA domain appears to be involved in both an amino-pi interaction with Y184 and an ionic interaction with N182 of the PH domain. Similar interactions are observed at the interface in the trimer model. L62 (L54 in the Grb7RAPH model) of one RA domain (blue) engages in hydrophobic packing with A20 of a second RA domain (yellow) (Figure 7, right panel). R64 (R56 in the Grb7RAPH model) of the same RA domain (blue) is involved in an amino-pi stabilization with Y15 of the second RA domain (yellow). An additional ionic interaction is observed between E63 of the first RA domain (blue) and R22 of the third RA domain (green).

The similar stabilizing interactions seen at the trimer interface suggest RA domain oligomerization may result nonspecifically as a consequence of protein energetics. By forming dimers and trimers the RA domain may achieve lower overall energy, where residues normally at the RA/PH interface gain stability by forming similar interactions with other RA domains in the absence of the PH domain.

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Conclusions

These data point out oligomerization of the Grb7RA domain is transient and somewhat non-specific in nature. To investigate whether Grb7RA domain oligomerization occurs *in vivo*, one could potentially envision a BRET-based (Bioluminescence Resonance Energy Transfer) study whereby compatible fluorescent tags are linked to the RA domain of two populations of Grb7 sequentially introduced into mammalian cells. Such an approach would be complicated by the fact that full-length Grb7 dimerizes through its SH2 domain, and possibly additionally through the adjacent RAPH domains. In this case BRET signals may erroneously give “false positives” for Grb7RA domain oligomerization, caused by full-length Grb7 dimerization bringing the RA domains into close proximity (but not reflecting true RA domain oligomerization).

Although the functional significance of this oligomerization is not clear, the results seem to reiterate the importance of treating the Grb7RAPH module as a functional unit, thus strengthening our understanding of oligomerization in the full-length protein. Our findings add support to the view that the ***intact*** RAPH domain region is important in regulating the structure and function of the Grb7 protein.

In addition, our studies provide a cautionary tale for the potentially deleterious effect of non-native residues resulting from common protein expression systems. It is often assumed that one to several additional non-native residues at the N or C terminus of a protein domain will not substantially alter the domain’s function or physical properties. The study herein shows even two extra residues (the N-terminal GS residues remaining on the Grb7RA domain after GST cleavage with thrombin) can affect the oligomerization state of the expressed protein domain. Protein oligomerization state is often linked with specific functionality, therefore affecting the oligomerization state of a protein may produce further unwanted functional consequences, or possibly lead investigators down erroneous mechanistic pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

6His	six histidine residue protein tag used for purification
BPS	between pleckstrin and src
ErbB2	erythroblastosis oncogene B 2
Grb7	growth factor receptor bound protein 7
GST	glutathione s-transferase protein tag used for purification

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PH	pleckstrin homology
RA	ras associating
SEC	size exclusion chromatography
SH2	src homology 2

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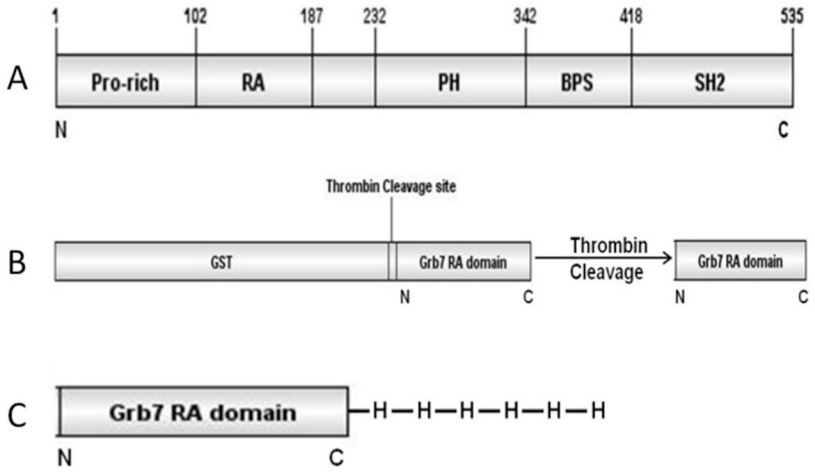

Figure 1.

A. Domain Topology of the Grb7 Protein. The Grb7 protein consists of an N terminal pro-rich region followed by the RA, PH domains, the BPS region, and a C-terminal SH2 domain. Approximate domain boundaries are indicated by residue numbers. **B. Topology of the GST cleaved Grb7RA domain.** The Grb7RA domain is expressed with a Glutathione S transferase (GST) protein tag at the N-terminus. The GST tag is cleaved by thrombin protease to obtain the GST cleaved Grb7RA domain. This process leaves two non-native amino acid residues remaining on the N-terminus: glycine and serine. **C. Topology of the 6His-Grb7RA domain.** The 6His-Grb7RA domain is expressed with 6 histidine residues remaining at the C terminus.

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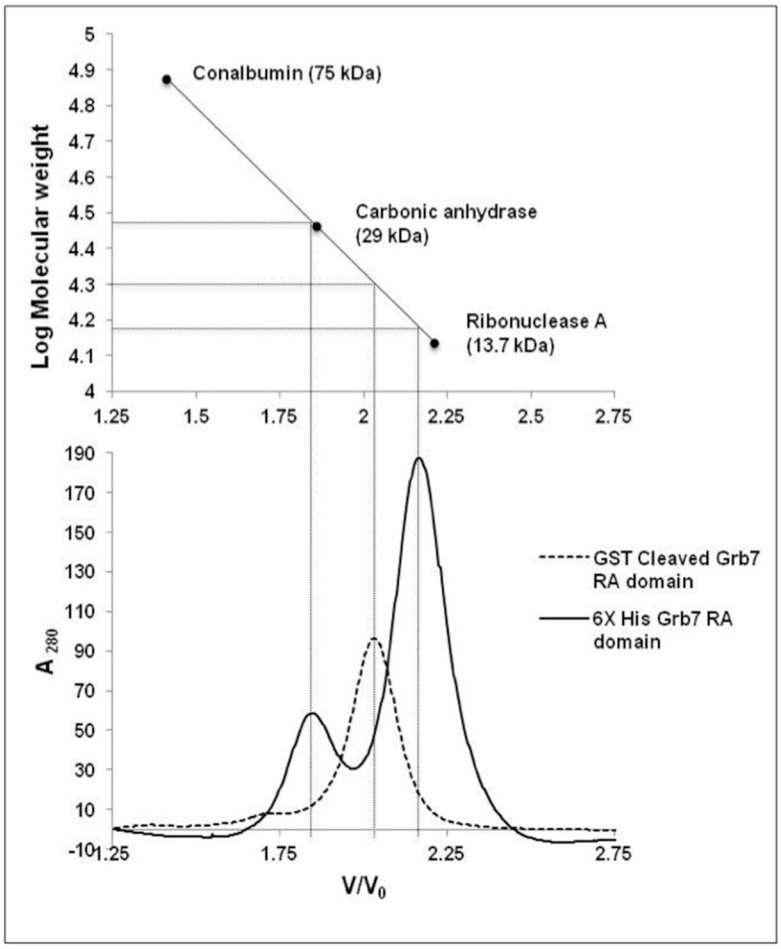

Figure 2. Size exclusion results for the GST cleaved and 6His-Grb7RA domains

The bottom panel represents the elution profiles of each Grb7RA domain. The x-axis is the ratio between the elution volume (V) and the void volume (V_0, 34 mL for the column used). The y-axis represents the absorbance at 280 nm. In the top panel is the calibration curve obtained with molecular weight standards as indicated, performed on the same size exclusion column. The y-axis represents the log of the molecular weight, and the x-axis represents the ratio of elution volume of each standard and the void volume.

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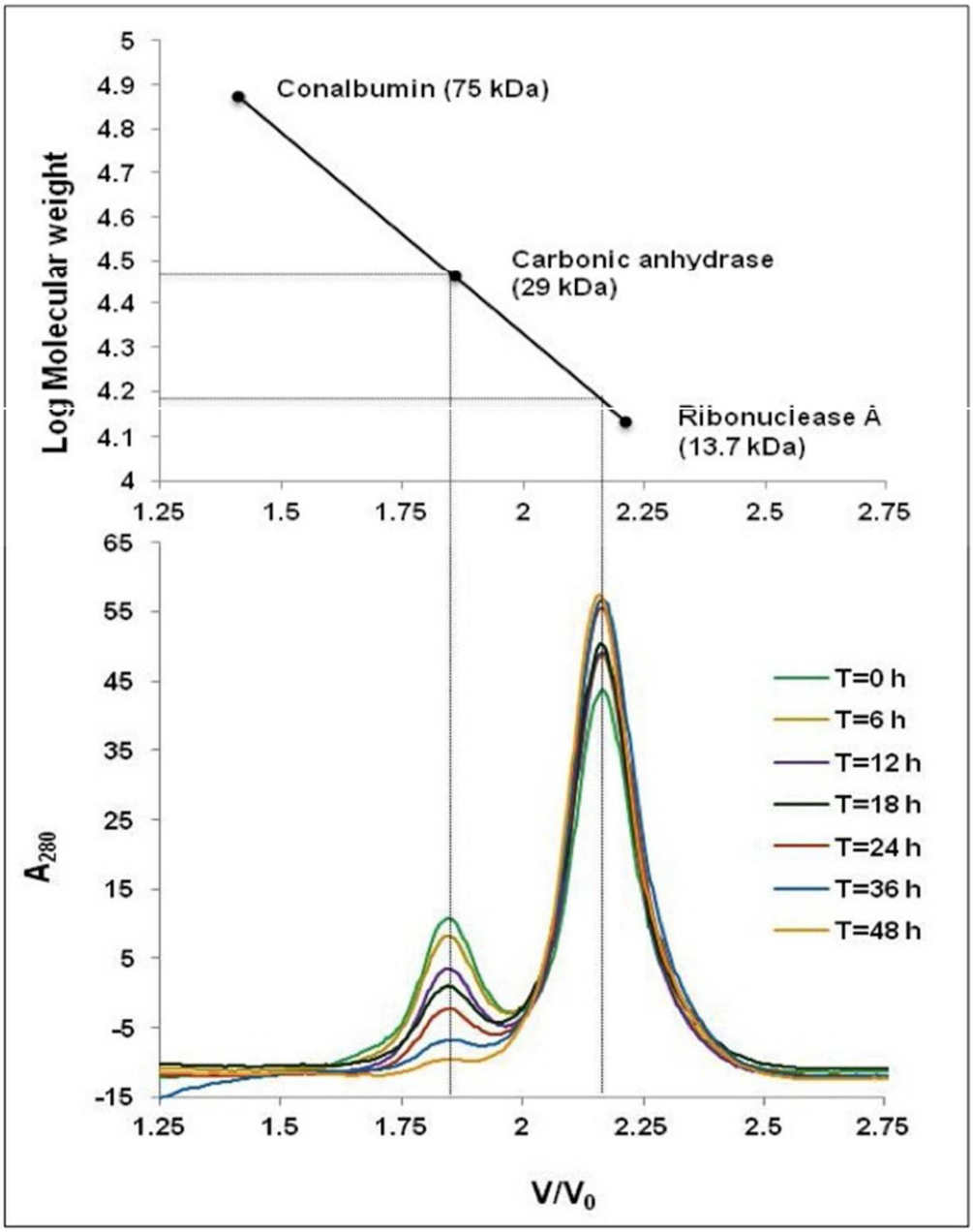

Figure 3. Size exclusion results for the 6His-Grb7RA domain at different time intervals

The bottom panel represents the elution profiles of the 6His-Grb7RA domain over time. Each coded line represents a different time point as indicated in the legend. The x-axis is the ratio between the elution volume (V) and the void volume (V_0, 34 mL for the column used). The y-axis represents the absorbance at 280 nm. In the top panel is the calibration curve obtained with molecular weight standards as indicated, performed on the same size exclusion column. The y-axis represents the log of the molecular weight, and the x-axis represents the ratio of elution volume of each standard and the void volume.

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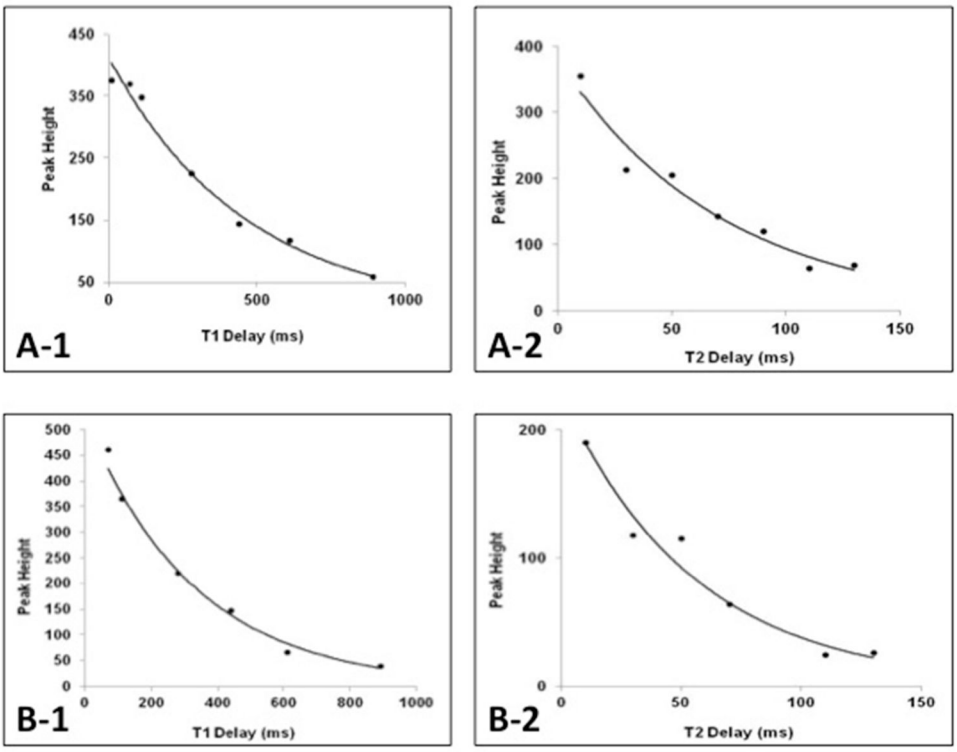

Figure 4.

6His-Grb7RA domain: A-1. T_1 decay curve for a representative Grb7RA domain residue (C36) of the 6His-Grb7RA domain. **A-2.** T_2 decay curve for a representative Grb7RA domain residue (C36) of the 6His-Grb7RA domain. **GST cleaved Grb7RA domain: B-1.** T_1 decay curve for the same representative Grb7RA domain residue (C36) of the GST cleaved Grb7RA domain. **B-2.** T_2 decay curve for the same representative Grb7RA domain residue (C36) of the GST cleaved Grb7RA domain.

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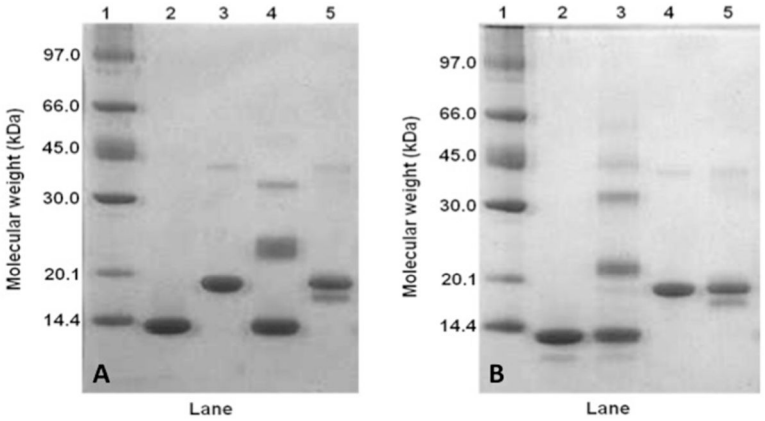

Figure 5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the cross-linked Grb7RA domains. Left: 6His-Grb7RA cross-linking results

Lane 1: Molecular weight marker. Lanes 2 and 3 represent 6His-Grb7RA and Horse heart Myoglobin (respectively) prior to cross-linking. Lane 4 represents 6His-Grb7RA treated with Glutaraldehyde, lane 5: represents horse heart myoglobin treated with Glutaraldehyde under the same conditions. **Right: GST cleaved Grb7RA cross-linking results**. Lane 1: Molecular weight marker. Lane 2 represents GST cleaved Grb7RA prior to cross-linking, lane 3 represents the GST cleaved Grb7RA domain after Glutaraldehyde treatment. Lanes 4 and 5 represent horse heart myoglobin before and after Glutaraldehyde treatment respectively. It is noted the higher molecular weight species present in the horse heart myoglobin samples is present in all instances, regardless of cross-linking status. The lower molecular weight species seen in the Glutaraldehyde treated myoglobin samples could represent a degradation product under the cross-linking conditions.

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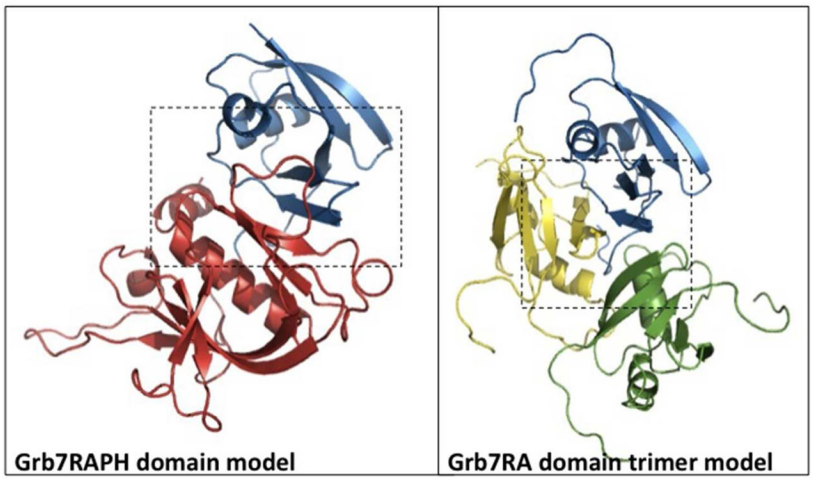

Figure 6.

Left Panel: Homology model of the Grb7RAPH domain. The Grb10RAPH domain structure (PDB# 3HK0) served as a template for homology modeling using the software Swiss-pdb viewer as described under the Methods section. The RA domain is represented in blue, while the PH domain is in red. The approximate region enlarged in Figure 7 (left panel) is indicated by a dashed rectangle. **Right Panel: Docking model of the Grb7RA domain trimer.** The software program Hex was utilized to generate potential dimer and trimer models of the Grb7RA domain as described in the Methods section. A representative trimer model is shown. Grb7RA domain “1” (as discussed in the text) is represented in blue, Grb7RA domain “2” is shown in yellow, and Grb7RA domain “3” is shown in green. The approximate region enlarged in Figure 7 (right panel) is indicated by a dashed rectangle.

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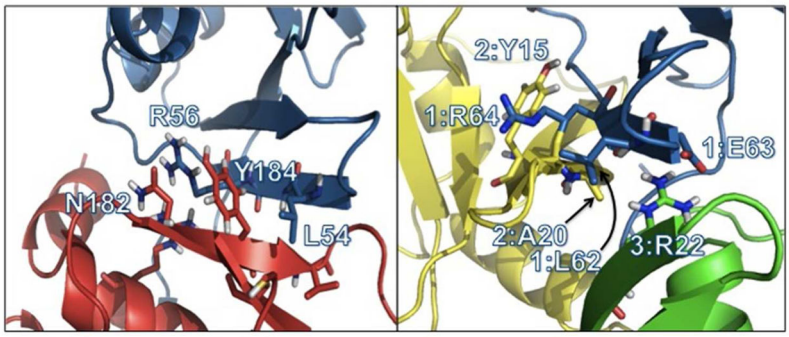

Figure 7.

Left Panel: Enlarged region of the Grb7RAPH domain homology model. Representative RA/PH domain interface contacts between residues are indicated in a stick format. Notable interactions include L54 (L62 in the trimer model) of the RA domain with Y184 of the PH domain, and R56 (R64 in the trimer model) of the RA domain with N182 of the PH domain.

Right Panel: Enlarged region of the Grb7RA domain trimer model. Representative Grb7RA domain trimer interface contacts between residues are indicated in a stick format. Notable interactions include L62 (L54 in the Grb7RAPH model) of Grb7RA domain “1” (blue) with A20 of Grb7RA domain “2” (yellow), R64 (R56 in the Grb7RAPH model) of Grb7RA domain “1” (blue) with Y15 of Grb7RA domain “2” (yellow), and E63 of Grb7RA domain “1” (blue) with R22 of Grb7RA domain “3” (green).

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Table 1

Perrin Factor, Prolate Axial Ratio and Oblate Axial Ratio for the Grb7RA domain expressed constructs.

Construct	Perrin Factor	Prolate Axial Ratio	Oblate Axial Ratio
1) 6× His Grb7RA	1.31	5.8	6.52
2) 6× His Grb7RA Cross-linked	2.34	29.9	47.98
3) GST cleaved Grb7RA	1.32	5.99	6.75
4) GST cleaved Grb7RA Cross-linked	2.12	23.44	34.82

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Table 2

Diameters and estimated molecular weights of the Grb7-RA constructs.

Construct	Diameter (nm)	Molecular Weight (kDa)
1) 6× His Grb7 RA	4.25	19.6
2) 6× His Grb7-RA Cross-linked	8.97	112.8
3) GST cleaved Grb7 RA	4.18	18.9
4) GST cleaved Grb7-RA Cross-linked	7.94	84.8