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ARTICLE

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Grb7-SH2 domain dimerisation is affected by a single point mutation

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Abstract Growth factor receptor bound protein 7 (Grb7) is an adaptor protein that is co-overexpressed and forms a tight complex with the ErbB2 receptor in a number of breast tumours and breast cancer cell lines. The interaction of Grb7 with the ErbB2 receptor is mediated via its Src homology 2 (SH2) domain. Whilst most SH2 domains exist as monomers, recently reported studies have suggested that the Grb7-SH2 domain exists as a homodimer. The self-association properties of the Grb7-SH2 domain were therefore studied using sedimentation equilibrium ultracentrifugation. Analysis of the data demonstrated that the Grb7-SH2 domain is dimeric with a dissociation constant of approximately 11 μM. We also demonstrate, using size-exclusion chromatography, that mutation of phenylalanine 511 to an arginine produces a monomeric form of the Grb7-SH2 domain. This mutation represents the first step in the engineering of a Grb7-SH2 domain with good solution properties for further biophysical and structural investigation.

Keywords Growth factor receptor bound protein 7 · Src homology 2 domain · Analytical ultracentrifugation · Size-exclusion chromatography · Protein engineering

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Abbreviations DTT: Dithiothreitol · EDTA: Ethylenediaminetetraacetic acid · EGF-R: Epidermal growth factor receptor · Grb: Growth factor bound protein · GST: Glutathione S-transferase · Hepes: *N*-(2-Hydroxyethyl)piperazine-*N*′-ethanesulfonic acid · IPTG: Isopropyl-*β*-D-thiogalactopyranoside · MES: 2-Morpholinoethanesulfonic acid · PBS: Phosphate-buffered saline · PH: Plekstrin homology · PMSF: Phenylmethylsulfonyl fluoride · SDS-PAGE: Sodium dodecyl sulfate–polyacryamide gel electrophoresis · SH2: Src homology 2

Introduction

Human growth factor receptor bound protein 7 (Grb7) is a 532 amino acid protein that was originally identified as a binding partner for activated epidermal growth factor receptor (EGF-R) using the cloning of receptor targets technique (Margolis et al. 1992). Grb7 is a member of a family of proteins that includes two other homologous proteins, Grb10 (Ooi et al. 1995) and Grb14 (Daly et al. 1996). These proteins share a conserved multidomain structure and contain several modular domains that mediate both protein–protein and protein–lipid interactions. These domains include an N-terminal proline-rich domain, a Ras-associating-like domain, a plekstrin homology (PH) domain, a C-terminal Src homology 2 (SH2) domain and the recently identified region between the PH and SH2 domains termed the BPS domain (Shen and Guan 2004; Han et al. 2001).

Grb7-family proteins function as adaptors, often coupling activated tyrosine kinase receptors to downstream signaling pathways. In addition to the EGF-R (also known as ErbB1), Grb7 binding partners include the ErbB2, ErbB3 and ErbB4 receptors (Fiddes et al.

1998; Stein et al. 1994), the platelet-derived growth factor receptor (Yokote et al. 1996) and focal adhesion kinase (Han and Guan 1999). While a large number of binding partners functioning upstream of Grb7 have been identified, little is known about the downstream events in these pathways. As a consequence, the precise biological function of Grb7 is poorly understood. Studies have shown, however, that Grb7 is involved in the regulation of cell migration (Shen et al. 2002; Shen and Guan 2001) and it has been implicated in tumour progression (Tanaka et al. 1997, 2000). The discovery that Grb7 is co-overexpressed and forms a tight complex with the ErbB2 receptor in breast cancer (Stein et al. 1994) has made the Grb7:ErbB2 complex an attractive target in the development of therapeutics to treat breast cancer.

The interaction between Grb7 and the activated ErbB2 receptor is mediated by the SH2 domain of Grb7 (Stein et al. 1994). Approximately 100 amino acids in length, SH2 domains are small, modular domains, which bind to phosphorylated tyrosine residues in a sequence-dependent manner (Bradshaw and Waksman 2002; Kuriyan and Cowburn 1997). Binding specificity arises from residues in both the SH2 domain and in the target protein, with the SH2 domain able to recognise the 3–6 residues immediately C-terminal to the phosphotyrosine (Songyang et al. 1993; Lee et al. 1994). These residues are recognised by amino acids located in the αB helix and both BG and EF loops of the SH2 domain (nomenclature by Eck et al. 1993; Waksman et al. 1993).

Whilst SH2 domains are typically monomeric, the recently determined crystal structure of the Grb10γ-SH2 domain at 1.65-Å resolution revealed that it formed a noncovalent dimer (Stein et al. 2003). Using both analytical ultracentrifugation and size-exclusion chromatography, Stein et al. confirmed that the both the SH2 domain and the full-length protein were multimeric in solution. It was proposed that dimerisation contributed to the binding specificity of this domain by stabilising the position of both the BG and EF loops. Given the similarity between the SH2 domains of Grb7 and Grb10 (approximately 67% amino acid identity) they hypothesised that the Grb7-SH2 domain also dimerises in solution. This is supported by the strict conservation within the Grb7 family of residues (with the exception of Phe-496) involved in the dimerisation interface. It has also been noted that the solution behaviour of the Grb7-SH2 domain is consistent with its existence as a dimer. NMR titration data have suggested a dimer-to-monomer transition upon peptide ligand binding (Brescia et al. 2002). Upon peptide binding, chemical shift perturbations were noted for residues at the putative dimer interface, as well as at the ligand binding site and the spectral properties improved (as would be expected for a lower molecular weight species), allowing the determination of the solution structure of the Grb7-SH2/peptide complex (Ivancic et al. 2003). The uncomplexed Grb7-SH2 has not been amenable to structure determination by NMR, and this may be due to it, in fact, existing as a dimer, limiting the spectral quality of multidimensional heteronuclear experiments (Barbara Lyons, personal communication).

In the current study, we used sedimentation equilibrium ultracentrifugation to demonstrate unambiguously that the Grb7-SH2 domain forms a dimer in solution. In addition, we have undertaken the first step in engineering a monomeric form of uncomplexed Grb7-SH2 suitable for structural studies using NMR. Based upon a mutation reported to convert a dimeric SH2 domain to its monomeric form (Stein et al. 2003), we created the mutant F511R. We report successful conversion to the monomer using size-exclusion chromatography as well as other insights that will assist the engineering of this system for future biophysical and structural studies.

Experimental

Preparation of Grb7-SH2

The pGex2T plasmid containing the human Grb7-SH2 insert (encoding residues 415–532) was obtained from Roger Daly (Janes et al. 1997). *E. coli* strain BL21(DE3).pLysS was used as the expression host. All cultures were grown in Luria–Bertani medium containing 100 μg ml^{-1} ampicillin and 25 μg ml^{-1} chloramphenicol at 37°C with shaking at 180 rpm. The glutathione S-transferase (GST) fusion protein was induced at an A_{600} of 0.8 by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a concentration of 0.3 mM. After 4 h, the cells were harvested by centrifugation. The cell pellet was resuspended in phosphate-buffered saline (PBS) at pH 7.4 containing 2 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT), 0.5% Triton-X 100 and 1 mM phenylmethylsulfonyl fluoride (PMSF) and lysed by three cycles of freeze–thaw and sonication. The lysate was centrifuged at 20,000*g* for 1 h and the supernatant incubated with glutathione beads (Amersham Biosciences, NJ, USA) overnight at 4°C. After washing with resuspension buffer lacking PMSF and EDTA, the fusion protein was eluted from the glutathione beads with PBS (pH 7.4) containing 5 mM DTT, 0.5% Triton-X and 25 mM glutathione. Grb7-SH2 was cleaved from GST by incubating the purified fusion protein with thrombin (5 U ml^{-1} final concentration) at 4°C for 24 h. The protein solution was dialysed against 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (Hepes) (pH 7.6), 20% glycerol and 1 mM DTT and applied to a Pharmacia MonoS 10/10 cation-exchange column equilibrated in the same buffer. Grb7-SH2 was eluted using a linear gradient of 0–0.3 M NaCl in 20 mM Hepes (pH 7.6), 20% glycerol and 1 mM DTT over a volume of 35 ml. The absorbance of the eluate was monitored at 280 nm. The purified SH2 domain was dialysed into 50 mM 2-morpholinoethanesulfonic acid (MES) (pH 6.6), 100 mM NaCl and 1 mM DTT and concentrated. The purity of the protein preparation was

confirmed by sodium dodecyl sulfate–polyacryamide gel electrophoresis (SDS-PAGE) analysis with the purified protein migrating as a single band at the expected molecular weight.

Preparation of Grb7-SH2.F511R

Site-directed mutagenesis of Phe511 to Arg was performed by polymerase chain reaction, using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, CA, USA). pGex2T-Grb7-SH2 was used as the template with the following primers 5′-GCA GCT CGT GGA GCG CCA CCA GCT GAA CCG-3′ and 5′-CGG TTC AGC TGG TGG CGC TCC ACG AGC TGC-3′ used for mutagenesis. The mutation was verified by DNA sequencing. Grb7-SH2.F511R was expressed and purified as described before for the Grb7-SH2 domain with the exception that all cultures were incubated at 30°C.

Protein concentration determination

Protein concentration was determined spectrophotometrically (Gill and von Hippel 1989). The molar extinction coefficient at 280 nm was 8,250 M^{-1} cm^{-1} for both Grb7-SH2 and Grb7-SH2.F511R. Protein concentrations were confirmed using SDS-PAGE analysis and bovine serum albumin as a standard.

Analytical ultracentrifugation

Sedimentation equilibrium experiments were performed using a Beckman Optima XL-A analytical ultracentrifuge. Cells fitted with conventional Yphantis 12-mm six-channel equilibrium centrepieces (Yphantis 1964) and quartz windows were used. Samples of the Grb7-SH2 domain were prepared by dialysis against 50 mM MES pH 6.6, 100 mM NaCl and 1 mM DTT. The dialysate was used to dilute the samples to final concentrations of 12 and 36 μM. The samples were centrifuged at 18,000, 22,000 and 31,000 rpm and at 4°C. Absorption profiles were recorded at 280 and 360 nm with automatic dialysate absorption compensation. The data were recorded in 0.001-cm steps and ten scans were averaged to produce each profile. The system was deemed to be in equilibrium when profiles recorded 4 h apart were identical.

The absorption profiles were trimmed to exclude any data measuring above 1 absorption unit or displaying any sign of light scattering due to a buildup of pelleted protein. The data from four absorption profiles (representing the two different dilutions of the protein solution run at 18,000 and 22,000 rpm) were then analysed by the calculation of the apparent weight-average molecular weight ($M_{w,app}$) (Teller 1973) using the OMMENU program suite (Ralston 1994) and by fitting models of association to the absorbance versus the radial position distribution using the program NONLIN (Yphantis 1964; Johnson et al. 1981).

Sedimentation equilibrium data were fitted by the NONLIN program to the following equation:

$$c_{\text{total}} = \delta c + \sum_{i=1}^{n} C_i(r) = \delta c + \sum_{i=1}^{n} K_{1,i} C_1(r)^{q(i)},$$

where δc is the concentration offset of the first data point, $q(i)$ the degree of association for the ith associated species, $C_i(r)$ the concentration of the ith species at radius r, $K_{1,i}$ the equilibrium constant for the association of monomer to the $q(i)$-mer, $C_1(r)$ the concentration of the monomer at radius r, and n is the total number of species present in the model used for fitting the data. Using values of $q(2) = 2$, 3, and 4, it is possible to fit the data to models of monomer–dimer, monomer–trimer, and monomer–tetramer, respectively.

A value of 0.729 ml g^{-1} was used for the partial specific volume of the Grb7-SH2 domain, calculated from its reported primary sequence (Margolis et al. 1992). An absorbance coefficient (280 nm) of 8,250 M^{-1} cm^{-1} and a molecular weight of 13,672 for Grb7-SH2 were used in all calculations. The density of the buffer solution was calculated to be 1.00557 g ml^{-1} using the program SEDNTERP v1.08 (Hayes et al. 2003).

Size-exclusion chromatography

Size-exclusion chromatography was performed on a Superdex 75 HR 10/300 GL (Amersham Biosciences) column pre-equilibrated with 50 mM MES pH 6.6, 100 mM NaCl and 1 mM DTT. An amount of 500 μl of protein sample was injected onto the column and eluted with the same buffer at 0.5 ml min^{-1}. The absorbance of the eluate was monitored at 280 nm. The molecular weight of the SH2 domains was estimated by comparison of the elution volumes with those of gel filtration standards (Bio-Rad, CA, USA).

Results

Grb7-SH2 is a dimer in solution

The ability of the purified Grb7-SH2 domain to self-associate in solution was monitored using equilibrium analytical ultracentrifugation. The sedimentation equilibrium profiles acquired were used to construct an $M_{w,app}$ versus concentration plot (Fig. 1). Extrapolation of $M_{w,app}$ to zero concentration gave a value of approximately 26 kDa, just below the theoretical mass of a Grb7-SH2 dimer (theoretical monomer molecular mass 13.7 kDa). The absorbance verses r data collected at loading concentrations of 36 and 12 μM and two different speeds were simultaneously analysed using the nonlinear regression program NONLIN (Johnson et al.

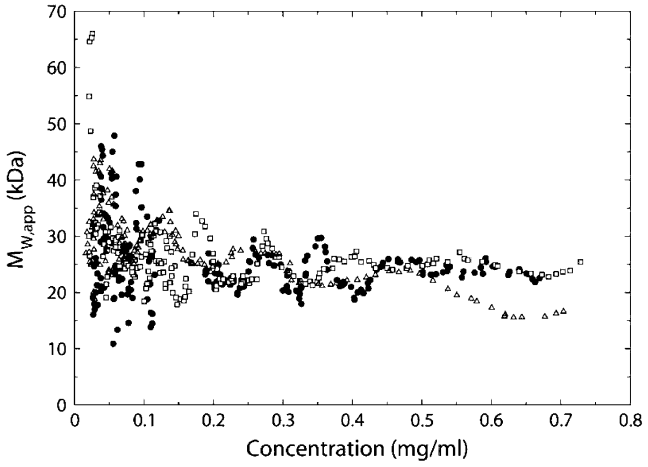

Fig. 1 Apparent weight-average molecular weight, $M_{w,app}$, verses protein concentration data obtained from sedimentation equilibrium experiments of the growth factor receptor bound protein 7 (*Grb7*) Src homology 2 (*SH2*) domain conducted at 4°C. Samples of Grb7-SH2 in 50 mM 2-morpholinoethanesulfonic acid (*MES*) pH 6.6, 100 mM NaCl and 1 mM dithiothreitol (*DTT*) were analysed by analytical ultracentrifugation at 18,000 rpm (*circles*), 22,000 rpm (*squares*) and 31,000 rpm (*triangles*). The initial loading concentrations were 12 and 36 μM

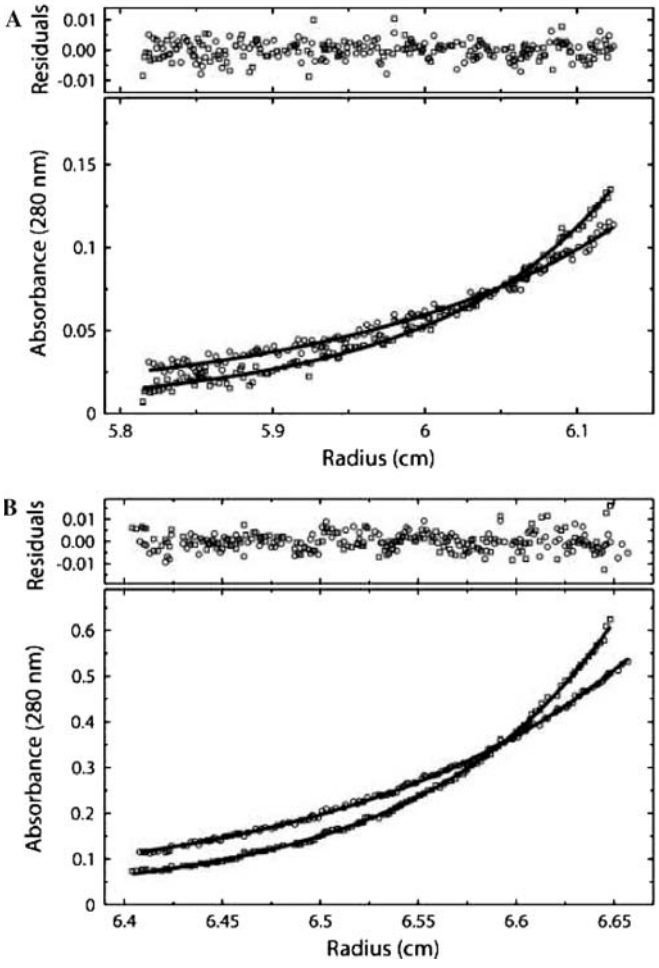

Fig. 2 Absorbance at 280 nm verses radius data at sedimentation equilibrium for Grb7-SH2 at initial loading concentrations of 12 μM (**a**) and 36 μM (**b**). Samples were in 50 mM MES pH 6.6, 100 mM NaCl and 1 mM DTT. They were centrifuged at 18,000 rpm (*circles*) and 22,000 rpm (*squares*) and 4°C. The four data sets were fitted simultaneously using the nonlinear regression program NONLIN (Johnson et al. 1981). The *solid line* represents the calculated fit to a monomer–dimer model. The residuals of the fit are shown in the *upper panels*

1981). The data were fitted to models of a 13.7 kDa species in equilibrium with multimeric species in order to derive the association constant. The best fit was obtained by employing a monomer–dimer self-association model. This yielded a dissociation constant of 10.7 μM with 95% confidence intervals of 5–16 μM (Fig. 2). This value is approximately fivefold weaker than the K_d obtained for dimerisation of the Grb10-SH2 domain calculated using the same technique (Stein et al. 2003)

Choice of mutation and protein production

With the aim of producing a monomeric form of Grb7-SH2 that is more amenable to NMR studies, mutation of a residue predicted to form part of the dimer interface was undertaken. The residue chosen was based on the Grb10-SH2 domain crystal structure (PDB ID: 1NRV; Stein et al. 2003). In the crystal structure of the Grb10-SH2 domain the dimer interface consists primarily of residues located in the αB helix of the domain (Fig. 3). The side chain of Phe-515 occupies a key position in this interface, projecting into its centre and packing against the side chains of Phe-515 and Thr-504 from the other protomer (Stein et al. 2003). Mutation of this residue to either an arginine or an alanine abrogated dimerisation of the domain (Stein et al. 2003). As the majority of residues implicated in the dimerisation of the Grb10-SH2 domain are conserved in Grb7 it was predicted that the equivalent mutations would have a similar effect on Grb7-SH2 dimerisation. The phosphopeptide binding face of the Grb7-SH2 domain is positioned away from the putative dimerisation interface, and would be predicted to be unaffected by this mutation. The F511R mutant was initially prepared in the same way as the wild-type Grb7-SH2 domain. It was notable that at 37°C similar levels of overexpression were achieved to that seen for Grb7-SH2. Grb7-SH2.F511R, however, displayed much lower solubility and/or folding efficiency as demonstrated by the high percentage present in the insoluble fraction of the cell lysate. The percentage of the mutant SH2 domain present in the soluble fraction was increased by growing the *E. coli* culture containing the pGex2T-Grb7-SH2.F511R plasmid at 30°C instead of 37°C. When compared with the wild-type Grb7-SH2 domain, approximately fivefold less Grb7-SH2.F511R was produced per litre of culture. Circular dichroism spectra collected between 200 and 300 nm for wild-type Grb7-SH2.F511R and Grb7-SH2 were essentially identical (data not shown), confirming the nondisruption of secondary structure due to mutation.

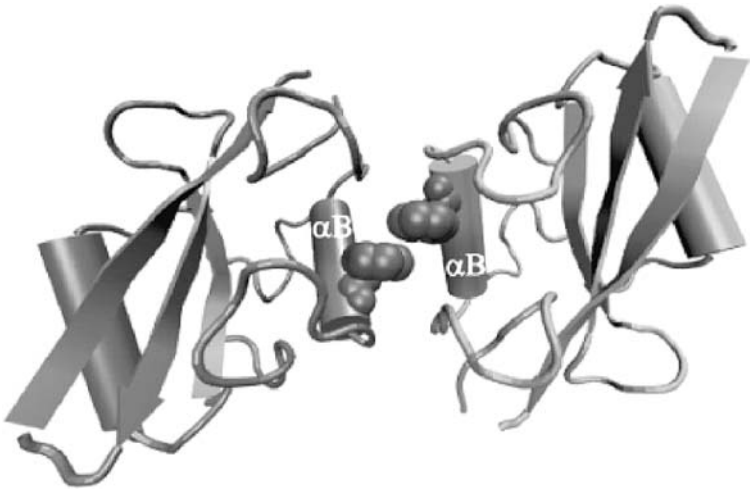

Fig. 3 Cartoon representation of the Grb10-SH2 domain dimer (PDB ID: 1NRV; Stein et al. 2003). The dimerisation interface is formed by residues located in the αB helix together with residues in the BG and EF loops. The side chain of Phe-515 (Phe-511 in Grb7) occupies a central position in this interface and is shown in a van der Waals representation in *dark grey*. The figure was generated using VMD (Humphrey et al. 1996)

Phenylalanine 511 required for dimer formation

Size-exclusion chromatography was used to assess the dimerisation state of Grb7-SH2.F511R. As a comparison, wild-type Grb7-SH2 was also subjected to size-exclusion chromatography under identical conditions. Both SH2 domains eluted from the column as a single peak although some ''tailing'' was observed at the end of the Grb7-SH2 peak, consistent with a fairly rapid monomer–dimer exchange. Notably, no tailing was observed for the mutant protein (Fig. 4). The two proteins eluted at different elution volumes, with the Grb7-SH2.F511R mutant eluting more slowly than the wild-type protein. Estimates of the apparent molecular weight for the two SH2 domains were obtained by comparison of the elution volumes of Grb7-SH2 and Grb7-SH2.F511R with the elution volumes of gel filtration standards (as indicated in Fig. 4 by the broken lines). Assuming that Grb7-SH2 domain is roughly globular, as shown by NMR structural information (Ivancic et al., 2003), this analysis yields apparent molecular mass estimates of 23 and14 kDa for the wild-type and mutant SH2 domains, respectively. As mentioned previously, the molecular mass of the Grb7-SH2 domain monomer is 13.7 kDa, suggesting that under the current chromatography conditions mutation of phenylalanine to an arginine abrogates dimerisation of the Grb7-SH2 domain.

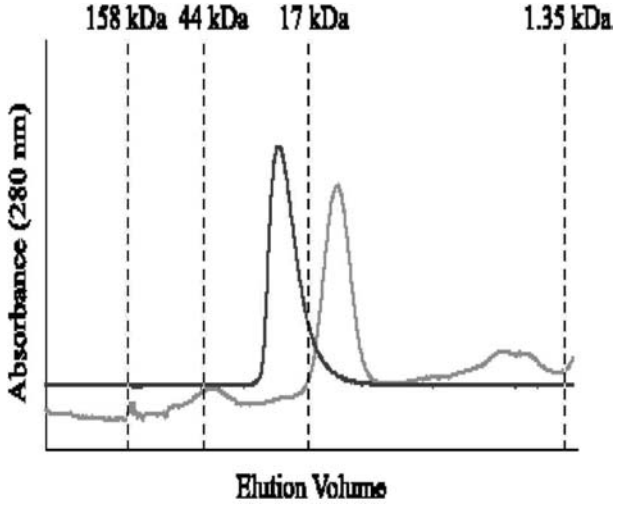

Fig. 4 Size-exclusion elution profiles of Grb7-SH2 (*black line*) and Grb7-SH2.F511R (*grey line*). The elution volume of the column calibration standards are indicated by the *dashed lines* with the molecular masses in kilodaltons indicated *above*. Samples were in 50 mM MES pH 6.6, 100 mM NaCl and 1 mM DTT. The data shown are representative of five independent experiments

Discussion

The results from the sedimentation equilibrium experiments indicate that the Grb7-SH2 domain dimerises in solution with a dissociation constant of approximately 11 μM. This is approximately fivefold weaker than that of the Grb10-SH2 domain (Stein et al. 2003). Interestingly, the key Grb10 dimerisation residues (Thr-504, Gln-511, Phe-515, Leu-518, Asn-519 and Asp-500), with the exception of Phe-496 (Tyr in Grb7), are strictly conserved between the Grb10 and Grb7. There are, however, several differences immediately adjacent to Phe-515 in Grb10 (Asp-514 to Glu and Tyr-516 to His). These differences may underlie the differences in the dissociation constants—but a more rigorous biophysical and structural examination would be needed to explore this.

The dimerisation of Grb7-SH2 domain represents a novel aspect of this signaling molecule and potentially confers an extra level of regulatory control to the Grb7 signaling pathways. While the dimerisation properties of full-length Grb7 have yet to be addressed, the calculated K_d for the SH2 domain suggests that significant dimerisation of the Grb7-SH2 domain would potentially occur when the local Grb7 concentration in the cell is in the micromolar range. While there are no data available on the levels of the Grb7 protein in cells, localisation of this protein in either the focal contacts (Han et al. 2000) or at

the membrane (Shen et al. 2002) may increase the concentration to the levels required for dimerisation.

The ability of the Grb7-SH2 domain to dimerise in solution is in contrast to the solution behaviour observed for the majority of SH2 domains (over 100 known SH2 domains) with only four reported cases of SH2 domain dimerisation in the literature (Stein et al. 2003; Hu et al. 2003; Réty et al. 1996; Schiering et al. 2000). As the predicted Grb7-SH2 dimer interface consists of secondary structure elements that also contribute to the binding specificity of SH2 domains, dimerisation may represent a novel mechanism used by members of the Grb7 family to modulate binding to the target protein.

This study has also demonstrated that mutation of phenylalanine 511 to an arginine prevents dimerisation of the Grb7-SH2 domain. This mutation was performed with the aim of producing monomeric Grb7-SH2 suitable for structural studies of the domain. The mutation of single residues to improve the solution properties of a molecule has been used successfully in a number of studies (Varley et al. 1997; Vivian et al. 2003; Saïda et al. 2004). In this case this mutation represents the first step in production of a Grb7-SH2 domain with improved solution properties. Additional mutations are likely to be necessary since the lower solubility of Grb7-SH2.F511R suggests that the exposure of the dimer interface is unfavourable. This is unsurprising, as several hydrophobic residues are predicted to exist at this interface. These are the clear targets for further mutation designed to create a monomeric Grb7-SH2 domain with excellent properties for structural studies.

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