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Dimerization in the Grb7 Protein

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Abstract

In previous studies, we showed that the tyrosine phosphorylation state of growth factor receptor-bound protein 7 (Grb7) affects its ability to bind to the transcription regulator FHL2 and the cortactin-interacting protein, human HS-1-associated protein-1. Here, we present results describing the importance of dimerization in the Grb7–Src homology 2 (SH2) domain in terms of its structural integrity and the ability to bind phosphorylated tyrosine peptide ligands. A tyrosine phosphorylation-mimic mutant (Y80E–Grb7–SH2) is largely dimerization deficient and binds a tyrosine-phosphorylated peptide representative of the receptor tyrosine kinase (RTK) erbB2 with differing thermodynamic characteristics than the wild-type SH2 domain. Another dimerization-deficient mutant (F99R–Grb7–SH2) binds the phosphorylated erbB2 peptide with similarly changed thermodynamic characteristics. Both Y80E–Grb7–SH2 and F99R–Grb7–SH2 are structured by circular dichroism measurements but show reduced thermal stability relative to the wild type–Grb7–SH2 domain as measured by circular dichroism and nuclear magnetic resonance. It is well known that the dimerization state of RTKs (as binding partners to adaptor proteins such as Grb7) plays an important role in their regulation. Here, we propose the phosphorylation state of Grb7–SH2 domain tyrosine residues could control Grb7 dimerization, and dimerization may be an important regulatory step in Grb7 binding to RTKs such as erbB2. In this manner, additional dimerization-dependent regulation could occur downstream of the membrane-bound kinase in RTK-mediated signaling pathways.

Keywords

adaptor proteins; Grb7; tyrosine kinase; erbB2; HER2; neu; cell migration; oligomerization; calorimetry

INTRODUCTION

Growth factor receptor-bound protein 7, 10 and 14 (Grb7, Grb10 and Grb14, respectively) comprise a family of Src homology 2 (SH2) domain-containing Grb proteins discovered through CORT screening (Ooi *et al.*, 1995; Daly *et al.*, 1996). A homologous *Caenorhabditis elegans* protein, Mig-10, is involved in neuronal migration during development (Manser *et al.*, 1997). The members of this family all contain an N-terminal Pro-rich region, an Ras associating-like domain (Wojcik *et al.*, 1999), a pleckstrin homology domain, a between pleckstrin and Src (BPS) domain and a C-terminal SH2 domain. The Ras associating, pleckstrin homology and BPS domains are also known as the Grbs and Mig domain. Mig-10 does not contain a C-terminal SH2 domain but instead replaces this with a Pro-rich sequence at the C-terminal (Manser *et al.*, 1997).

Grb7 has been shown to bind to erbB2 (avian erythroblastosis oncogene B 2) and focal adhesion kinase (FAK) through its SH2 domain. ErbB2 and Grb7 are both overexpressed in a subset (20–30%) of breast cancer patients who share a poor long-term survival rate and a statistically high incidence of metastases (Slamon, 1987; Seshadri *et al.*, 1993). The FAK/Grb7 association results in FAK phosphorylation of Grb7 at tyrosine residues and enhanced cell migration effects (Han *et al.*, 2000). Chu *et al.* (Chu *et al.*, 2009) have also shown that the tyrosine phosphorylation state of Grb7 is important in Grb7-mediated cell migration. The overexpression of Grb7 can lead to increased cell migration and development of metastases in cancer, as seen in the overexpression of Grb7 in lymph node metastases (Tanaka *et al.*, 1998). In addition, a Grb7 protein variant, lacking its C-terminal region and termed Grb7V, has been linked to invasive esophageal carcinoma (Tanaka *et al.*, 1998) and high-grade ovarian cancer (Wang *et al.*, 2010).

The tyrosine phosphorylation state of Grb7 has been shown important in Grb7's ability to interact with other signaling molecules. We have shown that the Grb7 protein binds to both FHL2 and human HS-1-associated protein X-1, and the tyrosine phosphorylation state of Grb7 is an important determinant in binding to both proteins (Siamakpour-Reihani *et al.*, 2009; Siamakpour-Reihani *et al.*, 2011). Tsai *et al.* (2007) showed that Grb7 acts as a translational repressor by binding to the mRNA 5' UTR of the kappa opioid receptor and Grb7 binding to kappa opioid receptor mRNA is controlled by its tyrosine phosphorylation state.

There is evidence that members of the Grb7 protein family can exist in a multimeric form, and this form could participate in a regulatory mechanism. The Grb10-SH2 domain crystal structure (PDB ID No. 1NRV) indicates the SH2 domain exists as a dimer (Stein *et al.*, 2003), whereas the gel filtration of Chinese hamster ovary cell lysates overexpressing full-length Grb10 implied the protein may exist in a tetrameric form, that is, a dimer of dimers (Dong *et al.*, 1998). Depetris *et al.* (2005) showed the high-affinity binding of the Grb14 protein to the insulin receptor requires the BPS domain and a dimerized SH2 domain, based on the decreased inhibitory action of an SH2 dimerization defective Grb14 mutant.

Our own previous results show that the Grb7-SH2 domain undergoes a competing dimerization reaction in solution (PDB ID No. 1MW4; Ivancic *et al.*, 2005), and a Grb7 crystal structure also presents the Grb7-SH2 domain as a dimer (PDB ID No. 2QMS; Porter *et al.*, 2007). The presence of dimerized molecules by X-ray crystallographic methods can often result from crystallization artifacts and not correlate with the existence of actual dimers. In the case of the Grb7 protein family, however, the previous studies substantiate multimeric (primarily dimeric) forms of these proteins exist in solution.

The studies presented here seek to lay the groundwork for clarifying the role of dimerization and tyrosine phosphorylation state in Grb7 regulation and function.

MATERIALS AND METHODS

Protein expression and purification

The expression and the purification of the human Grb7-SH2 domain have been described previously (Brescia *et al.*, 2002). Uniformly ^{15}N -labeled Grb7-SH2 domain was produced in minimal media containing 1 g/L $(^{15}\text{NH}_4)_2\text{SO}_4$ (Cambridge Isotope Labs, Woburn, MA, USA). Typical yields of purified Grb7-SH2 domain are 4–6 mg/l of culture. The expression and the purification of the Y80E and F99R Grb7-SH2 domain mutants are identical to that of the wild type (WT)-Grb7-SH2 domain.

Mutagenesis

The site-directed mutagenesis of the F99 and Y80 residues of the Grb7-SH2 domain was performed by polymerase chain reaction according to the manufacturer's guidelines, using the Quick-Change II mutagenesis kit (Invitrogen, Grand Island, NY, USA). Mutations were verified by DNA sequencing. The tyrosine 80 residue was selected for mutation based on its prediction as a possible phosphorylation site using the software NetPhos 2.0 (www.cbs.dtu.dk/zservices/NetPhos/).

Sample preparation for nuclear magnetic resonance, isothermal titration calorimetry and circular dichroism

Sample conditions for nuclear magnetic resonance (NMR) spectroscopy were as follows: 0.1–0.2 mM of protein, 1× phosphate-buffered saline and 5 mM of dithiothreitol, pH 7.4. Sample conditions for the isothermal titration calorimetry (ITC) studies were as follows: 30–50 μM Grb7-SH2 domain and 300–500 μM pY1139 peptide ligand, with replacement of dithiothreitol with *tris*(2-carboxyethyl)phosphine. The ITC conditions for the F99R and Y80E Grb7-SH2 domain mutants are the same as that for WT. The circular dichroism (CD) sample conditions were 0.5 mg/ml SH2 domain (WT, F99R, Y80D or Y80E) in 1× phosphate-buffered saline, pH 7.4.

Circular dichroism

CD spectra were recorded on a Jasco (Nantes, France) J-810 spectropolarimeter using 1-mm-thick quartz cells. In general, the Grb7-SH2 domain samples (WT, Y80E, F99R and Y80D) were analyzed in 1× phosphate buffer saline, at pH 7.4 and 25 °C. CD spectra were recorded with 0.5 mg/ml Grb7SH2 domain sample between 185 and 250 nm using a wavelength width of 2.0 nm and a scanning rate of 50 nm/min. For all spectra, the background spectrum of the buffer was subtracted.

Size exclusion chromatography

Size exclusion chromatography was performed on a Sephacryl S-200 HR (Amersham Biosciences, Pittsburgh, PA, USA) column pre-equilibrated with 1× phosphate-buffered saline, pH 7.40 at 4 °C. A volume of 0.5 ml of protein sample was injected onto the column and eluted with the same buffer at 0.5 ml/min. 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, Hercules, CA, USA). Initial concentrations on application to the column were as follows: WT, 756 μM ; Y80F, 666 μM ; Y80E, 974 μM ; F99R, 678 μM ; and Y80D, 1120 μM . Note that at these concentrations, more than 99.9% of the WT-Grb7-SH2 domain is in the dimerized form (Porter *et al.*, 2005).

Isothermal titration calorimetry

Titration experiments were performed using a VP-ITC ultrasensitive isothermal titration calorimeter (MicroCal Corp., Northampton, MA, USA) (Wiseman *et al.*, 1989). The Grb7-SH2 domain (WT, F99R or Y80E) was dialyzed with three exchanges of buffer (1/1000 v/v). To reduce errors arising from heats of dilution due to buffer differences between samples in the syringe and the reaction vessel, the pY1139 peptide ligand was dialyzed concurrently with the Grb7-SH2 sample. Both peptide and protein concentrations were determined using measured A280 values before loading into the syringe and reaction vessel. For each titration, 10 μ l of peptide ligand (300–500 μ M) was delivered into the protein sample (30–50 μ M) during the 20-s duration with an adequate interval (250 s) allowing complete equilibration. Binding curves involved the addition of 30 injections, which enabled 50% saturation to occur by the fifteenth injection. The heats of dilution, obtained by titrating the identical peptide solution into the reaction cell containing only the sample buffer, were subtracted before analysis. Data were recorded at a baseline of 20 μ cal/s and at a high gain mode, thus providing the fastest re-equilibration between injections. The syringe mixing speed was 300 rpm to ensure sufficient mixing while keeping baseline noise at a minimum. The data were collected automatically and analyzed with a one-site model via the Origin software provided from Microcal (ITC Origin, V7.0). The origin uses a nonlinear least-squares algorithm (minimization of χ^2), the concentration of the titrant and sample and an equilibrium equation to provide best fit values of the binding curve thus providing stoichiometry (n), change in enthalpy (ΔH) and binding constant K (Zhang *et al.*, 2000).

NMR spectroscopy

All NMR spectra of the 15 N-labeled Grb7-SH2 domains (WT, F99R or Y80E) were obtained at the annotated temperatures (Results section) on a Varian Unity Plus 500 MHz spectrometer. The spectrometer was equipped with a $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ resonance pulsed-field gradient probe. Spectra were recorded in the States-TPPI mode for quadrature detection with carrier frequencies for ^1H and ^{15}N at 4.73 and 120.0 ppm, respectively. In the direct dimension, 1024 actual data points were collected, and in the indirect dimension, 128 actual data points were collected, with 64 scans per increment. Spectral widths were 2200 Hz in the F1 dimension and 8000 Hz in the F2 dimension. All ^1H - ^{15}N correlation spectra were processed identically using instrument-supplied Varian VNMR software with sinebell squared (direct dimension) or sinebell (indirect dimension) apodization functions and zero filling to the next power of 2 in both dimensions.

Molecular dynamics simulations

Molecular dynamics simulations were carried out using chain A of the ligand-free Grb7-SH2 domain crystal structure (2QMS) (Porter *et al.*, 2007) to generate starting models. The F99R and the Y80E point mutations were introduced *in silico* using the Swiss-PdbViewer software (Guex and Peitsch, 1997). All-atom molecular dynamics simulations were performed using AMBER software (version 9) (Case *et al.*, 2006). Production simulations were conducted with constant pressure at 300 K (using Langevin dynamics to control temperature; Loncharich *et al.*, 1992), following energy minimization and equilibration of the starting models. Simulations of the F99R-Grb7-SH2 mutant used a 1-femtosecond time step with a modified (Onufriev *et al.*, 2000; Onufriev *et al.*, 2004) generalized Born implicit solvent model (Still *et al.*, 1990) with a distance cutoff of 16 Å for nonbonded interactions and a salt concentration of 150 mM (determined based on the solution conditions used to determine the wild-type Grb7-SH2 domain crystal structure; Porter *et al.*, 2007). Production simulations for the F99R-Grb7-SH2 mutant were carried out with constant temperature for 1.8 ns, after 1000 cycles of energy minimization, 20 ps of heating and equilibration (from 0 to 300 K). Independent simulations of the Y80E-Grb7-SH2 mutant were conducted (i) in implicit solvent, as described previously for the F99R-Grb7-SH2 mutant, and (ii) in explicit

solvent, with a 10-Å water box and with neutralizing ions. The additional simulations with explicit solvent were carried out for the Y80E mutant but not for the F99R mutant because we identified a putative stabilizing salt bridge involving the Y80E residue, and the generalized Born implicit solvent model is known to overestimate the strength of salt bridges (Okur *et al.*, 2008). Explicit solvent production simulations of the Y80E–Grb7–SH2 mutant were carried out with constant pressure and temperature for 5 ns, after 3500 cycles of energy minimization, 20 ps of heating from 0 to 300 K and 200 ps of unrestrained equilibration. In all simulations, a Langevin thermostat, with a collision frequency of 1 per picosecond, was used for temperature control.

Analytical ultracentrifugation

Sedimentation velocity experiments were run in a Beckman XL-I analytical ultracentrifuge. Sample protein and buffer solution (400 µl each) were loaded into the appropriate sides of a two-sector shaped cell. After allowing thermal equilibration at 4 °C for >1 h, the sample was spun at 40 000 rpm. Absorbance scans were taken continuously for 17 h. The data were analyzed with SEDFIT, allowing for a continuous $c(s)$ distribution (Schuck *et al.*, 2002). The partial specific volume of the protein and the buffer density and viscosity were calculated from SEDNTERP (Harding *et al.*, 1992; Lebowitz *et al.*, 1998). Initial concentrations were 93 µM for Y80E and 79 µM for F99R. Note that at these concentrations, more than 99.9% of the WT–Grb7–SH2 domain is in the dimerized form (Porter *et al.*, 2005).

RESULTS

A tyrosine phosphorylation mimic, Y80E–Grb7–SH2, is dimerization deficient

The Y80 residue of the Grb7–SH2 domain (Y495 in the full-length protein) is a predicted tyrosine phosphorylation site. Gel filtration studies of the Y80E–Grb7–SH2 mutant indicate that the domain is at least partially monomeric (Figure 1). Specifically, the Y80E–Grb7–SH2 domain elutes from a gel filtration column in a discreet band, at 71.1 ml elution volume, which converts to a molecular weight of 19 000. This is in comparison with the WT–Grb7–SH2 domain, which elutes over a broad volume range (65–76 ml), with a mean elution volume (65.9 ml) corresponding to an average molecular weight of 29 000. The previously characterized Grb7–SH2 dimerization-deficient mutant, F99R–Grb7–SH2 (Porter *et al.*, 2005), eluted from the same column at 74.6 ml, corresponding to a molecular weight of 11 000 (molecular weight of WT–Grb7–SH2, 13 800). The conservative mutation, Y80F, elutes at the same volume as the WT–Grb7–SH2 domain, demonstrating that loss of dimerization is specific to the glutamate mutation (i.e. negating the possibility that any mutation at the Y80 position causes loss of dimerization).

Analytical ultracentrifugation studies of the Y80E–Grb7–SH2 and F99R–Grb7–SH2 domain mutants agree with the gel filtration findings. Figure 2 shows the sedimentation velocity results for the F99R–Grb7–SH2 (dotted line) and Y80E–Grb7–SH2 (solid line), as a function of the sedimentation coefficient distribution ($c(s)$, y -axis) versus the Svedberg constant ($[S]$, x -axis). Fitting the data yields an apparent MW of 12.2 kDa for the F99R–Grb7–SH2 domain and 13.3 kDa for the Y80E–Grb7–SH2 domain. In sedimentation equilibrium experiments (data not shown), both mutants can be fit to a monomer–dimer equilibrium; however, the determined K_d s are 1600 µM (F99R) and 300–590 µM (Y80E). These K_d s are considerably larger than the K_d determined for the WT–Grb7–SH2 domain dimerization (11–22 µM; Porter *et al.*, 2005; Porter *et al.*, 2007). A small peak at a Svedberg value $[S]$ of approximately 2.9 (indicated by an asterisk) is due to a glutathione S-transferase-dimer impurity, resulting from the expression and purification of the recombinant Grb7–SH2 domains (band at approximately 27–28 kDa in the denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis; lane 4, Figure 2).

Dimerization-deficient mutants of the Grb7-SH2 (F99R-Grb7-SH2 and Y80E-Grb7-SH2) domain are structurally destabilized

CD spectra were recorded for the WT-Grb7-SH2, F99R-Grb7-SH2 and Y80E-Grb7-SH2 domains. The spectra are similar (Figure S2, top panel), indicate all three protein domains are folded and consist of a mixture of α -helical and β -sheet secondary structure. The results for the WT-Grb7-SH2 and F99R-Grb7-SH2 are in agreement with a previous report that the F99R-Grb7-SH2 domain mutant is structured (Porter *et al.*, 2005). In addition, the ^{15}N - ^1H correlation [nitrogen heteronuclear single quantum coherence (Nhsqc)] NMR spectra of all three SH2 domains show that the proteins are structured, displaying well-defined resonances and large chemical shift dispersion (Figure S1).

CD spectra were recorded in 10 °C increments from 25 °C to 65 °C for all three Grb7-SH2 domains (WT, F99R or Y80E). An accepted approach for monitoring protein denaturation (structural loss) by CD temperature melting is to plot the normalized ellipticity at 222 and/or 218 nm (Greenfield, 2006; Orwig and Lieberman, 2011) (Figure 3). In addition, the full CD traces for all three SH2 domains at different temperatures are provided in Figure S2. Residual secondary structure remains at 55 °C for the WT-Grb7-SH2 domain (Figures 3 and S2, center panel), whereas all secondary structure is absent for the Y80E-Grb7-SH2 and F99R-Grb7-SH2 domain mutants at this temperature. By 65 °C, all three Grb7-SH2 domains (WT, F99R or Y80E) are unstructured by CD measurement (Figures 3 and S2, lower panel). It is unlikely the changes in ellipticity for any of these SH2 domains is simply due to aggregation in that all three SH2 domains reach a constant ellipticity value at higher temperatures (i.e. the denaturation curve flattens at the top; John and Weeks, 2000).

An Nhsqc NMR analysis of all three Grb7-SH2 domains (WT, F99R or Y80E) was performed at temperatures ranging in 5 °C increments from 25 °C to 60 °C and revealed residues that experienced early loss of structure at lower temperatures (40 °C–45 °C) versus residues whose resonances remained at higher temperature (50 °C–55 °C) (Figure S1). Early loss of structure is indicated in residues 1–27 and 60–77, whereas residues 30–52 remained structured at 50 °C (Figure 4). The regions showing early loss of structure are the N-terminus, βD and $\beta\text{D}'$ strands and $\text{D}'\text{E}$ loop of the SH2 domain. The regions of the SH2 domain that remain structured at 50 °C include most of the αA helix and βB strand. By 60 °C, the distinctive Nhsqc fingerprint representative of a folded polypeptide had essentially collapsed and/or disappeared for WT-Grb7-SH2, indicating a primarily random coil and/or denatured peptide (Figure S1). This loss of the Nhsqc finger-print pattern occurred approximately 10 °C earlier for the F99R-Grb7-SH2 and Y80E-Grb7-SH2 domains, indicating reduced thermal stability for these mutants that corroborated the CD thermal denaturation results.

The binding of a tyrosine phosphorylated peptide to dimerization-deficient Grb7-SH2 domain mutants is entropically disfavored

Quantitative binding affinity measurements by ITC were recorded for the F99R-Grb7-SH2 (Figure 5A) and Y80E-Grb7-SH2 (Figure 5B) domains binding to a 10 amino acid phosphorylated tyrosine peptide (pY1139) representative of the Grb7 binding site on erbB2 at 25 °C (Table 1).

The F99R-Grb7-SH2 domain binds the erbB2 peptide with somewhat lower affinity (average $K_d = 9.29 \mu\text{M}$), whereas the Y80E-Grb7-SH2 binds the erbB2 peptide with similar affinity (average $K_d = 2.29 \mu\text{M}$) compared with the WT-Grb7-SH2 domain ($K_d = 2.28 \mu\text{M}$; Ivancic *et al.*, 2005). For both mutants, the interaction is enthalpically favored (F99R-Grb7-SH2, $\Delta H = -34.0 \text{ kcal/mol}$; Y80E-Grb7-SH2, $\Delta H = -46.4 \text{ kcal/mol}$) and entropically disfavored (F99R-Grb7-SH2, $-\Delta S = +26.9 \text{ kcal/mol}$; Y80E-Grb7-SH2,

$-T\Delta S = +38.7$ kcal/mol). This is in contrast to the WT-Grb7-SH2 domain binding pY1139, which is both enthalpically and entropically favored ($\Delta H = -4.66$ kcal/mol, $-T\Delta S = -3.04$ kcal/mol; Ivancic *et al.*, 2005).

Because the Grb7 protein (and its SH2 domain in particular for this study) has no enzymatic activity, no enzymatic assay exists to assess the functionality of the SH2 domain mutants. However, the ability of the mutants F99R and Y80E to bind tyrosine phosphorylated peptides can act as a demonstration of functionality. Therefore, Table 1 demonstrates that both Y80E-Grb7-SH2 and F99R-Grb7-SH2 domains bind the erbB2-representative peptide pY1139 within a factor of 5 compared with the WT-Grb7-SH2 domain.

Furthermore, it is assured that the WT-Grb7-SH2 domain exists essentially as a dimer (i.e. no monomeric WT-Grb7-SH2 domain present) for the ITC studies. The K_d for dimerization of the Grb7-SH2 domain is 11 μ M (Porter *et al.*, 2005). All ITC experiments were performed at concentrations considerably greater than this, for example, 30 μ M. At 11 μ M greater than 99.9% of the WT-Grb7-SH2 domain is in the dimerized form.

Finally, these studies provide the first steps for establishing the dimerization characteristics of these Grb7-SH2 domain mutants. It is acknowledged that not all protein for the Y80E mutant may be binding the pY1139 peptide (as evidenced by the binding stoichiometry), and the F99R mutant may suffer from aggregation effects (Porter *et al.*, 2005).

Molecular dynamics simulations indicate phosphorylation could favor the monomeric form of the Grb7-SH2 domain

The 2QMS Grb7-SH2 crystal structure (Porter *et al.*, 2007) provided a starting model for a dimerized form of the SH2 domain, as opposed to the monomeric NMR-derived structure (Ivancic *et al.*, 2003). The computational mutation of the tyrosine 80 residue (tyrosine 492 in the 2QMS Grb7-SH2 domain structure) to glutamate and energy minimization was performed on chain A before molecular dynamics simulations (described in Materials and Methods section). Molecular dynamics simulations in implicit solvent (1.8 ns) and in explicit solvent (independent simulations, 5 ns) suggest that conformational fluctuations in the β D'/EF-loop region may be minimized because of the formation of a salt-bridge interaction between Arg89 and Glu80 (Figure 6B). Such salt-bridge stabilization would discourage opening in the region necessary for establishment of a possible domain-swapped dimeric form (proposed by Pias *et al.*, 2010) and would favor the monomeric conformation of the SH2 domain.

We additionally modeled the F99R-Grb7-SH2 domain mutant using *in silico* mutation along with molecular dynamics simulations (1.8 ns in implicit solvent, described in Materials and Methods section). The *in silico* introduction of the F99R mutation demonstrates that the substitution of arginine for phenylalanine acts as a partially 'conservative' mutation. In the wild-type protein, a key hydrophobic interaction occurs between F90 and F99, in which the F99R-Grb7-SH2 mutant is replaced with a hydrophobic interaction involving F90 and the aliphatic region of the R99 side chain (Figure 6A).

DISCUSSION

The identification of a drug target is not sufficient for development of a successful treatment. This is amply demonstrated by the hundreds of 'targets' identified, coupled with the small number of actual targeted therapies available. For successful drug development, a basic understanding of how the target molecule functions and is regulated in its physiological state is needed. With this in mind, it is logical to think that the oligomerization state of a molecule can affect its ability to engage in signaling and also its binding to

inhibitors. Our studies here have sought to begin understanding the role of dimerization in the Grb7 protein and provide a foundation for exploitation of this knowledge in the development of Grb7 signaling modulators.

The wild-type Grb7 protein exists primarily in dimeric form, both as the full-length protein and in a truncated form consisting of only the Grb7-SH2 domain (Ivancic *et al.*, 2003; Porter *et al.*, 2005; Porter *et al.*, 2007). The binding of the Grb7-SH2 domain to the pY1139 peptide representative of erbB2 results in at least partial loss of dimerization (Ivancic *et al.*, 2005) and is both an enthalpically and entropically favored process. It can be argued that pY1139 binding, driving monomerization of the SH2 domain, results in the entropically favored situation of change from a one-molecule form of Grb7 (i.e. dimerized) to a two-molecule Grb7 form. This favorable increase in entropy could potentially offset the disfavorable change in entropy that must result from ordering of residues at the erbB2/Grb7 binding interface. In the Grb7-SH2 dimerization-deficient mutants F99R and Y80E, binding to the pY1139 erbB2 peptide is enthalpically favored but entropically disfavored. This is consistent with an inability to monomerize on erbB2 peptide binding and subsequent entropic compensation for residue ordering at the Grb7/erbB2 binding interface. Thus, the loss of Grb7 dimerization may supply favorable entropic compensation during or after binding to the erbB2 receptor tyrosine kinase.

Dimerization itself and the structural stability afforded by dimerization over the monomeric state (as evidenced by our CD and NMR denaturation studies) may provide a form of Grb7 autoregulation by enhancing the lifetime of the nonsignaling form of the protein. In this model, upon binding to a signaling partner, such as erbB2, phosphorylation drives the Grb7 protein to the monomeric state, with the binding interaction to erbB2 potentially compensating for the loss of dimerization structural stability. The unequivocal demonstration of the effect of phosphorylation on dimerization state in the Grb7-SH2 domain would be demonstrated by successful *in vitro* tyrosine phosphorylation followed by the characterization of the SH2 domain molecular weight by size exclusion chromatography and/or AUC. *In vitro* phosphorylation of the full-length Grb7 protein has been achieved by Tsai *et al.* (2007). *In vitro* phosphorylation of the Grb7-SH2 domain alone using both FAK and Src kinases is presently underway in our laboratory.

We have recently performed molecular dynamics simulations on the wild-type Grb7-SH2 domain, using the 2QMS Grb7-SH2 domain crystal structure (Porter *et al.*, 2007) as a starting model for the dimerized form (Pias *et al.*, 2010). These calculations showed that the Grb7-SH2 domain could potentially undergo a conformational change resulting in an energetically stable domain-swapped form, akin to the domain-swapped conformational form of the Grb2-SH2 domain (Schiering *et al.*, 2000; Nioche *et al.*, 2002; Benfield *et al.*, 2007). Further molecular dynamics simulations on the Y80E-Grb7-SH2 domain, in this study, provide an argument for the preferred monomeric state of this mutant. From these simulations, we have hypothesized that a salt bridge formed between Arg89 and Glu80 stabilizes the β D'/EF-loop region of the Grb7-SH2 domain, pushing the reaction equilibrium towards the monomeric instead of the domain-swapped dimeric form (Figure 6B).

It is not readily apparent how the Y80E phosphorylation mimic mutation would disrupt 'unswapped' dimerization. The dimerization interface in the 2QMS Grb7-SH2 crystal structure appears to be composed almost entirely of the F99 and F90 residues (F502 and F511 in the 2QMS structure numbering) in both the WT and Y80E Grb7-SH2 domains. These four residues (two Phe residues from each monomer) form a hydrophobically packed dimer interface and may also engage in stabilizing π -stacking interactions. The molecular dynamics simulations on the Y80E-Grb7-SH2 mutant suggest that these interactions are

maintained in the unswapped dimer form, suggesting no stabilization of the monomer over the unswapped dimer. The results are unequivocal, however, that the Y80E–Grb7–SH2 domain phosphorylation mimic is dimerization deficient. These simulation results do not preclude the possibility that Grb7 may simultaneously exist in three forms: monomer, unswapped dimer and swapped dimer.

Molecular dynamics simulations on the F99R–Grb7–SH2 domain indicate that the dimerized form would be disfavored in both the swapped and unswapped dimeric cases because of the disruption of the hydrophobic packing among the four phenylalanine residues that form the dimer interface.

Another SH2-containing protein, Grb2, has been the subject of targeted therapy development, with little clinical success. Inhibitors to the Grb2-SH2 domain suffer from low physiological binding and/or lack of specificity. It has been shown that the dimerized form of the Grb2-SH2 domain binds with lower affinity than the monomer form to tyrosine phosphorylated peptides representative of intracellular binding partners (Benfield *et al.*, 2007). In addition, the existence of a domain-swapped dimerized form of the Grb2-SH2 domain has been shown to exist (Schiering *et al.*, 2000; Nioche *et al.*, 2002). The potentially important contribution of the dimerized form of the Grb2-SH2 domain to binding has been largely disregarded in the design and development of Grb2 inhibitors.

Our study suggests that dimerization in the Grb7 protein may be controlled by its tyrosine phosphorylation state. The monomeric versus dimeric state of the Grb7–SH2 domain binds the pY1139 erbB2 peptide with differing thermodynamic characteristics and demonstrates decreased structural stability. Such factors may be important considerations in the development of effective therapeutics targeting Grb7.

Abbreviations and symbols

erbB2	erythroblastic leukemia viral (v-erb-b) oncogene homolog 2, also known as HER2
CD	circular dichroism
FAK	focal adhesion kinase
Grb7,10,14	growth factor receptor–bound protein 7, 10 and 14 protein, respectively
ITC	isothermal titration calorimetry
Nhsqc	nitrogen heteronuclear single quantum coherence
NMR	nuclear magnetic resonance
pY	phosphorylated tyrosine
SH2	Src homology 2

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Figure 1.

Size exclusion chromatography results for the WT-Grb7-SH2, F99R-Grb7-SH2, Y80E-Grb7-SH2, Y80F-Grb7-SH2 and Y80D-Grb7-SH2 domains. The bottom panel contains the elution profile for each Grb7-SH2 domain. The y-axis is the absorption signal at 280 nm, whereas the x-axis is the ratio of the protein elution volume divided by the column void volume (34 ml). The top panel shows the calibration curve determined by running the following molecular weight standards through the column: conalbumin, 75 000 MW; carbonic anhydrase, 29 000 MW; ribonuclease A, 13 700 MW. The y-axis plots the molecular weight, whereas the x-axis shows the molecular weight standard protein elution volume divided by the column void volume.

Figure 2.

Left panel: sedimentation velocity results for the F99R-Grb7-SH2 (dotted line) and Y80E-Grb7-SH2 (solid line) domains. Right panel: gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the F99R-Grb7-SH2 domain sample for analytical ultracentrifugation study. Lane 4 is the essentially pure F99R-Grb7-SH2 domain.

Figure 3.

CD temperature denaturation study of the WT-Grb7-SH2, F99R-Grb7-SH2 and Y80E-Grb7-SH2 domains monitored at 222 nm wavelength. Experimental details can be found in the Materials and Methods section. X, WT-Grb7-SH2 domain; O, F99R-Grb7-SH2 domain; +, Y80E-Grb7-SH2 domain.

Figure 4.

Ribbon representation of the NMR thermal denaturation study results for the WT-Grb7-SH2 domain. Two different orientations of the Grb7-SH2 are shown. Left panel: side view; right panel: top view. Red coloration represents regions that showed early loss of resonances (before 40 °C, Figure S1): residues 1–27 and 60–77. Blue coloration represents regions that retained resonances at 50 °C: residues 30–52. Grey coloration represents regions of mixed early loss of resonances at 40 °C and retention of resonances at 50 °C.

Figure 5.

(A, top) Exothermic heats of reaction ($\mu\text{cal/s}$) measured for 35 injections of the pY1139 peptide into an F99R-Grb7-SH2 domain sample. (Bottom) The solid line is the best fit to the data for a single binding site model using a nonlinear least squares fit to solve for K_b (equilibrium binding constant, $1/K_d$) and ΔH (change in enthalpy of reaction), as reported in the Results section and Table 1. (B, top) Exothermic heats of reaction ($\mu\text{cal/s}$) measured for 35 injections of the pY1139 peptide into a Y80E-Grb7-SH2 domain sample. (Bottom) The solid line is the best fit to the data for a single binding site model using a nonlinear least squares fit to solve for K_b ($1/K_d$) and ΔH , as reported in the Results section and Table 1.

Figure 6.

Comparison of interactions involving residue R89 in the wild-type Grb7-SH2 domain (green) and in the Y80E-Grb7-SH2 mutant (orange). (A) Amino- π stacking interaction involving the Y80 and R89 side chains in the wild-type domain (chain A of the Grb7-SH2 domain crystal structure, 2QMS). (B) Salt bridge formed by side chain atoms of residues E80 and R89 in the Y80E-Grb7-SH2 mutant. Shown is a snapshot collected at the end of a 5-ns explicit solvent molecular dynamics simulation. Figure rendered with PyMOL (<http://www.pymol.org/>).

Table 1

Thermodynamic parameters for the binding of the pY1139 peptide to the Grb7-SH2 domains at 25 °C

Grb7-SH2 domain	K_d (μM)	ΔG (kcal mol ⁻¹)	ΔH (kcal mol ⁻¹)	$-T\Delta S$ (kcal mol ⁻¹)
WT ^a	2.28 ± 0.15	-7.70 ± 0.04	-4.66 ± 0.05	-3.04 ± 0.09
F99R-Grb7-SH2 ^b	9.29 ± 2.17	-6.89 ± 0.13	-34.0 ± 10.2	26.9 ± 10.3
Y80E-Grb7-SH2 ^c	2.29 ± 0.43	-7.69 ± 0.10	-46.4 ± 12.5	38.7 ± 12.6

^aThe reported values are the average for five separate experiments.

^bThe reported values are the average for three separate experiments.

^cThe reported values are the average for two separate experiments.