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Multivalent Binding and Facilitated Diffusion Account for the Formation of Grb2-Sos1 Signaling Complex in a Cooperative Manner

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Abstract

Despite its key role in driving cellular growth and proliferation through receptor tyrosine kinase (RTK) signaling, the Grb2-Sos1 macromolecular interaction remains poorly understood in mechanistic terms. Herein, using an array of biophysical methods, we provide evidence that although Grb2 adaptor can potentially bind to all four PX Ψ PXR motifs — designated herein S1, S2, S3 and S4 — located within the Sos1 guanine nucleotide exchange factor, the formation of Grb2-Sos1 signaling complex occurs with a 2:1 stoichiometry. Strikingly, such bivalent binding appears to be driven by the association of Grb2 homodimer to only two out of a four potential PX Ψ PXR motifs within Sos1 at any one time. Of particular interest is the observation that out of a possible six pairwise combinations in which S1–S4 motifs may act in concert for the docking of Grb2 homodimer through bivalent binding, only S1/S3, S1/S4, S2/S4 and S3/S4 do so, while S1/S2 and S2/S3 pairwise combinations appear to only afford monovalent binding. This salient observation implicates the role of local physical constraints in fine tuning the conformational heterogeneity of Grb2-Sos1 signaling complex. Importantly, the presence of multiple binding sites within Sos1 appears to provide a physical route for Grb2 to hop in a flip-flop manner from one site to the next through facilitated diffusion and such rapid exchange forms the basis of positive cooperativity driving the bivalent binding of Grb2 to Sos1 with high affinity. Collectively, our study sheds new light on the assembly of a key macromolecular signaling complex central to cellular machinery in health and disease.

Keywords

SH3-ligand interactions; Intrinsic disorder; Multivalent binding; Flip-flop hopping; Facilitated diffusion; Positive cooperativity

INTRODUCTION

Grb2-Sos1 interaction plays a central role in relaying external signals from receptor tyrosine kinases (RTKs) at the cell surface to downstream effectors and regulators such as Ras and Akt within the cytosol (1–4). Comprised of the ubiquitous nSH3-SH2-cSH3 signaling module, where the nSH3 and cSH3 are respectively the N-terminal and the C-terminal SH3 domains flanking the central SH2 domain, Grb2 recognizes activated RTKs by virtue of its SH2 domain's ability to bind to tyrosine-phosphorylated (pY) sequences in the context of pYXN motif located within the cytoplasmic tails of a diverse array of receptors, including

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EGF and PDGF receptors (5–7). Upon binding to RTKs, the SH3 domains of Grb2 present an opportunity for a wide spectrum of proline-rich proteins to be recruited to the inner membrane surface, the site of initiation of a plethora of signaling cascades (3, 8–15). Among them, the Sos1 guanine nucleotide exchange factor and the Gab1 docker are by far the best characterized downstream partners of Grb2 (8–10, 16, 17). Upon recruitment to the inner membrane surface, Sos1 facilitates the GDP-GTP exchange within the membrane-bound Ras GTPase and thereby switches on a key signaling circuit that involves the activation of MAP kinase cascade central to cellular growth and proliferation (18, 19). In contrast, the recruitment of Gab1 to the inner membrane surface provides docking platforms for the Shp2 tyrosine phosphatase and the PI3K kinase, which respectively account for further amplification of Ras activity, as sustained activation of Ras requires both the Sos1-dependent and Gab1-dependent pathways (20–23), and the activation of Akt serine-threonine kinase, which plays a pivotal role in cell growth and survival (24).

How exactly does Grb2 recruit Sos1 to the inner membrane surface? Although seminal work implicated the role of both SH3 domains of Grb2 in the recruitment of Sos1 to the inner membrane surface (3, 8, 17), recent studies have shown that only the nSH3 domain binds to Sos1 in an allosteric manner such that the cSH3 domain is freed up for binding to Gab1 so as to generate the Sos1-Grb2-Gab1 ternary signaling complex in a non-competitive fashion (25, 26). It is important to note that Sos1 contains four distinct sites within its proline-rich (PR) domain for binding to the nSH3 domain of Grb2 (Figure 1). These sites, designated herein S1, S2, S3 and S4, share the PX ψ PXR consensus motif, where X is any residue and ψ is valine, leucine or isoleucine. On the basis of structural studies of the nSH3 domain of Grb2 in complex with peptides containing the PX ψ PXR motif in Sos1 (27–31), the nSH3 domain displays a characteristic β -barrel fold harboring a hydrophobic cleft on one face of the domain for accommodating the incoming peptide. While the β -barrel is comprised of a pair of nearly-orthogonal β -sheets, with each β -sheet containing three anti-parallel β -strands, the peptide adopts a relatively open left-handed polyproline type II (PPII) helical conformation upon binding. Although our previous studies have shown that the isolated nSH3 domain of Grb2 can potentially bind to peptides derived from all four S1–S4 motifs in a physiologically-relevant manner (26, 32, 33), the precise mechanism of the assembly of Grb2-Sos1 signaling complex remains hitherto poorly understood. In light of the knowledge that Grb2 exists in a dimer-monomer equilibrium in solution (34), it is tempting to postulate that Grb2 could bind to Sos1 in a multivalent manner so as to generate higher-order Grb2-Sos1 multimers rather than a simple binary complex.

In an effort to further elucidate the mechanism underlying the assembly of Grb2-Sos1 signaling complex, the present study was conceived. Herein, using an array of biophysical methods, we provide evidence that although Grb2 adaptor can potentially bind to all four PX ψ PXR motifs — designated herein S1, S2, S3 and S4 — located within the Sos1 guanine nucleotide exchange factor, the formation of Grb2-Sos1 signaling complex occurs with a 2:1 stoichiometry. Strikingly, such bivalent binding appears to be driven by the association of Grb2 homodimer to only two out of a four potential PX ψ PXR motifs within Sos1 at any one time. Of particular interest is the observation that out of a possible six pairwise combinations in which S1–S4 motifs may act in concert for the docking of Grb2 homodimer through bivalent binding, only S1/S3, S1/S4, S2/S4 and S3/S4 do so, while S1/S2 and S2/S3 pairwise combinations appear to only afford monovalent binding. This salient observation implicates the role of local physical constraints in fine tuning the conformational heterogeneity of Grb2-Sos1 signaling complex. Importantly, the presence of multiple binding sites within Sos1 appears to provide a physical route for Grb2 to hop in a flip-flop manner from one site to the next through facilitated diffusion and such rapid exchange forms the basis of positive cooperativity driving the bivalent binding of Grb2 to Sos1 with high

affinity. Collectively, our study sheds new light on the assembly of a key macromolecular signaling complex central to cellular machinery in health and disease.

MATERIALS and METHODS

Sample preparation

Full-length human Grb2 (residues 1–217) as well as the wildtype and various mutant constructs of PR domain of human Sos1 (residues 1141–1300) were cloned into pET30 bacterial expression vectors with an N-terminal His-tag using Novagen LIC technology. Notably, the mutant constructs of the PR domain of Sos1 were generated through alanine substitution of consensus ψ and arginine residues, located within the corresponding PX ψ PXR motifs, through de novo cDNA synthesis courtesy of GenScript Corporation (Figure 1). All recombinant constructs were expressed in *Escherichia coli* BL21 Star (DE3) bacterial strain (Invitrogen) and purified on a Ni-NTA affinity column using standard procedures. Briefly, bacterial cells were grown at 20°C in Terrific Broth to an optical density of greater than unity at 600nm prior to induction with 0.5mM isopropyl β -D-1-thiogalactopyranoside (IPTG). The bacterial culture was further grown overnight at 20°C and the cells were subsequently harvested and disrupted using a BeadBeater (Biospec). After separation of cell debris at high-speed centrifugation, the cell lysate was loaded onto a Ni-NTA column and washed extensively with 20mM imidazole to remove non-specific binding of bacterial proteins to the column. The recombinant proteins were subsequently eluted with 200mM imidazole and dialyzed against an appropriate buffer to remove excess imidazole. Further treatment on a Hiload Superdex 200 size-exclusion chromatography (SEC) column coupled in-line with GE Akta FPLC system led to purification of Grb2 and various constructs of the PR domain of Sos1 to an apparent homogeneity as judged by SDS-PAGE analysis. Final yields were typically between 10–20mg protein of apparent homogeneity per liter of bacterial culture. Protein concentration was determined by the fluorescence-based Quant-It assay (Invitrogen) and spectrophotometrically on the basis of extinction coefficients calculated for each recombinant construct using the online software ProtParam at ExPasy Server (35). Results from both methods were in an excellent agreement.

Analytical light scattering

Analytical light scattering (ALS) experiments were conducted on a Wyatt miniDAWN TREOS triple-angle static light scattering detector and Wyatt QELS dynamic light scattering detector coupled in-line with a Wyatt Optilab rEX differential refractive index detector and interfaced to a Hiload Superdex 200 size-exclusion chromatography (SEC) column under the control of a GE Akta FPLC system within a chromatography refrigerator at 10°C. Protein samples of full-length Grb2 and various constructs of the PR domain of Sos1 were prepared in 50mM Tris, 200mM NaCl, 1mM EDTA and 5mM β -mercaptoethanol at pH 8.0 and loaded onto the column at a flow rate of 1ml/min and the data were automatically acquired using the ASTRA software. The starting concentrations injected onto the column were typically between 40–50 μ M of each protein construct. The angular- and concentration-dependence of static light scattering (SLS) intensity of each protein species resolved in the flow mode was measured by the Wyatt miniDAWN TREOS detector. The SLS data were analyzed according to the following built-in Zimm equation in ASTRA software (36, 37):

$$[Kc/R_\theta] = ((1/M) + 2A_2c) [1 + ((16\pi^2(R_g)^2/3\lambda^2)\sin^2(\theta/2))] \quad [1]$$

where R_θ is the excess Raleigh ratio due to protein in the solution as a function of protein concentration c (mg/ml) and the scattering angle θ (42°, 90° and 138°), M is the observed molar mass of each protein species, A_2 is the second virial coefficient, λ is the wavelength

of laser light in solution (658nm), R_g is the radius of gyration of protein, and K is given by the following relationship:

$$K = [4\pi^2 n^2 (dn/dc)^2] / N_A \lambda^4 \quad [2]$$

where n is the refractive index of the solvent, dn/dc is the refractive index increment of the protein in solution and N_A is the Avogadro's number ($6.02 \times 10^{23} \text{ mol}^{-1}$). Under dilute protein concentration ($c \rightarrow 0$), Eq [1] reduces to:

$$[Kc/R_\theta] = [1/M + ((16\pi^2 (R_g)^2 / 3M\lambda^2) \sin^2(\theta/2))] \quad [3]$$

Thus, a plot of $[Kc/R_\theta]$ versus $\sin^2(\theta/2)$ yields a straight line with slope $16\pi^2 R_g^2 / 3M\lambda^2$ and y-intercept $1/M$. Accordingly, molar mass can be obtained in a global analysis from the y-intercept of linear fits of a range of $[Kc/R_\theta] - \sin^2(\theta/2)$ plots as a function of protein concentration along the elution profile of each protein species using SLS measurements at three scattering angles. Herein, the weighted-average molar mass (M_w) and number-average molar mass (M_n) were calculated from the following relationships:

$$M_w = \sum (c_i M_i) / \sum c_i \quad [4]$$

$$M_n = \sum c_i / \sum (c_i / M_i) \quad [5]$$

where c_i is the protein concentration and M_i is the observed molar mass at the i th slice within an elution profile. It should however be noted that R_g could not be determined for any of the protein species due to the lack of angular-dependence of scattered light. The time- and concentration-dependence of dynamic light scattering (DLS) intensity fluctuation of each protein species resolved in the flow mode was measured by the Wyatt QELS detector positioned at 90° with respect to the incident laser beam. The DLS data were iteratively fit using non-linear least squares regression analysis to the following built-in equation in ASTRA software (38–40):

$$G(\tau) = \alpha \text{Exp}(-2\Gamma\tau) + \beta \quad [6]$$

where $G(\tau)$ is the autocorrelation function of dynamic light scattering intensity fluctuation I , τ is the delay time of autocorrelation function, Γ is the decay rate constant of autocorrelation function, α is the initial amplitude of autocorrelation function at zero delay time, and β is the baseline offset (the value of autocorrelation function at infinite delay time). Thus, fitting the above equation to a range of $G(\tau) - \tau$ plots as a function of protein concentration along the elution profile of each protein species computes the weighted-average value of Γ using DLS measurements at a scattering angle of 90° . Accordingly, the translational diffusion coefficient (D_t) of each protein species was calculated from the following relationship:

$$D_t = [(\Gamma\lambda^2) / (16\pi^2 n^2 \sin^2(\theta/2))] \quad [7]$$

where λ is the wavelength of laser light in solution (658nm), n is the refractive index of the solvent and θ is the scattering angle (90°). Additionally, the hydrodynamic radius (R_h) of each protein construct was determined from the Stokes-Einstein relationship:

$$R_h = [(k_B T) / (6\pi\eta D_t)] \quad [8]$$

where k_B is Boltzman's constant ($1.38 \times 10^{-23} \text{JK}^{-1}$), T is the absolute temperature and η is the solvent viscosity. We note that the R_h reported here represents the weighted-average value as defined by the following expression:

$$R_h = \sum (c_i R_{h,i}) / \sum c_i \quad [9]$$

where c_i is the protein concentration and $R_{h,i}$ is the observed hydrodynamic radius at the i th slice within an elution profile. It should also be noted that, in both the SLS and DLS measurements, protein concentration (c) along the elution profile of each protein species was automatically quantified in the ASTRA software from the change in refractive index (Δn) with respect to the solvent as measured by the Wyatt Optilab rEX detector using the following relationship:

$$c = (\Delta n) / (dn/dc) \quad [10]$$

where dn/dc is the refractive index increment of the protein in solution.

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) measurements were performed on a Microcal VP-ITC instrument and data were acquired and processed using the integrated Microcal ORIGIN software. Briefly, protein samples were prepared in 50mM Tris, 200mM NaCl, 1mM EDTA and 5mM β -mercaptoethanol at pH 8.0. The experiments were initiated by injecting $25 \times 10 \mu\text{l}$ aliquots of 0.5–1mM of each construct of the PR domain of Sos1 from the syringe into the calorimetric cell containing 1.8ml of 50–100 μM of full-length Grb2 solution at 25°C. The change in thermal power as a function of each injection was automatically recorded using the ORIGIN software and the raw data were further processed to yield binding isotherms of heat release per injection as a function of molar ratio of each PR construct to full-length Grb2. The heats of mixing and dilution were subtracted from the heat of binding per injection by carrying out a control experiment in which the same buffer in the calorimetric cell was titrated against each PR construct in an identical manner. To extract binding affinity (K_d) and binding enthalpy (ΔH), the ITC isotherms were iteratively fit using non-linear least squares regression analysis to the following built-in equation in the ORIGIN software:

$$q(i) = (\Delta HVP/2n) \{ [1 + (nL/P) + (nK_d/P)] - [1 + (nL/P) + (nK_d/P)]^2 - (4nL/P) \}^{1/2} \quad [11]$$

where $q(i)$ is the heat release (kcal/mol) for the i th injection, V is the effective volume of Grb2 solution in the calorimetric cell (1.46ml), P is the total concentration of Grb2 in the calorimetric cell, L is the total concentration of each PR construct added from the syringe for the i th injection, and n is the stoichiometry of full-length Grb2 bound to each PR construct at equilibrium. The above equation is derived from the binding of a ligand to a macromolecule using the law of mass action assuming a one-site model (41). The free energy change (ΔG) upon the binding of Grb2 to each PR construct was calculated from the relationship:

$$\Delta G = nRT \ln K_d \quad [12]$$

where n is the integral number of binding sites within the PR domain or the observed stoichiometry of binding of Grb2 to the PR domain, R is the universal molar gas constant (1.99 cal/K/mol), and T is the absolute temperature. The entropic contribution ($T\Delta S$) to the free energy of binding was calculated from the relationship:

$$T\Delta S = \Delta H - \Delta G \quad [13]$$

where ΔH and ΔG are as defined above. Thermodynamic parameters associated with cooperative binding of Grb2 to the observed pairwise combinations of S1–S4 sites within the PR domain of Sos1 with a 2:1 stoichiometry were calculated from the following relationships:

$$\Delta\Delta G_c = [(\Delta G_{ij}) - (\Delta G_i + \Delta G_j)] \quad [14]$$

$$\Delta\Delta H_c = [(\Delta H_{ij}) - (\Delta H_i + \Delta H_j)] \quad [15]$$

$$T\Delta\Delta S_c = [(T\Delta S_{ij}) - (T\Delta S_i + T\Delta S_j)] \quad [16]$$

where $\Delta\Delta H_c$ and $T\Delta\Delta S_c$ are respectively the underlying enthalpic and entropic components to the free energy of cooperativity ($\Delta\Delta G_c$), the subscript i denotes the corresponding thermodynamic parameters (ΔG_i , ΔH_i and $T\Delta S_i$) associated with the binding of Grb2 to site i (first site), the subscript j denotes the corresponding thermodynamic parameters (ΔG_j , ΔH_j and $T\Delta S_j$) associated with the binding of Grb2 to site j (second site), and the subscript ij denotes the corresponding thermodynamic parameters (ΔG_{ij} , ΔH_{ij} and $T\Delta S_{ij}$) associated with the binding of Grb2 to both sites i and j. $\Delta G_i/\Delta H_i/T\Delta S_i$ and $\Delta G_j/\Delta H_j/T\Delta S_j$ for each of the four S1–S4 sites were calculated from the binding of Grb2 to the triple-mutant PR constructs (PR_mS123, PR_mS124, PR_mS134 and PR_mS234). $\Delta G_{ij}/\Delta H_{ij}/T\Delta S_{ij}$ for any observed pairwise combination of S1–S4 sites (S1–S4, S1–S3, S2–S4 and S3–S4) were calculated from the binding of Grb2 to the PR constructs with a 2:1 stoichiometry, namely the wildtype PR construct (PR_WT), the single-mutant PR constructs (PR_mS1, PR_mS2, PR_mS3 and PR_mS4), and the double-mutant PR constructs (PR_mS12, PR_mS13, PR_mS23 and PR_mS24).

Circular dichroism

Far-UV circular dichroism (CD) measurements were conducted on a Jasco J-815 spectrometer thermostatically controlled at 25°C. Experiments were conducted on 5 μ M of wildtype PR domain of Sos1 in 10mM Sodium phosphate at pH 8.0. Data were collected using a quartz cuvette with a 2-mm pathlength in the 190–250nm wavelength range. Data were normalized against reference spectra to remove the contribution of buffer. Data were recorded with a slit bandwidth of 2nm at a scan rate of 10nm/min. Each data set represents an average of four scans acquired at 0.1nm intervals. Data were converted to molar ellipticity, $[\theta]$, as a function of wavelength (λ) of electromagnetic radiation using the equation:

$$[\theta] = [(10^5 \Delta\epsilon)/cl] \text{ deg.cm}^2.\text{dmol}^{-1} \quad [15]$$

where $\Delta\epsilon$ is the observed ellipticity in mdeg, c is the protein concentration in μ M and l is the cuvette pathlength in cm.

Molecular modeling

Molecular modeling (MM) was employed to construct structural models of Grb2 bivalently bound to S1/S4 sites within the PR domain of Sos1 either as two independent monomers (Grb2-PR-Grb2), or in the context of a homodimer ($[\text{Grb2}]_2$ -PR), using the MODELLER software based on homology modeling (42). Briefly, the atomic models were built in various stages. First, the intervening polypeptide chain spanning S1/S4 sites within the PR domain

of Sos1 was folded into a compact globular-like topology using the QUARK server based on ab initio modeling. The QUARK server can be accessed online at <http://zhanglab.ccmb.med.umich.edu/quark>. Notably, ab initio modeling was motivated by the fact that the PR domain of Sos1 appears to be structurally compact on the basis of our hydrodynamic data presented here. Next, the [Grb2]₂-PR structural model was built using the ab initio structural model of the PR domain in combination with the crystal structure of Grb2 homodimer alone (PDB# 1GRI) and the solution structure of isolated nSH3 domain of Grb2 bound to an PX ψ PXR motif homologous to S1 and S4 sites (PDB# 4GBQ) using multiple-template alignment strategy in MODELLER (42). Additionally, hydrogen bonding restraints between the consensus arginine (R1156) within the S1 site and D15 within the nSH3 domain of one monomer of Grb2 as well as between the consensus arginine (R1295) within the S4 site and D15 within the nSH3 domain of second monomer of Grb2 were introduced as described previously (43). The Grb2-PR-Grb2 structural model was derived from the pre-built structural model of [Grb2]₂-PR. Here, the intervening polypeptide chain spanning S1 and S4 sites within the PR domain of Sos1 was excised out and the two monomers within Grb2 homodimer bound to isolated S1 and S4 sites were spatially displaced and moved apart laterally in a rigid-body fashion so as to devoid them of any inter-monomer contacts in MOLMOL (44). The resulting conformation of two non-interacting Grb2 monomers in complex with isolated S1 and S4 sites in combination with the ab initio structural model of the PR domain were subsequently used as templates to construct the Grb2-PR-Grb2 structural model in MODELLER (42). In each case, a total of 100 structural models were calculated and the structure with the lowest energy, as judged by the MODELLER Objective Function, was selected for further analysis. The modeled structures were rendered using RIBBONS (45). All calculations and data processing were performed on a Linux workstation equipped with a dual-core processor.

Molecular dynamics

Molecular dynamics (MD) simulations were performed with GROMACS (46, 47) using the integrated OPLS-AA force field (48, 49). Briefly, the modeled structures of Grb2 in complex with the PR domain of Sos1 through bivalent binding at S1/S4 sites as two independent monomers (Grb2-PR-Grb2) or in the context of a homodimer ([Grb2]₂-PR), were centered within a cubic box and hydrated using the extended simple point charge (SPC/E) water model (50, 51). The hydrated structures were energy-minimized with the steepest descent algorithm prior to equilibration under the NPT ensemble conditions, wherein the number of atoms (N), pressure (P) and temperature (T) within the system were respectively kept constant at $\sim 10^5$, 1 bar and 300 K. The Particle-Mesh Ewald (PME) method was employed to compute long-range electrostatic interactions with a 10Å cut-off (52) and the Linear Constraint Solver (LINCS) algorithm to restrain bond lengths (53). All MD simulations were performed under periodic boundary conditions (PBC) using the leap-frog integrator with a time step of 2fs. For the final MD production runs, data were collected every 10ps over a time scale of 50ns. All simulations were run on a Linux workstation using parallel processors at the High Performance Computing facility within the Center for Computational Science of the University of Miami.

RESULTS and DISCUSSION

PR domain of Sos1 adopts a monomeric conformation in solution

In order to understand the assembly of Grb2-Sos1 complex, we first conducted ALS analysis on the full-length Grb2 and the PR domain of Sos1 so as to assess their propensities to associate into higher-order oligomers in solution (Figure 2 and Table 1). Importantly, in addition to the wildtype construct of the PR domain (PR_WT), ALS analysis was also conducted on various single, double and triple mutant constructs, mutated with respect to

one or more of the S1–S4 sites, in order to determine their effect on hydrodynamic properties of the PR domain (Figure 1b). Our data suggest that while Grb2 exists in a monomer-dimer equilibrium in agreement with our previous study (34), the wildtype and various mutant constructs of the PR domain are all monomers in solution. In an attempt to gain insights into the macromolecular polydispersity and shape of the various species, we also determined the corresponding M_w/M_n ratio and hydrodynamic radius (R_h) from our data. Our analysis reveals that both the monomeric and dimeric forms of Grb2 as well as the PR monomers display M_w/M_n ratio close to unity, implying that they are all highly monodisperse in solution. Furthermore, the hydrodynamic radii observed for the Grb2 monomer and dimer are consistent with their tightly packed $\alpha\beta$ -folds (54).

Remarkably, the hydrodynamic radii observed for the various PR constructs of Sos1 lie somewhere between that of monomeric and dimeric Grb2, implying that the PR domain of Sos1 adopts a compact shape in a manner akin to that adopted by globular proteins rather than an extended random coil-like conformation devoid of any structural features. This observation is particularly surprising in that the proline-rich proteins such as the PR domain of Sos1 are believed to be devoid of any intrinsic structure in solution. This is due to the rigidity of proline sidechain that is structurally-destabilizing but nevertheless allows proline-rich proteins to adopt a rigid conformation ideally suited for binding to cognate ligands at the expense of little entropic penalty, which is often the bottleneck in protein-protein interactions pertinent to cellular signaling cascades. The fact that the PR domain of Sos1 appears to be compact and globular in solution suggests strongly that the lack of secondary structural elements such as α -helices and β -strands alone may not necessarily equate to lack of intrinsic structure. On the contrary, our data support the notion that proline-rich proteins may be able to adopt a compact shape and that such adoption may be a necessity to avoid chaos within the cellular environment, where the high concentration of proline-rich sequences and proteins in general may otherwise increase cellular entropy through intrinsic disorder and formation of structural knots.

PR domain of Sos1 is intrinsically-disordered

The PR domain of Sos1 is abundant in residues, such as proline as well as polar and charged residues, that are usually found in structurally-disordered proteins. Given its key role in the Ras/MAPK pathway, it is fitting that the PR domain of Sos1 shares such a virtue with other structurally-disordered proteins believed to play a central role in mediating cellular signaling cascades (55–59). In an attempt to further analyze the extent of structural disorder within the PR domain of Sos1, we performed secondary structure prediction analysis using POODLE (60). As shown in Figure 3a, our analysis reveals that the PR domain is indeed predominantly disordered and lacks any identifiable α -helical and/or β -strand features characteristic of well-folded $\alpha\beta$ proteins. This salient observation is further corroborated by our far-UV CD analysis (Figure 3b), wherein the spectrum of the PR domain shows a minimum centered around 208nm, a signature usually characteristic of proteins containing random coil and polyproline type II (PPII) helical conformations (61, 62). Additionally, the rather broad far-UV CD spectrum of the PR domain suggests that it is conformationally heterogeneous as would be expected of structurally-disordered proteins devoid of a well-defined compact fold.

Grb2 binds to the PR domain of Sos1 with a 2:1 stoichiometry

Although seminal work implicated the role of both SH3 domains of Grb2 in the recruitment of Sos1 to the inner membrane surface (3, 8, 17), recent studies have shown that only the nSH3 domain binds to Sos1 in an allosteric manner such that the cSH3 domain is freed up for binding to Gab1 so as to generate the Sos1-Grb2-Gab1 ternary signaling complex in a non-competitive fashion (25, 26). Importantly, our previous studies have shown that the

isolated nSH3 domain of Grb2 can potentially bind to peptides derived from all four S1–S4 motifs within Sos1 in a physiologically-relevant manner (26, 32, 33). To understand stoichiometry and the underlying thermodynamic forces driving the formation of Grb2–Sos1 signaling complex, we next measured the binding of full-length Grb2 to the PR domain of Sos1 using ITC (representative data are shown in Figure 4 and all data summarized in Table 2). Our analysis reveals that the formation of Grb2–Sos1 complex is driven by favorable enthalpic changes accompanied by entropic penalty. This implies that specific intermolecular hydrogen bonding and ion pairing interactions predominate over hydrophobic forces in mediating the association of Grb2 with Sos1 in agreement with our previous reports (26, 32, 33).

Of particular note is the observation that Grb2 binds to the wildtype PR domain (PR_WT), containing all four S1–S4 sites, with a 2:1 stoichiometry. In light of previous studies (25, 26), the most straightforward interpretation of this finding is that Grb2 binds in a bivalent manner to Sos1 to form the Grb2–Sos1 complex with a 2:1 stoichiometry and that this interaction is mediated through the binding of nSH3 domain within each Grb2 molecule to only two out of a possible four available S1–S4 sites within Sos1. Given that the PR domain of Sos1 adopts a compact shape and that Grb2 displays the propensity to homodimerize in solution (Figure 2), the 2:1 stoichiometry observed here however may not necessarily be due to the binding of nSH3 domain within each molecule of Grb2 to the PR domain of Sos1. On the contrary, it is highly plausible that the binding of PR domain through one of its four S1–S4 sites to the nSH3 domain of one monomer within Grb2 homodimer sterically blocks the binding of second Grb2 monomer to another PR domain so as to prevent the formation of Grb2–Sos1 complex with a 2:2 stoichiometry. To test the extent to which the formation of Grb2–Sos1 complex with a 2:1 stoichiometry may be influenced by steric hindrance, we next measured the binding of full-length Grb2 to various single, double and triple mutant constructs of the PR domain, mutated with respect to one or more of the S1–S4 sites (Figure 1 and Table 2). Our data show that Grb2 binds to all single-mutant PR constructs (PR_mS1, PR_mS2, PR_mS3 and PR_mS4) with a 2:1 stoichiometry, implying that none of the four S1–S4 sites is critical for the bivalent binding of Grb2 to Sos1. Remarkably, Grb2 binds in a bivalent manner to only four (PR_mS12, PR_mS13, PR_mS23 and PR_mS24) out of the six (PR_mS12, PR_mS13, PR_mS14, PR_mS23, PR_mS24 and PR_mS34) double-mutant PR constructs. This salient observation suggests that bivalent binding of Grb2 to Sos1 can only be afforded by specific pairwise combinations of S1–S4 sites (S1/S4, S1/S3, S2/S4 and S3/S4) but not others (S1/S2 and S2/S3). Close examination of the relative physical location of S1–S4 sites within the PR domain suggests that the length of the intervening linker spanning various pairwise combinations of these sites is likely to play a key role in determining the stoichiometry of Grb2–Sos1 complex (Figure 1a). Thus, while this intervening linker is comprised of 55–133 residues for the pairwise combinations of S1–S4 sites that favor bivalent binding of Grb2 to Sos1 (S1/S4, S1/S3, S2/S4 and S3/S4), it merely spans 23–26 residues for the pairwise combinations that result in the formation of Grb2–Sos1 complex with a 1:1 stoichiometry (S1/S2 and S2/S3). The failure of S1/S2 and S2/S3 pairwise combinations to afford bivalent binding of Grb2 to Sos1 thus most likely results from steric hindrance in that binding of one molecule of Grb2 to S1 site physically blocks the binding of a second molecule of Grb2 to S2 site and vice versa. In a similar manner, binding of one molecule of Grb2 to S2 site would be expected to physically block the binding of a second molecule of Grb2 to S3 site and vice versa. Finally, Grb2 binds to all four triple-mutant PR constructs with a 1:1 stoichiometry (PR_mS123, PR_mS124, PR_mS134 and PR_mS234). This finding strongly argues that the formation of Grb2–Sos1 complex with a 2:1 stoichiometry is not due to steric hindrance and that it strictly requires the integrity of at least two native sites in one of the four pairwise combinations (S1/S4, S1/S3, S2/S4 and S3/S4).

Role of facilitated diffusion in the formation of Grb2-Sos1 complex

Within the living cell, multivalent binding has evolved as a common mechanism to augment the affinity and specificity of macromolecular interactions. Such enhancement is possible because intermolecular binding at one site not only increases the effective local concentration at the second site but subsequent intramolecular binding also lowers entropic penalty. Interestingly, our ITC data suggest that binding of Grb2 to two or more sites within the PR domain of Sos1 does not necessarily have to be mutually inclusive in order to enhance the affinity and specificity of macromolecular interactions (Table 2). Thus, for example, Grb2 binds in a monovalent manner to the PR_mS14 double-mutant construct with an affinity that is comparable to that observed for bivalent binding to PR_mS12, PR_mS13, PR_mS23 and PR_mS24 double-mutant constructs and, apparently, only marginally weaker than affinities associated with bivalent binding to the wildtype (PR_WT) and single-mutant constructs (PR_mS1, PR_mS2, PR_mS3 and PR_mS4). In other words, bivalent binding seemingly does not result in any enhancement in binding affinity relative to monovalent binding of Grb2 to PR_mS14 construct containing two native sites. That this is so is further corroborated by the observation that the monovalent binding of Grb2 to the PR_mS34 double-mutant construct is stronger than bivalent binding to any of the PR constructs analyzed here. In a remarkable contrast, our analysis on triple-mutant constructs of the PR domain (PR_mS123, PR_mS124, PR_mS134 and PR_mS234) reveals that monovalent binding of Grb2 is up to an order of magnitude weaker relative to bivalent binding to PR constructs with two or more potential binding sites. The observation that monovalent binding is weaker than bivalent binding when the number of potential sites within the PR constructs is reduced to one but comparable and somewhat stronger than bivalent binding when more than one potential site is available unequivocally implicates the role of facilitated diffusion in mediating Grb2-Sos1 interaction.

In thermodynamic terms, Grb2 appears to be in equilibrium exchange between a bound state in association with the PR domain and a free state in solution. The presence of multiple binding sites within the PR domain likely facilitates hopping of Grb2 between these sites in a flip-flop fashion and such physically-mediated rapid exchange, as opposed to slow random diffusion in solution, accounts for high-affinity binding. In the presence of only one potential site within the PR domain, this equilibrium exchange occurs slowly due to the fact that as Grb2 dissociates from one molecule of PR domain, the random search for a second potential site to re-associate will likely be met by a separate molecule of PR domain and, such a scenario will slow the exchange rate between the bound state and free state, thereby drastically reducing the affinity of Grb2 to PR constructs which contain only a single potential binding site. In contrast, when more than one potential site within the PR domain is available, dissociation from one site will likely result in rapid re-association or exchange with a second potential site due to its spatial proximity as well as due to enhanced collisional probability through facilitated diffusion leading to high-affinity binding. However, if the two potential sites are not in steric hindrance with respect to spatial accessibility of Grb2, then binding will likely occur in a bivalent manner, wherein the exchange between the bound and free states will also be rapid due to the reasons discussed above. Taken collectively, our data suggest that bivalent binding is not a pre-requisite for an enhancement in the affinity of binding and that monovalent binding through a flip-flop mechanism can also account for such synergy given the availability of multiple binding sites so as to increase the exchange rate through facilitated diffusion. Strikingly, our data also suggest that ligand binding to two potential sites through monovalent binding in a flip-flop manner may be accompanied by higher affinity than bivalent binding. We believe that this scenario can be accounted for by the unfavorable entropic factors associated with bivalent binding but not monovalent binding through a flip-flop mechanism.

Bivalent binding of Grb2 to Sos1 is governed by positive cooperativity

Our ITC data suggest that bivalent binding of Grb2 to multiple sites within the PR domain of Sos1 results in enhancement of binding affinity by up to an order of magnitude relative to monovalent binding in the context of the availability of a single site (Table 2). That this is so strongly implicates the role of positive cooperativity in driving the assembly of Grb2-Sos1 signaling complex. In other words, binding at one site enhances binding at a second site in a synergistic manner such that the overall free energy associated with binding at both sites is greater than the sum of free energies associated with binding at each site individually. In order to further shed light on the nature of these cooperative interactions, we calculated the free energy of cooperativity ($\Delta\Delta G_c$) associated with bivalent binding at each potential pairwise combinations of S1–S4 sites within the PR domain as well as the underlying enthalpic ($\Delta\Delta H_c$) and entropic ($T\Delta\Delta S_c$) components associated with such cooperativity (Table 3). Our analysis reveals that $\Delta\Delta G_c$ associated with bivalent binding of Grb2 to the PR domain ranges from -1.36 to -3.36 kcal/mol. More importantly, the origin of this favorable $\Delta\Delta G_c$ lies in both enthalpic and entropic components. For example, bivalent binding at S1/S4, S1/S3 and S3/S4 sites within the PR_WT construct is driven by favorable $\Delta\Delta H_c$ accompanied by an unfavorable $T\Delta\Delta S_c$, while bivalent binding at S2/S4 sites is favored by both $\Delta\Delta H_c$ and $T\Delta\Delta S_c$. On the other hand, bivalent binding at S2/S4 and S1/S4 sites respectively within the PR_mS13 and PR_mS23 constructs is largely governed by favorable $T\Delta\Delta S_c$ accompanied by an unfavorable $\Delta\Delta H_c$. These salient observations indicate that the positive cooperativity associated with bivalent binding of Grb2 to the PR domain of Sos1 arises through both the formation of additional intermolecular contacts (favorable enthalpy) as well as burial of additional hydrophobic surfaces (favorable entropy). We also note that the favorable entropic contributions to the overall free energy of cooperativity are also likely to arise from the rapid exchange of Grb2 between multiple sites within the PR domain through facilitated diffusion. This invokes the possibility that although Grb2 can only occupy a maximum of two sites within the PR domain at any one moment, non-occupied sites are likely involved in modulating this protein-protein interaction.

Structural basis of the binding of Grb2 to the PR domain of Sos1 as two independent monomers versus a homodimer

Our thermodynamic data suggest that the bivalent binding of Grb2 to Sos1 with a 2:1 stoichiometry is under cooperative control in that the binding of nSH3 domain within one Grb2 molecule to one of the S1–S4 sites within Sos1 facilitates the binding of nSH3 domain within a second Grb2 molecule. On the other hand, our hydrodynamic data indicate that Grb2 exists in a monomer-dimer equilibrium in solution. In light of these observations, it is thus conceivable that Grb2 could bind to the PR domain of Sos1 either as two independent monomers (Grb2-PR-Grb2), or alternatively as a homodimer ($[\text{Grb2}]_2\text{-PR}$). To gain insights into the structural basis of the binding of Grb2 to Sos1 as two independent monomers and to compare this mode of binding to that of a homodimer, we built appropriate structural models of Grb2 bivalently bound to PX Ψ PXR motifs located within the S1 and S4 sites in the PR domain of Sos1 (Figure 5). Our structural models suggest that the canonical hydrophobic grooves within the nSH3 domains of Grb2 that accommodate the PX Ψ PXR motifs are fully exposed to solution in the context of both the monomers (Figure 5a) and homodimer (Figure 5b), implying that it is indeed physically feasible for Grb2 to bind to the PR domain both as two independent monomers or as a homodimer. Importantly, the monomers within Grb2 homodimer adopt a two-fold axis of symmetry such that the SH2 domain of one monomer docks against the cSH3 domain of the other and vice versa in a head-to-tail fashion as observed in the crystal structure determined by Ducruix and co-workers (54).

Our structural modeling also reveals that each PX Ψ PXR motif located within S1 and S4 sites latches onto the hydrophobic groove running parallel to the RT loop within the β -barrel fold

of each nSH3 domain and adopts a left-handed PPII-helical conformation in a canonical fashion (27–30). Within each nSH3 hydrophobic groove, the PX ψ PXR motifs are stabilized by an extensive network of intermolecular contacts as reported earlier (26, 32, 33). In particular, the aliphatic sidechains of consensus ψ residue within S1 (V1153) and S4 (V1292) sites are sandwiched between benzyl rings of F9 and Y52 located within each nSH3 hydrophobic groove through van der Waals contacts. Additionally, the guanidine moieties of consensus arginine within S1 (R1156) and S4 (R1295) sites hydrogen bond and/or ion pair with the carboxylic groups of D15 located within each nSH3 hydrophobic groove. Importantly, the aliphatic sidechains of R1156 (S1) and R1295 (S4) are further stabilized through van der Waals contacts with the indole moieties of W36 located within each nSH3 hydrophobic groove.

We add that although the spatial orientations of both PX ψ PXR motifs relative to the nSH3 domains within Grb2 monomers or homodimer can be relied upon with a high degree of confidence at atomic level, due to the fact that they were modeled on the basis of high sequence identity with corresponding templates, there is a high probability of uncertainty in the relative orientation and conformation of the intervening polypeptide chain spanning S1 and S4 sites within the PR domain of Sos1. Notably, this intervening polypeptide chain was folded into a compact globular-like topology, in agreement with our hydrodynamic data, on the basis of *ab initio* modeling without any template. Despite such shortcomings, our structural models lend physical insights into the bivalent binding of Grb2 adaptor to Sos1 guanine nucleotide exchange factor and provide a much anticipated structural framework for further understanding the assembly of this key signaling complex.

MD simulations support the binding of Grb2 to the PR domain of Sos1 as a homodimer in lieu of two independent monomers

In an attempt to test the validity of our structural models and to shed light on macromolecular dynamics underlying the assembly of Grb2-Sos1 signaling complex, we performed MD simulations on structural models of Grb2 bivalently bound to PX ψ PXR motifs located within the S1 and S4 sites in the PR domain of Sos1 either as two independent monomers (Grb2-PR-Grb2), or alternatively as a homodimer ([Grb2]₂-PR) (Figure 6). Importantly, we assessed the stability and dynamics of various complexes and their constituent components in terms of both the root mean square deviation (RMSD) of backbone atoms as a function of simulation time and root mean square fluctuation (RMSF) of backbone atoms as a function of residue number along each protein chain. As shown in Figure 6a, the MD trajectories reveal that while the [Grb2]₂-PR complex reaches structural equilibrium with an RMSD of $\sim 7\text{\AA}$ after about 30ns, the Grb2-PR-Grb2 complex displays structural instability with a continually increasing RMSD of greater than $15\text{\AA}$ even after 50ns of simulation time. Simply put, these observations strongly argue that the Grb2 homodimer bound to PR domain of Sos1 is structurally more stable than Grb2 monomers.

To understand the origin of such differential structural stabilities, we next deconvoluted the overall RMSD of each complex into its three constituent components: the PR domain of Sos1 and Grb2 monomers, designated MonA and MonB. Remarkably, our analysis reveals that the PR domain and Grb2 monomers individually are also much more structurally stable in the context of [Grb2]₂-PR complex versus the Grb2-PR-Grb2 complex. Thus, while Grb2 monomers in the context of a Grb2 homodimer bound to the PR domain display relatively high stability with an RMSD of $\sim 1.5\text{\AA}$ over the entire course of MD simulation, they appear to be unstable with an RMSD of $\sim 3\text{\AA}$ when bound to the PR domain as two independent monomers. Additionally, while the PR domain in the context of [Grb2]₂-PR complex reaches structural equilibrium with an RMSD of $\sim 10\text{\AA}$ after about 30ns, the PR domain in the context of Grb2-PR-Grb2 complex remains structurally unstable with an RMSD in excess of $15\text{\AA}$. The distinctly higher structural stabilities of the PR domain of Sos1 and Grb2

monomers in the context of [Grb2]₂-PR complex are further demonstrated through our RMSF analysis (Figure 6b), wherein the distribution of atomic fluctuations within each residue of corresponding protein chain is monitored over the entire course of MD simulation. Thus, while a majority of residues within Grb2 monomers in the context of [Grb2]₂-PR complex fluctuate with an RMSF of less than 2 Å, this value rises to greater than 5 Å in the context of Grb2-PR-Grb2 complex. Strikingly, our RMSF analysis also reveals that the residues within the PR domain of Sos1 in the context of [Grb2]₂-PR complex display lower fluctuations than those in the context of Grb2-PR-Grb2 complex (Figure 6c).

We note that one possibility for the rather large distance deviations and fluctuations observed for the Grb2-PR-Grb2 complex may be due to the highly flexible nature of the PR domain tethering the Grb2 monomers. Additionally, Grb2 monomers in isolation are also likely to be more dynamic than in the context of Grb2 dimer alone or when bound to the PR domain. Our previous studies have indeed shown that Grb2 monomers most likely undergo structural rearrangement, with respect to the spatial orientation of various domains within the nSH3-SH2-cSH3 modular cassette, so as to adopt a conformation that is somewhat topologically distinct and thermodynamically more stable from that observed in the context of Grb2 homodimer (34). In an attempt to overcome the additional instability introduced by the rather large intervening linker (~130 residues) spanning S1/S4 sites within the PR domain, we also performed MD simulations on Grb2 monomers in isolation and in the context of Grb2 dimer in complex with PR polypeptides spanning S1/S2 and S2/S3 sites, which harbor rather shorter intervening linkers (~20 residues). Our results nonetheless reach a similar conclusion — both the PR domain and Grb2 monomers display higher structural stability within the [Grb2]₂-PR complex versus the Grb2-PR-Grb2 complex. Taken together, our MD analysis suggests that the [Grb2]₂-PR complex is structurally more stable than the Grb2-PR-Grb2 complex, though the possibility that Grb2 may bind to the PR domain as isolated monomers equally as effectively as a homodimer cannot be excluded.

CONCLUSIONS

In response to mitogenic stimulation of RTKs, the Grb2-Sos1 interaction facilitates the activation of Ras GTPase and Akt kinase that in turn drive a plethora of cellular signaling cascades central to health and disease (1–4). Despite such urgency, the assembly of Grb2-Sos1 signaling complex remains poorly understood in mechanistic terms. Although structural studies provided insights into how the nSH3 domain of Grb2 recognizes the PX Ψ PXR motifs within Sos1 over a decade ago (27–31), further progress in elucidating how these two proteins come together has been largely hampered by the flexible nature of the PR domain of Sos1. Herein, our biophysical analysis sheds new light into the assembly of Grb2-Sos1 signaling complex at macromolecular level.

We have demonstrated here that the formation of Grb2-Sos1 signaling complex occurs with a 2:1 stoichiometry in agreement with previous studies reported by Samelson and co-workers (63). Of particular note is the observation that Grb2 binds in a bivalent manner to S1/S4, S1/S3, S2/S4 and S3/S4 pairwise combination of sites within the PR domain of Sos1 but not to S1/S2 and S2/S3. We attribute the inability of Grb2 to bind to S1/S2 and S2/S3 pairwise combination of sites in a bivalent manner to the physical constraints arising from the rather shorter length of the intervening linker spanning these sites relative to other pairwise combinations. Nonetheless, the demonstration that the bivalent binding of Grb2 to Sos1 through a variety of pairwise combinations of S1–S4 sites is feasible strongly argues that conformational heterogeneity may be a hallmark of Grb2-Sos1 signaling complex — formation of conformationally-distinct complexes through the employment of distinct pairwise combinations of S1–S4 sites. We believe that such conformational heterogeneity may be an important regulator of the cellular signaling cascades under the control of Grb2-

Sos1 complex. Thus, for example, binding of Grb2 to one or more of the S1–S4 sites may be blocked through post-translational modification of these sites within certain tissue types, which in turn could provide a mechanism to gauge the conformational heterogeneity of Grb2-Sos1 complex. Alternatively, binding of other cellular partners within the vicinity of S1–S4 sites may also sterically hinder the extent to which Grb2 can recognize these sites and thereby fine-tune the degree of conformational heterogeneity of Grb2-Sos1 complex. More importantly, within the living cell, multivalent binding has evolved as a common mechanism to augment the affinity and specificity of macromolecular interactions. Remarkably, our data suggest that bivalent binding is not a pre-requisite for an enhancement in the affinity of binding and that monovalent binding in a flip-flop fashion can also lead to such enhancement given the availability of multiple binding sites so as to increase the exchange rate through facilitated diffusion. Our structural models in combination with MD simulations suggest that although it is physically feasible for Grb2 to bind to Sos1 as two independent monomers, it most likely does so as a homodimer. This scenario is further consistent with our finding that the formation of Grb2-Sos1 complex is governed by positive cooperativity — binding of nSH3 domain within one monomer of Grb2 to Sos1 facilitates binding of nSH3 domain within the second monomer of Grb2.

In short, our study provides new insights into the assembly of a key protein-protein interaction central to cellular machinery and argues for a better understanding of the role of multivalent interactions and flip-flop binding coupled with facilitated diffusion and cooperativity in driving cellular signaling cascades central to health and disease. Importantly, current strategies for the rationale development of next-generation therapies are based on tethering two potential inhibitors with the intent to enhance target specificity. Our findings presented here suggest that the design of drugs that can potentially exploit the flip-flop mechanism may provide an alternative and perhaps even more robust strategy for the treatment of disease with more streamlined efficacy coupled with low toxicity.

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ABBREVIATIONS

ALS	Analytical light scattering
CD	Circular dichroism
DLS	Dynamic light scattering
EGF	Epidermal growth factor
Gab1	Grb2-associated binder 1
Grb2	Growth factor receptor binder 2
ITC	Isothermal titration calorimetry
LIC	Ligation-independent cloning
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MD	Molecular dynamics
MM	Molecular modeling

PDGF	Platelet-derived growth factor
PPII	Polyproline type II
PR	Proline-rich
RTK	Receptor tyrosine kinase
SEC	Size-exclusion chromatography
SH2	Src homology 2
SH3	Src homology 3
SLS	Static light scattering
Sos1	Son of sevenless 1

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Figure 1.

Domain organization of Sos1 and the maps of various constructs of its proline-rich (PR) domain. (a) The PR domain of Sos1 lies at the extreme C-terminal end and contains four distinct sites, herein designated S1, S2, S3 and S4, characterized by the presence of PX Ψ PXR consensus motif. The complete sequences of these four sites are shown. The position of various residues relative to the first proline within the PX Ψ PXR motif, which is designated zero, is also indicated. Other domains within Sos1 are HF (histone fold), DH (Dbl homology), PH (pleckstrin homology), REM (Ras exchange motif) and Cdc25. (b) Maps of wildtype (WT) and various mutant constructs of the PR domain of Sos1 used in this study. The wildtype PR construct (PR_WT) contains all four S1–S4 motifs. In the single-

mutant PR constructs (PR_mS1, PR_mS2, PR_mS3 and PR_mS4), one of the four S1–S4 motifs is disrupted individually with the mutated site indicated by the prefix m. In contrast, the double-mutant PR constructs (PR_mS12, PR_mS13, PR_mS14, PR_mS23, PR_mS24 and PR_mS34) contain pairwise disruptions of all possible combinations of S1–S4 motifs. Finally, the triple-mutant PR constructs (PR_mS123, PR_mS124, PR_mS134 and PR_mS234) were designed to disrupt all but one of the S1–S4 motifs. Note that the disruption of each of the four S1–S4 motifs, as indicated by a saltire (X), was achieved through alanine substitution of ψ and arginine residues within the corresponding PX ψ PXR consensus sequence, the integrity of which is required for their binding to the nSH3 domain of Grb2. The numerals at the ends of each construct indicate amino acid sequence number within human Sos1.

Figure 2.

ALS analysis of full-length Grb2 and the PR domain of Sos1 (Sos1_PR). (a) Elution profiles as monitored by the differential refractive index (Δn) plotted as a function of elution volume (V) for Grb2 (top panel) and Sos1_PR (bottom panel). Note that Grb2 elutes as two distinct species corresponding to a dimer and a monomer, while Sos1_PR domain elutes as a single monomeric species. (b) Partial Zimm plots obtained from analytical SLS measurements at a specific protein concentration for Grb2 dimer and monomer (top panel) and Sos1_PR monomer (bottom panel). The solid lines through the data points represent linear fits. (c) Autocorrelation function plots obtained from analytical DLS measurements at a specific protein concentration for Grb2 dimer and monomer (top panel) and Sos1_PR monomer (bottom panel). The solid lines through the data points represent non-linear least squares fits to Eq [6].

Figure 3. Secondary structure analysis of the PR domain of Sos1. (a) In silico prediction of intrinsic disorder within the PR domain. (b) Experimentally-determined far-UV CD spectrum of the PR domain.

Figure 4.

ITC analysis of the binding of full-length Grb2 to PR_WT wildtype construct (a), PR_mS4 single-mutant construct (b), PR_mS34 double-mutant construct (c) and PR_mS234 triple-mutant construct (d) of Sos1. The upper panels show raw ITC data expressed as change in thermal power with respect to time over the period of titration. In the lower panels, change in molar heat is expressed as a function of molar ratio of the corresponding PR construct to Grb2. The solid lines in the lower panels show the fit of data to a one-site model, as embodied in Eq [11], using the ORIGIN software.

Figure 5.

Structural models of Grb2 bound to S1/S4 sites within the PR domain of Sos1 either as two isolated monomers, Grb2-PR-Grb2 (a), or in the context of a homodimer, [Grb2]₂-PR (b). One monomer of Grb2 is shown in green (MonA) and the other in blue (MonB). The PR domain of Sos1 is colored brown. The Grb2-Sos1 interfaces between the hydrophobic grooves within the nSH3 domains of Grb2 monomers accommodating the PX ψ PXR motifs within S1/S4 sites are marked by dashed circles. The sidechain moieties of ψ and arginine residues within the PX ψ PXR motifs at S1/S4 sites are colored yellow. The sidechain moieties of residues within the nSH3 domains of Grb2 that interact with ψ and arginine residues within the PX ψ PXR motifs are colored red.

Figure 6. MD analysis of Grb2 bound to S1/S4 sites within the PR domain of Sos1 either as two independent monomers (Grb2-PR-Grb2) or in the context of a homodimer ([Grb2]₂-PR). (a) Root mean square deviation (RMSD) of backbone atoms (N, C α and C) within each simulated structure relative to the initial modeled structures of Grb2-PR-Grb2 (top panel) and [Grb2]₂-PR (bottom panel) complexes as a function of simulation time. Note that the overall RMSD for each complex (black) is deconvoluted into PR domain (brown) and each of the two Grb2 monomers, designated MonA (green) and MonB (blue). (b) Root mean square fluctuation (RMSF) of backbone atoms (N, C α and C) averaged over the entire course of corresponding MD trajectories of Grb2-PR-Grb2 (top panel) and [Grb2]₂-PR (bottom panel) complexes as a function of residue number within each of the two Grb2 monomers, designated MonA (green) and MonB (blue). (c) Root mean square fluctuation (RMSF) of backbone atoms (N, C α and C) averaged over the entire course of corresponding MD trajectories of Grb2-PR-Grb2 (top panel) and [Grb2]₂-PR (bottom panel) complexes as a function of residue number within PR domain (brown). Note that the vertical arrows indicate the location of S1 and S4 motifs within the PR domain.

Hydrodynamic parameters obtained from ALS measurements for full-length Grb2 and various constructs of the PR domain of Sos1

Table 1

	Associativity	M _{cal} (kD)	M _w (kD)	M _n (kD)	M _w /M _n	D _t (μm ² .s ⁻¹)	R _h (Å)
Grb2	Monomer	30	31 ± 1	30 ± 2	1.02 ± 0.03	63 ± 1	39 ± 1
	Dimer	60	64 ± 3	63 ± 4	1.01 ± 0.01	45 ± 1	55 ± 2
PR_WT	Monomer	22	22 ± 1	22 ± 1	1.00 ± 0.01	53 ± 1	46 ± 1
PR_mS1	Monomer	22	21 ± 1	21 ± 1	1.00 ± 0.01	54 ± 1	45 ± 1
PR_mS2	Monomer	22	25 ± 1	25 ± 2	1.01 ± 0.01	54 ± 2	45 ± 1
PR_mS3	Monomer	22	26 ± 1	25 ± 2	1.03 ± 0.04	50 ± 4	48 ± 3
PR_mS4	Monomer	22	22 ± 1	22 ± 2	1.03 ± 0.04	57 ± 4	44 ± 2
PR_mS12	Monomer	22	27 ± 1	26 ± 2	1.01 ± 0.01	59 ± 1	42 ± 1
PR_mS13	Monomer	22	24 ± 1	23 ± 1	1.05 ± 0.01	58 ± 1	43 ± 1
PR_mS14	Monomer	22	24 ± 1	23 ± 2	1.03 ± 0.03	58 ± 3	42 ± 1
PR_mS23	Monomer	22	26 ± 1	26 ± 1	1.01 ± 0.01	56 ± 1	44 ± 1
PR_mS24	Monomer	22	24 ± 1	24 ± 1	1.01 ± 0.02	57 ± 2	43 ± 1
PR_mS34	Monomer	22	25 ± 1	24 ± 1	1.01 ± 0.01	54 ± 1	45 ± 1
PR_mS123	Monomer	22	26 ± 1	25 ± 1	1.03 ± 0.03	49 ± 2	50 ± 2
PR_mS124	Monomer	22	22 ± 1	22 ± 2	1.01 ± 0.01	55 ± 4	44 ± 3
PR_mS134	Monomer	22	23 ± 2	22 ± 3	1.04 ± 0.05	53 ± 3	46 ± 2
PR_mS234	Monomer	22	22 ± 1	22 ± 1	1.02 ± 0.02	51 ± 2	47 ± 1

Note that M_{cal} is the molar mass of each recombinant construct calculated from the corresponding amino acid sequence alone. Errors were calculated from at least three independent measurements. All errors are given to one standard deviation.

Thermodynamic parameters obtained from ITC measurements for the binding of wildtype (WT) and mutant constructs of the PR domain of Sos1 to full-length Grb2

Table 2

	n	K _d (μM)	ΔH (kcal.mol ⁻¹)	TΔS (kcal.mol ⁻¹)	ΔG (kcal.mol ⁻¹)
PR_WT	2.12 ± 0.03	7.02 ± 0.04	-39.85 ± 0.28	-25.77 ± 0.29	-14.08 ± 0.01
PR_mS1	1.94 ± 0.01	8.99 ± 0.51	-39.81 ± 0.59	-26.03 ± 0.66	-13.78 ± 0.07
PR_mS2	2.02 ± 0.05	6.98 ± 0.30	-34.11 ± 0.25	-20.03 ± 0.20	-14.08 ± 0.05
PR_mS3	2.04 ± 0.02	2.40 ± 0.03	-38.39 ± 0.33	-23.04 ± 0.32	-15.35 ± 0.01
PR_mS4	2.01 ± 0.02	6.18 ± 0.27	-38.17 ± 0.10	-23.94 ± 0.05	-14.23 ± 0.05
PR_mS12	2.05 ± 0.04	19.57 ± 0.27	-37.06 ± 0.11	-24.20 ± 0.09	-12.86 ± 0.02
PR_mS13	2.14 ± 0.02	10.14 ± 0.05	-36.55 ± 0.08	-22.91 ± 0.08	-13.64 ± 0.01
PR_mS14	1.14 ± 0.01	15.48 ± 0.10	-17.55 ± 0.22	-11.06 ± 0.21	-6.57 ± 0.01
PR_mS23	2.00 ± 0.01	12.67 ± 0.15	-34.56 ± 0.04	-21.18 ± 0.05	-13.37 ± 0.01
PR_mS24	1.89 ± 0.02	11.76 ± 0.70	-32.52 ± 0.64	-19.05 ± 0.71	-13.46 ± 0.07
PR_mS34	1.00 ± 0.01	2.29 ± 0.12	-17.53 ± 0.42	-9.82 ± 0.44	-7.70 ± 0.03
PR_mS123	1.07 ± 0.05	37.28 ± 3.62	-20.09 ± 0.03	-14.04 ± 0.03	-6.05 ± 0.06
PR_mS124	1.03 ± 0.04	101.44 ± 2.04	-14.61 ± 0.13	-9.15 ± 0.12	-5.45 ± 0.01
PR_mS134	1.05 ± 0.01	29.98 ± 1.65	-18.14 ± 0.25	-11.96 ± 0.28	-6.18 ± 0.03
PR_mS234	1.03 ± 0.02	44.96 ± 1.29	-16.88 ± 0.12	-10.94 ± 0.10	-5.94 ± 0.02

Note that n is the stoichiometry of full-length Grb2 bound to each PR construct. Errors were calculated from at least three independent measurements. All errors are given to one standard deviation.

Thermodynamic parameters associated with cooperative binding of full-length Grb2 to wildtype (WT) and various mutant constructs of the PR domain of Sos1 with a 2:1 stoichiometry

Table 3

	Bivalent Sites	$\Delta\Delta H_c$ (kcal.mol ⁻¹)	$T\Delta\Delta S_c$ (kcal.mol ⁻¹)	$\Delta\Delta G_c$ (kcal.mol ⁻¹)
PR_WT	S1/S4	-2.88	-0.79	-2.09
	S1/S3	-8.36	-5.68	-2.69
	S2/S4	-1.62	+0.23	-1.85
	S3/S4	-5.15	-2.58	-2.58
PR_mS1	S2/S4	-1.58	-0.03	-1.55
	S3/S4	-5.11	-2.84	-2.28
PR_mS2	S1/S4	+2.86	+4.95	-2.09
	S1/S3	-2.62	+0.06	-2.69
	S3/S4	+0.59	+3.16	-2.58
PR_mS3	S1/S4	-1.42	+1.94	-3.36
	S2/S4	-0.16	+2.96	-3.12
PR_mS4	S1/S3	-6.68	-3.85	-2.84
PR_mS12	S3/S4	-2.36	-1.01	-1.36
PR_mS13	S2/S4	+1.68	+3.08	-1.41
PR_mS23	S1/S4	+2.41	+3.80	-1.38
PR_mS24	S1/S3	-1.03	+1.04	-2.07

Note that thermodynamic parameters associated with cooperativity are provided for all observed pairwise combinations of S1–S4 sites within each PR construct to which Grb2 may bind in a bivalent manner.