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Regions outside of conserved PxxPxR motifs drive the high affinity interaction of GRB2 with SH3 domain ligands

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ABSTRACT

SH3 domains are evolutionarily conserved protein interaction domains that control nearly all cellular processes in eukaryotes. The current model is that most SH3 domains bind discreet PxxPxR motifs with weak affinity and relatively low selectivity. However, the interactions of full-length SH3 domain-containing proteins with ligands are highly specific and have much stronger affinity. This suggests that regions outside of PxxPxR motifs drive these interactions. In this study, we observed that PxxPxR motifs were required for the binding of the adaptor protein GRB2 to short peptides from its ligand SOS1. Surprisingly, PxxPxR motifs from the proline rich region of SOS1 or CBL were neither necessary nor sufficient for the in vitro or in vivo interaction with full-length GRB2. Together, our findings show that regions outside of the consensus PxxPxR sites drive the high affinity association of GRB2 with SH3 domain ligands, suggesting that the binding mechanism for this and other SH3 domain interactions may be more complex than originally thought.

1. Introduction

SH3 domain-containing proteins regulate a large number of cellular functions in humans from signal transduction to metabolism to differentiation [1,2]. SH3 domains are evolutionally conserved in eukaryotes from budding yeast, which produce 28 proteins with SH3 domains, to humans, which express over 300 proteins with SH3 domains [1,3]. Although sequence conservation is relatively low, SH3 domains have a conserved structure of 5 or 6 beta strands that form two anti-parallel beta sheets. The current model is that vast majority of SH3 domains bind to discrete sequences of (R/K)xPxxP (class I) or PxxPx(R/K) (class II) in a shallow groove between the beta sheets [2,3]. Peptide ligands containing these motifs associate in vitro with isolated SH3 domains with relatively weak affinities, ranging from 1 μM to 1 mM [2,4]. There are other atypical interaction motifs that associate with a small number of SH3 domains. The most well studied of these is these atypical interactions are class III binding reactions driven by RxxK motifs. In contrast to class I or class II SH3 domain binding reactions, the affinity of class III SH3 domain interaction appears to be much stronger [1,3,5]. In addition to relatively low affinities, isolated SH3 domains also have variable specificity for peptide ligands. Some isolated domains have relatively high specificity for individual ligands, notably the class III C-terminal SH3 domain of GADS and RxxK peptides from SLP-76, but the majority of class I and class II SH3 domains bind multiple PxxP containing peptides [2,4]. Based primarily on studies using isolated domains and peptide ligands, the current model is that SH3 domains are evolutionally and structurally conserved protein interaction domains that have weak affinity and low specificity for PxxP containing ligands.

In contrast to isolated SH3 domain/peptide interactions, the binding of full-length SH3 domain containing proteins to larger regions of their ligands has substantially stronger affinity. The binding affinity of the isolated C-terminal SH3 domain of GADS to a peptide derived from SLP-76 was ~250 nM, while the interaction of full-length GADS with the complete proline-rich region of SLP-76 is ~10-fold stronger [5,6]. Similarly, the SH3 domain of p67PHOX binds with 1000-fold increased affinity to a 32 amino acid peptide derived from p47PHOX compared to shorter peptides due to molecular interactions outside of the PxxP motif [7,8]. Finally, peptides derived from PxxP motifs found in SOS1interact with individual SH3 domains from GRB2 with affinities ranging from 20 μM to 1 mM [9–13], whereas the association of full-length GRB2 with full-length SOS1 or the complete proline rich regions (PRR) of SOS1 and CBL has affinities of 300–400 nM [14–16]. Together, these studies suggest that the interaction of full-length SH3 domain-containing proteins with in vivo ligands may have substantially stronger affinity than previously thought due to interactions outside of the conserved PxxP motifs.

Abbreviations: DLS, dynamic light scattering; ITC, isothermal titration calorimetry; PRR, proline rich region; TIRF, total internal reflection fluorescence.

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To more fully address this prediction, we utilized quantitative biophysical and imaging techniques to examine the binding of GRB2 to its physiological ligands SOS1 and CBL. GRB2 is an adaptor protein with a central SH2 domain, which binds to phosphorylated proteins, and two flanking SH3 domains [17,18]. GRB2 facilitates the interaction of phosphorylated receptors and adaptor proteins with SH3 domain ligands, including the RAS guanine nucleotide exchange factor SOS1 and the E3 ubiquitin ligase, CBL [17,18]. In this study, we found that similar to previous observations PxxPxR motifs were absolutely required for the interaction of full-length GRB2 with peptides derived from the proline rich C-terminal tail of SOS1. In contrast, these same PxxPxR motifs were neither necessary nor sufficient for the high affinity interaction of SOS1 or CBL with full-length GRB2 or required for the in vivo recruitment of SOS1 to the plasma membrane in activated T cells. These studies show conclusively that regions outside of the PxxPxR motifs are critical for the high affinity interaction of GRB2 with full-length ligands.

2. Material and methods

2.1. Protein purification

The bacterial expression constructs for full-length human GRB2, the complete proline rich region of murine SOS1 or human CBL were described previously in [6,15]. Deletions of individual sites in these constructs were performed with the QuickChange II XL Site Directed Mutagenesis kit from Stratagene using standard manufacturer protocol. Rosetta 2 cells expressing 6X-His tagged GRB2 were shaken for 36 h at 25 °C in Superbroth. Rosetta 2 cells expressing 6X-His tagged proline rich regions of SOS1 or CBL were shaken 24–48 h at 37 °C in ZYM-5052 [19]. The cells were pelleted, and pellets were then resuspended in His-tag purification buffer (50 mM $NaPO_4$, 250 mM NaCl, pH 7.4) and lysed using sonication. The supernatant was applied to a Ni^{2+} HisTrap HP affinity column and bound proteins were eluted using imidazole. Proteins were further purified by size exclusion chromatography using a 16/60 Superdex 75 gel filtration column and then concentrated using 10,000 MWCO centrifugal filters. As determined by dynamic light scattering, little aggregation was observed for any protein (data not shown).

2.2. Peptide array

The peptide array was produced as previously described in [20]. The array scans the entire proline rich region of SOS1 (amino acids 1117–1319) and contains 12 amino acid peptides that have 9 amino acid overlaps with the peptides on either side (see key for the array in Supplemental Fig. 1). The membrane was blocked with 5% milk in TBST and then incubated with His-tagged GRB2 for 1.5 h. The amount of bound GRB2 was examined by immunoblotting with an anti-GRB2 antibody diluted in 5% milk as previously described in [21] and the array was visualized by chemiluminescence using a Fuji imager.

2.3. Isothermal titration calorimetry (ITC)

The protein samples were degassed and ITC measurements recorded using a MicroCal VP-ITC System with SOS1 or CBL proline rich regions as the injected sample and GRB2 as the cell sample. For the peptide inhibition studies, GRB2 was incubated with varying molar ratios of a SOS1 site 1 peptide (EVPVPPPVPPRRRPE) purchased from EZ Biolabs. The chamber was kept under constant stirring at 350 rpm and all experiments were performed at 25 °C. Injection of SOS1 or CBL into buffer showed constant heats of dilution. The heat of dilution was determined by averaging the last 3–5 injections and subtracted from the raw value. The data are analyzed using the single site binding model using the Origin ITC analysis package. The values for affinity, stoichiometry and ΔH were averaged from at least four separate injections and statistically analyzed via ANOVA using GraphPad Prism. Outliers in the data were determined via the ROUT method using GraphPad Prism, with Q = 0.1 %.

2.4. Dynamic light scattering

Dynamic light scattering (DLS) data was collected at 25 °C using a DynaPro NanoStar (Wyatt Technology). The data was analyzed using Dynamic (version 7.1.7, Wyatt Technology). Each acquisition was an average of 10 scans and the values for radius and percent polydispersity were averaged from four individual acquisitions. The data was plotted and statistically analyzed via ANOVA with Tukey's multiple comparison test using GraphPad Prism.

2.5. Cell lines and transfections

SOS1 wild-type, SOS1 Δ1234Z, and SOS1 scrambled cDNA were PCR amplified from plasmid constructs and inserted into lentiviral expression vectors (Gift from Dr. Stephen Bunnell). The lentiviral expression vectors and packaging vectors were cotransfected into 293T cells using lipofectamine 2000. After 48 h, the virus-containing medium was removed and virus was concentrated using Lenti-X concentrator (Clontech). Jurkat E6.1 T cells were grown at 37 °C with 5% CO_2 in complete RPMI (RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin). They were transduced with the described vectors and selected using puromycin. YFP expression on stable cell lines was analyzed by flow cytometry using an Accuri C6 flow cytometer.

2.6. Immunoblotting

Proteins in cellular lysates were separated by polyacrylamide gel electrophoresis using 4–15% Criterion Precast polyacrylamide gels. The separated proteins were then transferred to polyvinylidene difluoride and the membrane was blocked for 1 h at room temperature in SEA BLOCK Blocking Buffer. The membranes were incubated overnight at 4 °C with anti-YFP followed by 30 min of incubation at room temperature with the respective secondary antibody. The blots were then developed using the Licor Odyssey Infrared Imager.

2.7. TIRF microscopy

Imaging was performed using a Leica AM TIRF MC imaging system as previously described [22,23]. Briefly, Jurkat E6.1 T cells stably expressing SOS1 wild-type PRR–YFP, SOS1 Δ1234Z PRR–YFP, or YFP alone were activated for 5 min on glass chamber slides coated with 10 μg/ml of anti-CD3 antibody. Cells were fixed with 3% paraformaldehyde and permeabilized with 0.25% Triton X-100. After blocking, cells were stained with anti-phospho LAT Y226 antibodies followed by incubation with secondary antibodies. The YFP fluorescence and LAT phosphorylation occurring at the membrane was imaged using TIRF microscopy. The total YFP fluorescence in the same cells was imaged by EPI fluorescence. All images were acquired using Leica AF software and processed using Fiji software package. To quantify YFP recruitment, the TIRF YFP fluorescence was normalized to the total YFP fluorescence in the EPI fluorescence channel in the imaged cell. The ratio of the values of TIRF YFP fluorescence over total YFP fluorescence in the EPI fluorescence channel was calculated for 40 randomly selected cells for each of the two independent experiments. The individual values and the mean ± 95% confidence intervals were plotted using GraphPad Prism. The statistical analysis was performed using GraphPad Prism via ANOVA analysis.

3. Results

3.1. PxxPxR motifs are required for the binding of GRB2 to peptide ligands

The proline rich C-terminal tail of SOS1 PRR contains four consensus PxxPxR SH3 domain binding motifs, and peptides containing these motifs bind individual GRB2 SH3 domains with affinities ranging from 20 μM to 1 mM [9–13]. Although these studies have shown that GRB2

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