The intramolecular allostery of GRB2 governing its interaction with SOS1 is modulated by phosphotyrosine ligands

Neda S. Kazemein Jasemi, Christian Herrmann, Eva Magdalena Estirado, Lothar Gremer, Dieter Willbold, Luc Brunsveld, Radovan Dvorsky, Mohammad R. Ahmadian

1Institute of Biochemistry and Molecular Biology II, Medical Faculty of the Heinrich-Heine University, 40225 Düsseldorf, Germany
2Department of Physical Chemistry I, Ruhr University Bochum, Bochum, Germany
3Laboratory of Chemical Biology, Department of Biomedical Engineering and Institute for Complex Molecular Systems (ICMS), Eindhoven University of Technology, P.O. Box 513, 5600MB, Eindhoven, The Netherlands
4Institute of Physical Biology, Heinrich Heine University Düsseldorf, 40204 Düsseldorf, Germany
5Institute of Biological Information Processing, Structural Biochemistry (IBI-7), Forschungszentrum Jülich, 52425 Jülich, Germany

Abstract

Growth factor receptor-bound protein 2 (GRB2) is a trivalent adaptor protein and a key element in signal transduction. It interacts via its flanking nSH3 and cSH3 domains with the proline-rich domain (PRD) of the RAS activator SOS1 and via its central SH2 domain with phosphorylated tyrosine residues of receptor tyrosine kinases (RTKs; e.g., HER2). The elucidation of structural organization and mechanistic insights into GRB2 interactions, however, remain challenging due to their inherent flexibility. This study represents an important advance in our mechanistic understanding of how GRB2 links RTKs to SOS1. Accordingly, it can be proposed that (1) HER2 pYP-bound SH2 potentiates GRB2 SH3 domain interactions with SOS1 (an allosteric mechanism); (2) the SH2 domain blocks cSH3, enabling nSH3 to bind SOS1 first before cSH3 follows (an avidity-based mechanism); and (3) the allosteric behavior of cSH3 to other domains appears to be unidirectional, although there is an allosteric effect between the SH2 and SH3 domains.

Keywords: GRB2, HER2, proline-rich domain, proline-rich motif, RAS, SH2, SH3, SOS1

Introduction

Allosteric regulation of multivalent proteins is widespread in a variety of biological processes [1]. In multivalent proteins, the signal can be propagated from one site to other sites by allosteric mechanisms. Allosteric sites facilitate the effector’s binding to the protein often by controlling the conformational changes within the protein enabled by protein dynamics [2, 3]. As the exact mechanism of the allosteric regulation of proteins is still elusive, we aimed to shed light on the allosteric behavior of multivalent growth factor receptor-bound protein 2 (GRB2) and its impact on its interaction with the RAS activator Son of Sevenless 1 (SOS1).

GRB2 is a ubiquitously expressed and evolutionarily conserved adaptor protein [4] that links extracellular signals to a variety of pathways, including the RAS signaling pathways, with
diverse signaling molecules as for instance SOS1 [5]. It is a 25-kDa protein and consists of three protein interaction modules, one SRC homology 2 (SH2) and two terminal SRC homology 3 domains (nSH3 and cSH3) (Fig. 1a). The SH3 domains typically bind to the proline-rich motifs (PRMs) of target proteins, e.g., RAS-specific guanine nucleotide exchange factor (RASGEF) SOS1 and GRB2-associated-binding protein 1 (GAB1) [5-12]. It has been recently shown that the specificity of the SH3 domain interactions is determined by their full-length protein [13]. The SH2 domain specifically binds to tyrosylphosphatase (pY)-containing proteins, such as activated receptor tyrosine kinases (RTKs; e.g., human epidermal growth factor receptor or HER2) and other transmembrane proteins [14-16]. The GRB2 SH2 domain has been shown to bind to a pY-Φ-N-Φ consensus motif (where Φ represents a hydrophobic residue) of activated receptors [17]. The binding of the GRB2-SOS1 complex to the tyrosine phosphorylation sites on cell surface proteins has been proposed to translocate SOS1 to the plasma membrane in the vicinity of RAS. This enables GDP/GTP exchange on RAS, leading to activation of the mitogen-activated protein kinase (MAPK) cascade [18, 19]. The GRB2-GAB1 complex is known to activate the protein tyrosine phosphatase SHP2. The GRB2-GAB1-SHP2 complex downregulates the PI3K pathway and induces RAS activation [20, 21].

The interaction of GRB2 with SOS1 was first described in the early 1990s [22-24]. The first study by Lemmon et al. showed that GRB2 forms a 1:2 complex with a SOS1 peptide (GTDEVPPVVPPRRPES) with a Kea value of 22 µM, and a 1:1 complex with HER1 pYP, with a Kev value of 0.4 µM [24]. This study and Cussac et al. proposed independent ligand binding to the SH2 and SH3 domains of GRB2 [24, 25]. Since then, many different groups have investigated GRB2 interactions with various SOS1 peptides with nanomolar to millimolar binding affinities for GRB2 FL, nSH3, and cSH3 using different methods and varying conditions [9, 26-32]. Moreover, GRB2 nSH3 has been generally appreciated as the main SOS1 binding module, and cSH3 may increase the overall stability of this protein complex. The GRB2-SOS1 interaction has been very recently proposed to induce a closed conformation in nSH3, while the cSH3 conformation remains unchanged [33]. McDonald et al. proposed the formation of the SOS1-GRB2-GAB1 complex [6, 20]. Accordingly, SOS1 binding to nSH3 induces a conformational change in GRB2, allowing GAB1 to access the cSH3 domain in a noncompetitive manner. This means that the association of one molecule GRB2 with its upstream ligands, e.g., HER2 or LAT, leads to the activation of two distinct pathways, namely, the PI3K and MAPK pathways [18-21].

GRB2-SOS1 complex formation requires the interaction of GRB2 SH3 domains with the C-terminal proline-rich domain (PRD) of SOS1 [23, 34]. The SOS1 PRD contains more than ten PRMs (Table S1), most of which do not seem to bind GRB2 SH3 domains [29, 30, 32, 35]. The best-investigated SOS1 PRM is VPVPPPVP (here called as reference peptide 1 or RP1). RP1 has been reported to bind GRB2 nSH3 more tightly than cSH3 [9, 25, 32, 36]. RP1 binding has been recently shown to induce a closed conformation of nSH3 [32]. Loss of the PRM-binding variants of GRB2 SH3 domains (substitution of W36 and/or W193 for K) has been shown to abolish the critical role of GRB2 in ERK activation via the SOS1-RAS-RAF-MEK axis [37, 38]. These mutations disrupt nSH3/cSH3 binding to SOS1. Thus, the GRB2-SOS1 interaction is a key step toward proliferation in normal and cancer cells [39]. Targeting the GRB2–SOS1 interaction has been suggested to offer new avenues for future therapeutic strategies for upstream mutations in cancer, such as in EGFR [35].

The notion that protein function is allosterically regulated by structural or dynamic changes in proteins has been extensively investigated for several proteins in solution [1]. A quantitative description of the communication between two distinct sites in a multivalent protein is still very challenging. In the case of SH3 domains of GRB2, while they have often been associated with allosteric mechanisms, the intradomain communication between residues has been poorly explored to date [40]. In this study, we investigated GRB2 and its interaction with proline-rich peptides of SOS1 in the presence and absence of HER2 phosphotyrosine peptide (pYP) (Table S1). Our data clearly indicate allosteric modulation of the GRB2-SOS1
interaction by HER2 pYP and a cooperative mechanism between cSH3 and nSH3 regarding binding to SOS1.

**Materials and Methods**

**Peptides.** Peptides used in this study are listed in [Table S1](#5890;).

**Constructs.** Constructs used in this study are listed in [Table S2](#).

**Proteins.** All proteins used in this study are listed in [Table S2](#).

**GST pull-down assay.** Pull-down of the SOS1 PRD was performed with GST fusion proteins of various GRB2 variants as previously described [41]. Purified GST was used as a negative control. Briefly, 20 µM GST-GRB2 FL and the respective SH3 domains were incubated with 30 µM SOS1 PRD and GSH Sepharose beads (GE Healthcare, UK) in a buffer containing 30 mM Tris-HCl at pH 7.5, 3 mM dithiothreitol, and 5 mM MgCl₂ for 1 h, at 4°C. After washing three times with the same buffer, the samples were denatured in Laemmli buffer for 10 min at 95°C and analyzed by SDS-PAGE and immunoblotting using a polyclonal anti-SOS1 antibody ([#5890; Cell Signaling Technology](#)).

**Fluorescence dot-blot analysis.** Pull-down of 10 µM FITC-labeled peptides with 5 µM GST fusion proteins of various GRB2 variants was performed under the same conditions as described for the GST pull-down assay. Purified GST was used as a negative control. After washing three times, bound proteins were eluted by incubating in the same buffer containing 20 mM reduced glutathione (GSH) for 15 min at 4°C, and the beads were separated by centrifugation. Bound FITC-labeled peptides were detected by dot blot analysis using 1 µL eluent at an emission wavelength of 600 nm and an Odyssey Fc imaging system (LI-COR Biosciences). Detected signals were densitometrically quantified using LI-COR Biosciences Image Studio version 5.2 imaging software.

**Fluorescence polarization (FP).** The interaction of FITC-labeled peptides (0.2 and 1 µM) with increasing concentrations of the SH3 domains (0.2 to 200 µM) was measured by fluorescence polarization using a Fluoromax 4 fluorimeter as previously described [42]. The excitation wavelength was 470 nm, and the emission wavelength was 560 nm. The dissociation constants (Kₐ) were obtained by fitting the concentration-dependent binding curve using a quadratic ligand binding equation.

**Isothermal titration calorimetry (ITC).** The thermodynamic parameters of GRB2-SOS1 and GRB2-GRB2 interactions were determined using an isothermal titration calorimeter (VP-ITC, MicroCal, Inc.) as described in [43]. In all experiments, the GRB2 proteins, including nSH3, cSH3, SH2, and FL, were placed into the sample cell at a concentration of 100 µM. The concentration of the proteins in the syringe was tenfold higher (1 mM) than the protein concentrations in the cell. For data evaluation, the manufacturer’s software was used as described in [44]. For all experiments, a buffer containing 50 mM Tris-HCl at pH 7.4, 5 mM MgCl₂ and 2 mM dithiothreitol was used.

**Analytical size-exclusion chromatography.** The homogeneity of purified proteins was determined using a Superdex 200 (10/300) column (GE Healthcare), an ÄKTA purifier (GE Healthcare), and a buffer containing 30 mM Tris-HCl at pH 7.5, 3 mM dithiothreitol, and 5 mM MgCl₂ in the presence and absence of 15 mM NaCl. The flow rate was sustained at 0.5 ml/min, uv absorbance detection was at 280 nm. The column was calibrated with a set of molecular weight protein standards (GE healthcare), comprising aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa) and RNase A (13.7 kDa). Fractions were collected at a volume of 0.5 ml, and then, peak fractions were visualized by 12.5% SDS-PAGE gel and staining using Coomassie brilliant blue.
Results

Both functional GRB2 SH3 domains are required for the interaction with SOS1. The interaction of the His-tagged SOS1 PRD with GST fusion proteins of GRB2 was analyzed with a pull-down assay. The PRD equally bound to GRB2 FL, nSH3, and cSH3 but not at all if conserved tryptophan (W36 in nSH3 and W193 in cSH3; Figs. 1b,c) was mutated to lysine (K); W36K in nSH3, W193K in cSH3, and W36K/W193K in GRB2 FL abrogated their binding to the SOS1 PRD (Fig. 2a). Interestingly, single mutations in GRB2 FL (W36K or W193K) also led to a significant impairment of the GRB2-RP1 interaction (Fig. 2a). This is surprising as both GRB2 FL$^{W36K}$ and GRB2 FL$^{W193K}$ had one SH3 domain intact and indicates that functional interdomain interactions exist for GRB2.

To examine the role of the GRB2 SH2 domain on the respective SH3 domains in SOS1 PRD interaction, we generated and purified SH3 deletion variants of GRB2, which contain a W-to-K variant in the remaining SH3 domain. The interaction of the GRB2 $\Delta$nSH3 and nSH3 domains with SOS1 PRD1 was analyzed using fluorescent polarization and pull-down assays (Fig. 2b). In both measurements, we detected SOS1 PRD interaction of GRB2 full-length wild type and $\Delta$SH3 (Fig. 2b) but not $\Delta$nSH3 or any of the tryptophan mutants (Fig. 4f). Our results signified that the presence of the SH2 domain or deletion of each of the SH3 domains did not affect the binding capability of the SH3 domain with the tryptophan mutation. Therefore, we concluded that tryptophan substitution affects SH3 function, which could not be restored upon addition of the SH2 domain. These data also showed that mutation or truncation of GRB2, which disrupts the GRB2 interaction with SOS1 RP1, also perturbs its interaction with all SOS1 proline-rich peptides (see below). Although GRB2 $\Delta$SH3 is wild type, it does not interact with SOS1 PRD compared to GRB2 FL (Fig. 2). Considering the interaction of SH3 domains with the SH2 domain and the auto-inhibitory role of the SH2 domain for cSH3 domain regulation, we concluded that there is a stronger interaction between the SH2 domain and the cSH3 domain in the absence of the nSH3 domain. Hence, the cSH3 binding site is not available for the interaction with SOS1, in the $\Delta$nSH3 construct. But the interaction of the nSH3 with SH2 domain in $\Delta$cSH3 is not strong enough for autoinhibition of the nSH3 domain, therefore it interacts with SOS1.

GRB2 FL and isolated SH3 domains exhibit varying affinities for distinct sites of the SOS1 PRD. Next, we analyzed the binding capacity of individual PRMs within the SOS1 PRD. The binding of ten FITC-labeled peptides (P1 – P10; Table S1; Fig. 3a) and two reference peptides (RP1 and RP2) to GST fusion proteins of GRB2 FL, nSH3, and cSH3, respectively, was qualitatively analyzed by combining GST pull-down and dot blot assays. As shown in Figure 3b, SOS1 peptides P3 and P4 and, to a certain extent, P5, as well as the reference peptides PR1 and PR2, bound strongly to GRB2-derived proteins. Signals observed for the other peptides were rather weak.

We next conducted fluorescence polarization measurements to obtain the affinities for the interaction between GRB2 and SOS1 PRMs. For this, increasing concentrations of the GRB2 proteins FL, nSH3, and cSH3 were titrated to the fluorescent peptides at a constant concentration of 1 $\mu$M. We monitored an increase in polarization for P3, P4, P5, and the reference peptides (Fig. S2a) but not for the other SOS1 peptides (data not shown). Equilibrium dissociation constants ($K_d$) for the respective GRB2-SOS1 interactions are summarized in Table S3 and shown in Figure 3c as bar charts. The highest affinity exhibiting a $K_d$ value of 1.2 $\mu$M was obtained for P3 binding to cSH3, which was 12.5-fold higher than that to nSH3. Relatively weak interactions with intermediate and low affinities were determined for P4 and P5 binding to cSH3 and nSH3, respectively. However, their dissociation constants of 15.5 $\mu$M and 13.2 $\mu$M, respectively, for GRB2 FL are still reasonable and suggest that P4 and P5 might constitute, in addition to P3, secondary binding sites for GRB2.
Isothermal titration calorimetry (ITC) measurements revealed that the GRB2-SOS1 interaction exhibits a two-site binding characteristic that is not surprising given the observations shown above. A fit to the ITC data using a one-site binding model yielded a GRB2-peptide stoichiometry of 1:1.88, suggesting that two peptide molecules bind to GRB2. A tentative fit according to a two-site model resulted in a first $K_d$ value of 1.2 µM and a second $K_d$ value of 4.0 µM (Figs. 5a, S4a). Note that such $K_d$ values can only be obtained with high precision when they are different by a factor of 10-100 or more. Nevertheless, our observations allow us to conclude that first, GRB2 uses both SH3 domains to bind SOS1 and second, the binding affinity of the SH3 domains is considerably higher in the context of GRB2 FL than in the isolated domains, suggesting intramolecular interactions and allosteric coupling between the domains.

Both functional SH3 domains of GRB2 FL are required for binding SOS1 RP1. Based on our previous observation, the SOS1 PRD interaction with GRB2 FL was sufficiently compromised when only one of its two SH3 domains contained a loss-of-function mutation (W36K or W193K; Fig. 2 and Table S2). We set out to investigate the impact of additional mutations that are likely to alter the interaction with SOS1 as well as those that indirectly affect the affinity of GRB2 toward fluorescent SOS1 RP1. We hypothesized that such variations reside in the intramolecular and/or interdomain interactions within GRB2. To this end, we determined the $K_d$ values for the binding of various GRB2 variants to SOS1 RP1 using fluorescence polarization under the same conditions as described above. The first analysis showed that not only a double but also a single tryptophan mutation of GRB2 SH3 domains completely abolished SOS1 RP1 binding (Fig. 4a). In contrast, the isolated nSH3 and cSH3 domains of GRB2 strongly bound RP1 with $K_d$ values of 3.4 and 11.5 µM (Fig. 4b), similar to the ITC results (Figs. 5b,c), respectively, but not their tryptophan to lysine mutations (Fig. 4b). To examine whether the lack of tryptophan affected SH3 function and not the presence of lysine, we further substituted one of the tryptophan residues, Trp193, for alanine and aspartic acid (W193A and W193D). These substitutions also completely impaired the RP1 interaction with cSH3 (Fig. 4b), underlines that the conserved tryptophan residue is essential for the interaction of the SH3 domain with the RPMs.

These data indicate that there is a mutual influence of the two SH3 domains in the context of GRB2 FL. Hence, we systematically investigated the impacts of the interconnecting regions, namely, (i) the SH2 domain, including its association with HER2, (ii) the linker regions of GRB2, and (iii) the allosteric interdomain communication of GRB2 on the GRB2-SOS1 interaction.

The GRB2 SH2 domain allosterically regulates the GRB2-SOS1 interaction. Possible upstream effects of HER2 on the GRB2-SOS1 interaction were examined. To mimic such a signaling event, we designed fluorescent peptides containing Tyr1193 (YP) and phospho-Tyr1193 (pYP) of human HER2 (Table S1) and determined their binding affinities for both GRB2 FL and its SH2 domain. Isolated SH2 and GRB2 FL bind fluorescent pYP 16- and 76-fold more strongly than nonphosphorylated fluorescent YP (Fig. 4c). The interdomain interaction in GRB2 FL strikingly enhances the HER2-GRB2 interaction, not only because of the high affinity of GRB2 FL for pYP but also because of the binding selectivity of pYP vs. YP.

Next, we addressed the impact of HER2 pYP binding to GRB2 FL on the SH3 interaction properties with fluorescently labeled SOS1 RP1. The experiments were conducted under the same conditions as before but in the presence of nonfluorescent HER2 pYP. Figure 4d shows that HER2 pYP binding to the GRB2 SH2 domain did not have any obvious effect on the RP1 interaction with GRB2 FL (see Fig. 4a). Remarkably, HER2 pYP induced a tight interaction of RP1 with GRB2W36K (Fig. 4d). As shown before, this GRB2 variant with one intact cSH3 domain was unable to bind SOS1 RP1 in the absence of HER pYP (Fig. 4a).
This effect of pYP was marginal for GRB2WT and even absent for GRB2W193K and subsequently for GRB2W36K/W193K (Fig. 4d). It seems that pYP association with SH2 uncouples potential interdomain interaction(s) within GRB2, suggesting, for the first time, an allosteric mechanism underlying the RTK-mediated regulation of the SOS1 interaction with GRB2.

To further examine the impact of the SH2 domain on SOS1 RP1 binding affinity, we generated GRB2 ΔSH2 variants (Table S2) and measured their RP1 binding ability. The results showed that GRB2 ΔASH2 binds not only RP1 with a high affinity (Fig. 4e)—higher than GRB2 FL in the absence and presence of HER2 pYP (Figs. 4a and 4d)—but, most remarkably, also ΔSH2W36K (Fig. 4e). Consistent with the data obtained with GRB2 FL, ΔSH2W193K and ΔSH2W36K/W193K did not show any RP1 binding.

These data indicate a modulatory impact of the pYP-SH2 complex on the GRB2-SOS1 interaction and may rather challenge the existing paradigm of GRB2-mediated SOS1 activation.

Physical interdomain interactions control the structural and functional switch of GRB2. To more deeply inspect the interdependency between the three GRB2 domains associated with the GRB2-SOS1 interaction, we generated GRB2 variants lacking either cSH3 or nSH3 (Table S2) and performed fluorescence polarization experiments using the same setup as before. Figure 4f shows that GRB2ΔcSH3 exhibited almost the same affinity for SOS1 RP1 as determined for GRB2 FL and that the presence of HER2 pYP did not show any meaningful increase in the GRB2ΔcSH3-SOS1 interaction (Fig. 4f). In contrast, and interestingly, a loss in SOS1 RP1 binding was observed for GRB2ΔnSH3, which was also disabled in SOS1 RP1 binding, even in the presence of HER2 pYP (Fig. 4f). In the next experiments, we checked the interaction of SH3 deletion variants possessing the W-to-K mutation RP1 and examined whether HER2 pYP binding to the SH2 domain allosterically induces a conformation in nSH3 that enables GRB2ΔcSH3W36K to bind RP1. Figures 3 and 4f show that neither GRB2ΔcSH3W36K nor GRB2ΔnSH3W193K bound RP1 and that the addition of HER2 pYP did not induce any allosteric effect on RP1 binding to GRB2ΔcSH3W36K.

To follow up on this observation, we next measured the impact of possible direct SH2-SH3 interactions on RP1 binding to individual SH3 domains. We carried out fluorescence polarization measurements to study the interaction of isolated cSH3 and nSH3 with SOS1 RP1 under the same conditions as shown in Fig. 4b but in the presence of excess amounts of the SH2 domain. Figure 4g shows that the SH2 domain "in trans" significantly interfered with the binding of RP1 to both the nSH3 and cSH3 domains. Whereas no binding was observed for nSH3, the binding affinity of cSH3 for SOS1 RP1 was reduced by 15-fold by the SH2 domain. Most remarkably, the presence of excess amounts of HER2 pYP-SH2 (200 µM) actually enabled nSH3 to bind RP1 (Kd = 11.3 µM) and strongly improved the cSH3-SOS1 RP1 interaction by 24-fold (Fig. 4g). Quite astonishing is that GRB2ΔnSH3 (SH2 domain "in cis") did not bind RP1 in the presence of HER2 pYP (Fig. 4f), suggesting that defined conformational constraints at the interface between SH2 and cSH3 may lock cSH3 in place for the interaction with SOS1 RP1.

These data suggest that binding of activated RTK and possibly other transmembrane proteins to the SH2 domain changes the overall conformation of the SH3 domains—most likely cSH3—and favors the association with SOS1.

As the above data indicated an unexpected interrelationship between GRB2 domains, we conducted a series of ITC experiments to uncover the direct or indirect interplay between isolated SH2 and SH3 domains as well as between isolated SH3 domains and SOS PR1 in the presence of the isolated SH2 domain. The data summarized in Figures 5d-g show that the isolated SH3 domains bind with very low affinity to the isolated SH2 of GRB2 and that the presence of HER2 pYP slightly attenuated these SH3-SH2 interactions. Moreover, in the
presence of SOS1 RP1, the SH2 domain neither binds nSH3 nor cSH3 (Figs. 5h-i). Interestingly, a different scenario emerged from titrating SOS RP1 to a mixture of isolated SH3 and SH2 domains (Figs. 5j-m). While RP1 did not bind to the nSH3-SH2 complex, it exhibited a high affinity for binding the mixture of nSH3, SH2, and HER pYP (Figs. 5j and 5k). These results are consistent with the fluorescence polarization data (Fig. 5g) and support the idea that activated RTK or other phosphotyrosyl-containing proteins binding to GRB2 facilitates its interaction with SOS1. In contrast, cSH3 binds RP1 with a slightly higher affinity in the presence of SH2 than in the absence of SH2 (Fig. 5c), and the addition of pYP did not significantly strengthen binding (Figs. 5i and 5m).

Taken together, the above findings expand our understanding of the principles of modular domain organization and the interdomain relationships of GRB2 and suggest a structural and functional switch that favors intramolecular interactions and allosteric coupling between its domains.

**Interdomain linkers of GRB2 confer highly dynamic interactions with SOS1.** We next addressed the question of whether the short linker segments of GRB2 interconnecting its globular domains (amino acids 54-63 and 149-159) play a role in the allosteric mechanism of GRB2 action. A closer look at the structure of GRB2 (PDB ID: 1GRI) revealed a kind of antiparallel structural arrangement of these two linkers, especially in the central regions with sequences 57-63 and 152-158 (Fig. 1a). To examine their impact, we generated deletion variants of GRB2 lacking both PHP and EQV. This variant was then combined with single and double tryptophan substitutions in the SH3 domains (Table S2). The data shown in Figure 4h indicate the critical role of the GRB2 linkers in the structural and functional features of GRB2 SH3 domains. Double truncation of the linker GRB2ΔPHP ΔEQV led to an immense decrease in SOS1 RP1 binding affinity by 22.5-fold. This impairment was not reversed in the presence of HER2 pYP (Fig. 4h). In contrast to experiments with GRB2 FL shown in Figure 4a, PHP/EQV deletion restored SOS1 RP1 binding to GRB2 W36K and GRB2 W193K. These observations clearly underscore the critical role of the linker in the SH3-mediated interaction with SOS1.

To further analyze the effect of single linker truncation, we generated GRB2ΔPHP and GRB2ΔEQV. Most remarkably, SOS1 RP1 binding was completely abolished upon PHP but not EQV deletion. The GRB2ΔEQV interaction with RP1 notably remained unchanged in the absence of HER2 pYP but was reduced 5-fold in the presence of pYP (Fig. 4i).

These data emphasize the critical role of the linker segments, which give GRB2 domains freedom and space to move and to orient with each other in the most suitable way. This becomes more difficult when the linkers are shorter.

**GRB2-SOS1 interaction underlies an allosteric mechanism.** An inspection of the GRB2 structure (PDB ID: 1GRI) showed that Y37, K50, K195, and T202 create an intramolecular interaction network between the two SH3 domains (Fig. 1d). We thought that replacing them with negatively charged aspartate residues may generate repulsive forces and thus abolish this interaction network. Another interesting contact site between the two SH3 domains is achieved by M1 in nSH3 and M186 in cSH3 (Fig. 1d). This methionine interaction is more of a van der Waals (hydrophobic) interaction, and its substitution by arginine or lysine may induce interdomain electrostatic repulsion. To investigate possible allosteric effects of these sites on SOS1 RP1 binding, we generated three sets of GRB2 variants, that altered GRB2 apart from the PRM binding site of the SH3 domains (Fig. 1; Table S2): (i) a sextuple variant with M1R, Y37D, K50D, M186R, K195D, and T202D substitutions (6allo); (ii) two triple variants with M1R, Y37D, and K50D substitutions (n3allo) and with M186R, K195D, and T202D (c3allo) substitutions; and (iii) a triple variant with the substitutions of Met1, Met186, and Thr202 with arginines (M1R/M186R/T202R; called 1-2allo).

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Fluorescence polarization data revealed that 6allo substitutions drastically impaired GRB2 SOS1 binding sites such that no RP1 association with GRB2\(^{6allo}\) was observed (Fig. 4j). This deficiency was slightly recovered in the presence of HER pYP. Curiously, isolated SH3 domains with the respective substitutions (3allo and 3allo, respectively) showed no binding with SOS1 RP1 (Fig. 4k). This raises the question of whether a physical interaction between two SH3 domains in GRB2\(^{WT}\) is a prerequisite for their interactions with SOS1 RP1. ITC measurements were performed to examine possible physical nSH3-cSH3 interactions. We observed that the two SH3 domains undergo very low-affinity binding (\(K_d = \geq 200 \mu M\)), which was abolished when the 3allo variants of nSH3 and cSH3 were used (Figs. 5n, 5o, S4, and S5). Moreover, analytical size exclusion chromatography of the SH3 proteins revealed that cSH3\(^{WT}\), in contrast to monomeric nSH3\(^{WT}\) and nSH3\(^{6allo}\), is a dimer and tetramer and is shifted to monomeric/dimeric populations in the case of the monomeric 3allo variant (Fig. S6). These observations confirmed the notion that an inter-SH3 domain interaction forms an alliance of its own. Certainly, these allosteric sites are not primary binding sites but are obviously critical in the optimal control of SOS1 association with GRB2.

To further follow up on the impact of the allosteric site, we measured the RP1 binding activity of GRB2\(^{3allo}\) and GRB2\(^{c3allo}\). GRB2\(^{n3allo}\) showed a 30-fold reduced \(K_d\) value for SOS1 RP1. However, in contrast to GRB2\(^{W36K}\), it was able to bind RP1 with a similar affinity in the background of W36K (Fig. 4l). RP1 binding was not observed for GRB2\(^{n3allo/W193K}\). Most remarkably, the GRB2\(^{n3allo}\)-SOS1RP1 interaction was greatly improved in the presence of HER pYP. This clearly supports the notion that the HER pYP-SH2 interaction induces a reorientation of the SH3 domains and optimizes the SH3-SOS1 interaction. In contrast, GRB2\(^{6allo}\) was able to efficiently bind RP1 with a \(K_d\) value of 1 \(\mu M\), which remained unchanged in the background of W193K but was completely abolished in the background of W36K (Fig. 4m).

To reduce the number of substitutions at the allosteric sites, we generated and analyzed GRB2\(^{2allo}\) using fluorescence polarization. As shown in Figure 4n, this protein binds SOS1 RP1 with high affinity and is able to bind RP1 with intermediate affinities in the background of W36K compared to GRB2\(^{WT}\). The presence of HER pYP did not change these binding affinities. GRB2\(^{2allo/W193K}\), however, exhibited a \(K_d\) value of 2.2 \(\mu M\) for RP1 binding (Fig. 4n), which interestingly resembles the high binding affinities of GRB2\(^{EQV}\) and GRB2\(^{c3allo/W193K}\) (Figs. 4i and 4m).

These data strongly suggest that a cooperative nSH3-cSH3 interaction is required to facilitate SOS1 binding and further support the idea of the opposing impacts of the SH3 domains on their RP1 binding capabilities. It seems that nSH3 allosterically facilitates RP1 association with cSH3 and cSH3 allosterically precludes the nSH3-RP1 interaction.

The unique C-terminal NRNV motif leverages GRB2 allostery. Inspection of the GRB2 structure revealed that the four very C-terminal amino acids NRNV undergo contacts with linker 2 (R215-Q156 interaction) and are in close vicinity to the SH2 domain. It was tempting to investigate their impact on the GRB2 interaction with SOS1 RP1 in the presence and absence of HER pYP that was addressed by deleting this motif. As shown in Figure 4o, NRNV deletion led to a drastic attenuation of the GRB2-RP1 interaction by more than 28-fold compared to GRB2 FL (Fig. 4a), which was recovered in the presence of HER2 pYP to the same extent as GRB2 FL (Fig. 4d). Most remarkably, GRB2\(^{LNRNV}\) variants with W36K or W193K substitutions were, in contrast to GRB2 FL, able to bind RP1 with even higher affinities than without tryptophan substitutions (Fig. 4o). Moreover, the addition of HER2 pYP resulted in the loss in RP1 binding to GRB2\(^{LNRNV/W36K}\) and gain in RP1 binding to GRB2\(^{LNRNV/W193K}\). The very high affinity of the latter resembles that of the wild-type GRB2 FL in the presence of pYP (Fig. 4d).
GRB2 FL has been reported to exist in equilibrium between dimeric and monomeric functional states [45][16, 46], which was confirmed by analytical size exclusion chromatography (Fig. S6). In contrast, GRB2YPNRNV interestingly eluted as a monomer in the same state as the monomeric control protein GRB2Y160E, which has been shown to mimic a phosphorylated form of GRB2 [46]. While a monomeric state was expected for GRB2Y160E, the GRB2YPNRNV monomer is remarkable and inexplicable at the same time. A database search showed that the NRNV motif in GRB2 is “singular” within the large human SH3 superfamily that contains 298 SH3 domains in 221 reviewed SH3 domain-containing proteins in the human proteome (data not shown). Moreover, a BLAST search showed that very few proteins contain an NRNV sequence, including the isoforms of the centromere-associated protein, CUB and sushi domain-containing protein 2, DMX-like protein 2, DNA polymerase zeta catalytic subunit, phosphatidylinositol 3-phosphate 5-kinase, reelin, and spectrin alpha chain.

With the very C-terminal NRNV, we identified a unique sequence motif that represents a very interesting element involved in the allosteric regulation of GRB2 and that seemingly leverages GRB2 allostery to direct RTK signaling toward SOS1 activation.

Discussion

One of the key steps in RAS activation at the plasma membrane is the bimodal interaction of the GRB2 adaptor protein with activated RTK and RASGEF SOS1. GRB2 carries one SH2 domain for association with a specific phosphotyrosine site of the activated RTK or nonreceptor tyrosine kinases and tyrosine-phosphorylated proteins and two SH3 domains responsible for binding to proline-rich motifs, such as the SOS1 PRD. This study provides comprehensive data on the molecular interactions of PRDs, PRMs, and Y-/pY-peptides with GRB2 in an attempt to shed light on the mechanism underlying RTKs linking with RASGEFs by GRB2 and demonstrates that the mechanism by which GRB2 functions as an adaptor protein is not based on a simple binding model. Using a systematic we found that (1) GRB2 binds with P3, P4, and P5, three out of ten SOS1 PRMs; (2) both functional GRB2 SH3 domains are required for the interaction with SOS1; (3) physical interdomain interactions of GRB2 structurally and functionally modulate a tight association with SOS1; (4) the two GRB2 linkers allow for highly dynamic interactions of the SH3 domains; (5) there is a reciprocal relationship between the two SH3 domains that determine their successive interactions with SOS1; and (6) the GRB2-SOS1 interaction underlies an allosteric mode of regulation that is leveraged by a unique C-terminal NRNV motif. Thus, GRB2 appears to undergo, upon upstream ligand binding (e.g., HER2 pYP), a series of structural transitions from one site to a physically distinct site that may reinforce a stepwise association of downstream ligands (e.g., SOS1 PRMs). Such signal propagation is on the one hand achieved via both interdomain structural changes and allosteric networks induced by HER2 pYP binding to the SH2 domain and, on the other hand, modulated by specific domain–domain rearrangements, which ultimately results in the engagement of both SH3 domains in binding and eventual activation of SOS1.

In an NMR structural study, Yuzawa et al. showed that GRB2 exists in multiple conformations in which two SH3 domains take different positions and orientations relative to the central SH2 domain [47]. Figure 6b shows the randomly selected conformation of GRB2 with the tendency to form an inter-SH3 domain interaction, which is proposed in this study to be a prerequisite for the SOS1 interaction. This assumption is based on the remarkable insight gained from the ITC measurements, which showed a direct interaction between nSH3 and cSH3 (Fig. 5n). Detailed structural inspection showed a physical interdomain interaction network (M1, Y37, K50 from nSH3 contacting M186, K195, and T202 of cSH3; Fig. 1d) that is obviously responsible for the allosteric inter-SH3 domain interaction and required for the GRB2-SOS1 interaction. The substitution of these residues, called 6allo in GRB2 FL or 3allo in each isolated SH3 domain, completely abrogated the nSH3-cSH3 interaction (Fig. 5o),
impaired the SOS1 RP1 binding capability of GRB2 FL and isolated Sh3 domains (Figs. 4j-k). The circumstances have considerably changed to a far more moderate extent but are also more complex at the same time when these residues were substituted only on one side, either nSH3\textsuperscript{3allo} (Fig. 4l) or cSH3\textsuperscript{3allo} (Fig. 4m). Uncoupling these inter-SH3 domain interactions only slightly improved the RP1 binding property of GRB2\textsuperscript{2allo} compared to GRB2 FL\textsuperscript{WT} (Fig. 4a) but strongly reduced the RP1-binding ability of GRB2\textsuperscript{3allo}, which was potentiated in the presence of HER2 \textit{pY} (Fig. 4l). Further reducing the number of amino acid substitutions, as in GRB2\textsuperscript{1,2allo} (M1R/M186R/T202R), showed only mild effects when we examined SOS1 RP1 binding (Fig. 4n).

This subject changed completely and became rather complicated when these allosteric site variations of GRB2 were additionally combined with the SOS1 binding site variations at either W36K or W193K substitutions. This means changes at two different sites of either nSH3, cSH3, or both. While 6allo variants with either W-to-K variants were disabled in binding RP1, the n3allo and 1-2allo variants with W36K regained the ability to bind RP1, although with low affinities and the addition of HER \textit{pY} in the case of 1-2allo did not show any effects (Figs. 4l-n). The hydrophobic interface is one of the key features of the SH3 domains and conserved W36 of nSH3 and W193 of cSH3 of GRB2 are located within this hydrophobic interface (Figs. 1b,c). This tryptophan directly interacts with proline residues, usually at position 4 of PRMs [48]. Thus, we assume that GRB2 W-to-K variants are disabled in SOS1 RP1 binding.

The c3allo and 1-2allo variants with W193K, however, revealed a high binding affinity for RP1, which increased slightly upon the addition of HER2 \textit{pY} (Figs. 4m-n). These data suggest that the allosteric inter-SH3 domain interaction promotes the nSH3-RP1 interaction but obviously attenuates or even blocks the association of RP1 with cSH3. Thus, there is reciprocal functional interdependence between SH3 domains that is required for SOS1 binding (Fig. 6b, 3ii). Additional integral elements, such as the linkers, the NRNV motif (see below), and the association of HER2 \textit{pY} with the SH2 domain are certainly required to obtain a complete picture of efficiently connecting GRB2 with SOS1. A missing element that most likely participates in the interdomain interaction dynamics of GRB2 is its proposed association with the lipid membrane (see below) [49].

A detailed inspection of the SOS1 PRD sequence revealed that the ten peptides selected for this study (P1-P10; Fig. 6a) cover 13 out of 14 identified types of proline-rich consensus sequence motifs (Table S4). However, the majority of studies have analyzed peptides with PXPPXR motifs that were derived from P3 or quite similar to RP1 (see Table S1), which has been proposed as a canonical GRB2-binding site. Liao et al. [32] also analyzed 10 SOS1 peptides, which partially cover the proline-rich motifs we selected for our study. Note that this article was published when we were finishing our study and preparing the manuscript for publication.

Sehti et al. calculated the intramolecular equilibrium constants for the interaction of GRB2 with SOS1 and determined a $K_4$ value of 0.4 \textmu M for the GRB2-SOS1 complex with a stoichiometry of 1:1 [50]. Liao et al. proposed two PRMs in the SOS1 PRD, corresponding to peptides P3 and P10 from our study (Fig. 3a), as binding sites for the GRB2 nSH3 and cSH3 domains, respectively [32]. They also considered but did not favor P4 and P3 because of their restricted structural flexibility compared to the distance between P3 and P10 (Fig. 6c, 1-3). We found only P3, P4, and P5 peptides interacting with GRB2 SH3 domains confirming merely partially this proposed model. We agree that the GRB2-SOS1 interaction involves the successive binding of both SH3 domains and that GRB2 binding to one site may enhance its binding to the other [9, 51, 52]. However, we think that the protein-protein interaction and complex formation process correlates with restricted/reduced structural flexibility, which is facilitated by adjacent or even tandem binding sites, such as P3-P5 or P3-P4 (Fig. 6c, 4-6). In this context, Vidal et al. demonstrated that the interaction of RP1 with GRB2 can be increased by 360- and 60-fold when RP1 is covalently linked to identical RP1 (a tandem RP1) or to a different peptide [53], i.e., PESPPLLPPR, the central part of P5; (see Fig. 3a).
Similarly, Yuzawa et al. showed that generated tandem peptides consisting of RP1-RHY peptides with varying numbers of linker residues exhibited much higher affinities, with $K_d$ values between 0.01 and 0.5 µM, for GRB2 FL compared to single peptides [47]. Accordingly, it has been suggested that GRB2 changes the relative position and orientation of the SH3 domains to enable efficient bivalent binding to noncontinuous binding sites and that binding affinity depends on the length of the linker between PRMs. In a very recent study, Landry and colleagues demonstrated that the SH3-containing full-length protein determines the specificity of its SH3 domain-mediated interactions [13]. We therefore propose that GRB2 undergoes stepwise, cooperative binding with SOS1, such as P3-P4 (Fig. 6c, 6). It is assumable that avidity can work in both directions. The mechanism described for GRB2 FL may hold true in the context of SOS1 FL if considering their physical interaction as a prerequisite for not only the recruitment of SOS1 to the membrane but also its activation (see below; Fig. 6d). Note that other SOS1 domains play a critical role in regulating SOS1 activation in the context of membrane proximity by sensing membrane lipids [54, 55].

Most multidomain proteins have at least some degree of segmental mobility facilitated by the flexibility of the interdomain linkers. This allows conformational changes that in some instances can be brought about by concerted domain movement from one distinct arrangement to another. The orientation of the globular domains in an SH3-SH2 or SH2-SH3 tandem may allow the formation of a compact rearrangement between them. Nussinov and colleagues proposed that linker sequences and lengths are optimized in the course of evolution for efficiency of protein functions [56]. They further argue that allosteric propagation of the energy that is generated by transient interactions and covalent modifications of multidomain proteins via flexible linkers can lead not only to conformational changes of a second binding site in another domain but also to a relatively large, allosterically driven reorientation of protein domains with respect to each other. Numerous studies have shown that linker segments between SH3 and SH2 domains in various proteins, including ABL [57], CRK [58], FAK [59], LCK [60], NCK [61], and PLCγ [62], modulate the binding of these domains to their targets and thus regulate corresponding cellular processes. Accordingly, the present study adds GRB2 to this list of proteins, whose domain arrangements and interaction dynamics are controlled, among others, by the flexibility of the linker segments. Our data revealed that truncation of one or both linkers affects GRB2 interaction with SOS1, most likely due to limited interdomain flexibility. An inspection of GRB2 structures [45, 47] revealed that both cSH3 and the very C-terminal NRNV motif are located in close proximity to linker 2 and may form stable intramolecular hydrogen bonds (see below). GRB2 SUMOylation by SUMO1 at K56, which is located in the first linker segment, has been shown to result in an increase in the formation of the GRB2-SOS1 complex sequentially in the activation of the Ras/MEK/ MAPK pathway [63]. Therefore, the linkers are not only flexible but are allosterically regulated [56].

The crystal structure of GRB2 FL, determined by Maignan et al. [45], has revealed that it exists as a homodimer with accessible binding sites for both SH2 and SH3 domains. Farooq and colleagues reported that GRB2 exists in dimer-monomer equilibrium in solution and that it is likely to undergo cSH3–SH2 domain swapping upon dimerization [64]. They proposed that although this equilibrium is likely to favor the monomer in quiescent cells, rapid expression and a rise in GRB2 concentration upon mitogenic stimulation are likely to shift this equilibrium in favor of the dimer [64]. Although both GRB2 monomers and dimers have been shown to bind to other active RTK dimers [16, 65-67], the literature consensus is that GRB2 binds EGFR dimers in the monomeric form [16, 24, 68]. It was reported that mutation of tyrosine 160 in the CSH3 domain changes GRB2 oligomerization toward the monomer state [16]. The monomer state of GRB2 Y160E, tryptophan mutants, or ΔNRNV does not interact with SOS1 in vitro; hence, we propose that the monomer state of GRB2 is the inactive form and that GRB2 monomer-dimer equilibrium is needed for the GRB2 interaction with SOS1. Ladbury and coworkers demonstrated that monomer-dimer equilibrium determines its function in cells [46]. Accordingly, we propose, monomeric GRB2 in the active
form associates with SOS1, whereas dimeric GRB2 represents its inactive inhibitory form. It has been suggested that dissociation of the dimer is facilitated by either phosphorylation of Y160 or through the binding of a tyrosylphosphate-containing ligand [46]. Liao et al. proposed that the autoinhibited GRB2 dimer undergoes a conformational change upon binding of its SH2 domain to pYP, which releases the flanking SH3 domains to bind and recruit SOS1 to the plasma membrane [32]. Two assumptions are implied in this study (Fig. 6d, 1): (1) the autoinhibited GRB2 is disabled in binding PRM-containing ligands, including SOS1 and (2) the GRB2-SOS1 complex forms upon stimulation of cells by a growth factor and the association of GRB2 with tyrosyl phosphate-containing ligands.

In this study, we found a novel motif, 214NRNV217, in the very C-terminal end of GRB2 that appears to play a key role in the allosteric regulation of GRB2 signaling from activated receptors to SOS1 activation. Database searches revealed that the NRNV sequence exists in a few human proteins and among human SH3-containing proteins, only in GRB2. Structural inspection of GRB2 showed that the NRNV motif is close to the SH2 domain and the linker region, which may have different consequences for GRB2 function. This may serve as a fine-tuning mechanism to maintain the dimer-monomer equilibrium of GRB2. We showed that deletion of NRNV resulted in the equilibrium shift of GRB2 dimer-monomer equilibrium toward the monomeric state study (Fig. S6) and, consequently, a 28-fold lower affinity for SOS1 RP1 (Fig. 4o). Moreover, GRB2^{NRNV/W36K} but not GRB2^{W36K} was able to interact with SOS1 RP1 (Figs. 4a and 4o), suggesting that the NRNV motif may be involved in the inhibitory effect of the SH2 domain on the cSH3 domain. Interestingly, the presence of HER2 pYP abolished the GRB2^{NRNV/W36K} interaction with RP1, supporting the notion that the physical association between the SH2 and cSH3 domains allosterically controls the GRB2-SOS1 interaction.

The prevailing paradigm, however, suggests that SOS1 undergoes a stable interaction with GRB2, resides in the cytoplasm and translocates to the plasma membrane upon stimulation (Fig. 6d, 2) [69-75]. Chook et al. showed that the GRB2-mSOS1 complex, copurified from insect cells, forms a molar ratio of 1:1 and binds pY peptides with higher affinity than GRB2 alone, suggesting that the proximity of SOS1 to GRB2 facilitates the interaction of the GRB2 SH2 domain with activated tyrosyl phosphorylated ligands [68]. Welham et al. reported that they did not detect GRB2 and SOS1 translocation to the plasma membrane upon cell stimulation by different growth factors, including EGF [76]. This raises the possibility of additional yet unknown mechanisms by which GRB2 and SOS1 form a complex that ultimately results in SOS1 activation at the plasma membrane.

While allosteric effects in SH3 domains have been previously explored to investigate the structural communication between contiguous domains in complex multidomain proteins [40], there is a lack of information regarding interdomain allosteric crosstalk within the GRB2 molecule. Groves and coworkers proposed an allosteric mechanism in which the binding affinity of LAT-GRB2 depends on phosphorylation at remote tyrosine sites [77]. As discussed above, it is still a matter of debate whether GRB2 association with its upstream pY molecules, such as HER2 or LAT, brings SOS1 `piggyback` to the plasma membrane, where it binds and activates membrane-associated RAS. In another study, Groves and coworkers demonstrated that the SOS1 catalytic RASGEF domain is inhibited by the PRD [78], suggesting that GRB2 binding to the PRD may induce SOS1 activation. This autoinhibitory role of the PRD limits GRB2-independent recruitment of SOS to the membrane through binding of RAS•GTP in the SOS1 allosteric binding site [79, 80]. Spatiotemporal specificity is further conferred by multiple intermolecular interactions on the membrane, such as with lipids and GRB2 that bind to the N- and C-terminal domains, respectively [78, 81].

Taken together, three scenarios for GRB2-mediated SOS1 translocation to the plasma membrane can be postulated under growth factor-stimulated conditions that lead to tyrosine phosphorylation of RTKs or other transmembrane proteins (Fig. 6d). (1) A dimer-to-monomer transition results in GRB2 association with tyrosyl phosphate-containing ligands via its SH2...
domain. The latter then associates upon a conformational change with the membrane phospholipids via surface cationic patches separated from pY-binding pockets [49]. This ultimately enables GRB2 SH3 domains to bind the SOS1 PRD. (2) A cytoplasmic bivalent GRB2-SOS complex binds to its activated ligands (pYP and phospholipids) and thereby recruits SOS1 to the plasma membrane-associated RAS. This model may represent simple recruitment of cytoplasmic SOS1 to the plasma membrane. (3) A cytoplasmic monovalent GRB2-SOS complex binds to its activated ligands (pYP and phospholipids), which in turn allosterically induces a structural rearrangement through GRB2 that makes it fully competent to bind a second binding site on the SOS1 PRD using the cSH3 domain and eventually activate SOS1. In this model, SOS1 recruitment to the plasma membrane is accompanied by its autoinhibition.
Data availability statement
All the data are in the manuscript.

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Additional information
Supplementary Information accompanies this paper.

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Figure 1. The GRB2 structure highlights the different regions, sites, and motifs investigated in this study. (a) Domain organization and signature motifs of GRB2. (b) The GRB2 structure (PDB code: 1GRI) is represented as a monomer from the front (left) and back sides (right). The SH2 domain (cyan) forming a complex with the HER2 pY peptide (magenta) links via linker 1 (red pearl-rope) and linker 2 (purple pearl-rope), the flanking nSH3 (yellow) and cSH3 (green) domains, and the very C-terminal NRNV motif (brown). The SH3 domains are in a complex with the SOS1 RP1 peptide (blue). The different sites relevant for this study are the two tryptophans W36 in nSH3 and W193 in cSH3 (both in orange), the two triplets PHP from linker 1 (red) and EQV from linker 2 (purple), and the six residues in the nSH3-cSH3 interface. (c) The GRB2 structure in b is shown from the top view to visualize W36 in nSH3 and W193 in cSH3 at the SH3-RP1 interface. (d) An open-book view of the two SH3 domains illustrates the relative position of the six interfacing residues of the allosteric site as indicated.

Figure 2. Inactivating mutation in one of the GRB2 SH3 domains also impairs the SOS1 PRD interaction of the other SH3 domain in an SH2-independent manner. Individual GST-GRB2 fusion proteins (20 µM) were used to pull down 30 µM purified SOS1 PRD (input). GST was used as a negative control. The binding of the SOS1 PRD to GRB2 proteins was visualized by immunoblotting using a polyclonal anti-SOS1 antibody (output), which specifically recognizes the SOS1 PRD as a single epitope (see Fig. S1). These experiments were performed three times (n = 3). The upper panel shows quantification of the GRB2 PRD interaction with the GRB2 variants (mean±s.e.m.); ***, p < 0.0001 to 0.001 and ****, p < 0.0001 (Student’s t-test); n.s., no signal. (a) Interdependent interaction of individual SH3 domains of GRB2 with the SOS1 PRD. W-to-K single mutations significantly impaired SOS1 PRD binding to GRB2 FL and completely abolished SOS1 PRD binding to isolated nSH3 and cSH3 domains (n.s., no signal). The SOS1 PRD did not show any binding to a W-to-K double mutation of GRB2 FL. (b) Significant inhibitory impact of the SH2 domain but not the nSH3 domain of GRB2 on SOS1 PRD binding by cSH3. SOS1 PRD pulldown experiments with nSH3 and cSH3 deletion variants of GRB2 revealed that GRB2ΔcSH2, lacking the cSH3 domain, binds the SOS1 PRD as efficiently as GRB2 FL and the isolated nSH3 protein shown in a. GRB2cSH2/W36K did not show any binding, indicating that the SH2 domain does facilitate SOS1 PRD binding of this variant. Deletion of the nSH3 domain (GRB2ΔnSH3), however, abrogated SOS1 PRD binding, and the W193K mutation did not restore this loss of function. GST-GRB2 FL and GST were used in these experiments as positive and negative controls.

Figure 3. The GRB2 binding properties for ten different SOS1 PRM-containing peptides. (a) The C-terminal PRD of SOS1 contains several PRMs, ten of which were investigated in this study as synthetic peptides, designated P1 to P10. The reference peptides RP1, a derivative of SOS1 and part of P1, and RP2, a derivative of the RHO GTPase WRCH1, were used as controls. (b) GRB2 selectively interacts with three out of ten PRM-containing peptides of SOS1. Dot blot analysis of fluorescent SOS1 peptides pulled down with GST fusion proteins clearly revealed the high binding selectivity of GRB2 FL and SH3 domains for P3 and P4, as well as the reference peptides RP1 and RP2. GRB2 FL also bound P5 much more strongly than isolated SH3 domains. (c) SOS1 P3 revealed the highest binding affinity for cSH3. Evaluated dissociation constants (Kd) of fluorescence polarization measurements for the SOS1 PRM-GRB2 interactions (for more detail, see Fig. S2A) are shown as bars. The colors of the bar charts highlight the Kd values (above the bars) for the SH3-PRM interaction, which are divided into high affinity (1–5 µM; green), intermediate affinity (6–20 µM; blue), and low affinity (>20 µM; red).

Figure 4. SOS1 peptide binding by GRB2 FL depends on multiple factors. Kd values (in µM) for the interaction of various GRB2 variants with SOS1 RP1 in the presence and absence of HER2 pY peptide were obtained using fluorescence polarization and FITC-
labeled (F) peptides (a-o). Determined $K_d$ values for the data shown in Figures S3, including the calculated error bars, were defined as high affinity (1 – 5 µM), intermediate affinity (6 – 20 µM), and low affinity (21 – 90 µM). The gray box in the lower panel indicates different labels and the specifications of the GRB2 variants, which are summarized and explained in Table S2.

**Figure 5.** The interdomain interactions of GRB2 determined by ITC measurements. Peptide-protein and protein-protein interactions were measured by isothermal titration calorimetry (ITC). Evaluated $K_d$ values in µM (a-o) for the ITC data shown in Figure S4 are divided into high affinity (1 – 5 µM), intermediate affinity (6 – 20 µM), low affinity (21 – 90 µM) and very low affinity (91 – >200 µM). The standard deviations for all $K_d$ values ranged from 10 to 15%. The gray box in the lower panel indicates different labels and the specifications of the GRB2 variants that are summarized and explained in Table S2.

**Figure 6.** Schematic models of GRB2 association with SOS1 and HER2. (a) Color-coded SOS1 PRD peptides P1 – P10 (see also Fig. 3a and Table S1). (b) Selected GRB2 models [47], where the relative position and orientation of the nSH3 and cSH3 domains are different (1 to 3). The nSH3-cSH3 interaction (i) and the PRM interaction capability of the SH3 domains (ii) are likely to be a functional prerequisite for potential bivalent SOS1 binding. (c) The selectivity of the GRB2-SOS1 PRD interaction. Items 1, 2 and 3 illustrate a recently proposed model [32] that favors P3 and P10 as distant binding sites for GRB2 nSH3/cSH3 due to structural flexibility. Our data do not support this model for two reasons. One is the lack of SOS1 P10 binding to GRB2, and the other is the allosteric interdomain communication of GRB2 required for the GRB2-SOS1 interaction. Accordingly, we propose that GRB2-SOS1 complex formation involves the successive binding of both SH3 domains (4 – 6). This presumes restricted structural flexibility, which is facilitated by adjacent or even tandem binding sites, such as P3 and P4. (d) HER2-GRB2-SOS1 complex formation at the plasma membrane (PM). Upper panels: RTKs or other transmembrane proteins are inactive (nonphosphorylated). Lower panels: Activation of RTKs or transmembrane proteins through tyrosine phosphorylation is shown by the arrow. (1) In this scenario, GRB2 exists in a dimeric autoinhibited state and does not bind SOS1. Growth factor stimulation induces sequential interaction of GRB2 with HER2 (i) and PM (ii), which leads to GRB2 monomerization and subsequent binding to and translocation of SOS1 (iii). (2) The cytosolic GRB2-SOS1 complex translocates to phosphorylated HER2, where pYP binding of the SH2 domain (i) induces its association with PM phospholipids (ii). (3) Another possible scenario, a cytosolic GRB2-SOS1 complex, is formed only by one SH3 domain, while the other SH3 domain (e.g., cSH3) is blocked intramolecularly. The complex translocates to the membrane upon phosphorylation of HER2, pYP binding of the SH2 domain (i) induces not only the SH2-PM interaction (ii) but also a conformational change in GRB2 that releases cSH3 and is now able to contact the SOS1 PRD at a second site (iii). In this model, the HER2-GRB2 association may also confer SOS1 activation. The gray box in the lower panel indicates different labels.
Figure 2

**a**

- Input: GST, WT, W36K, W193K, GRB2 FL
- Output: Anti SOS1 antibody signal

**b**

- Input: GST, GRB2 FL, WT, W193K
- Output: Anti SOS1 antibody signal

Legend:

- GST
- WT
- W36K
- W193K
- GRB2 FL

*Note: n.s. indicates not significant.*
Figure 4

- **a)** FL + RP1
- **b)** nSH3 or cSH3 + RP1
- **c)** FL or SH2 + YP or pYP
- **d)** FL + RP1/pYP
- **e)** ΔSH2 + RP1
- **f)** ΔcSH3 or ΔnSH3 + RP1/pYP
- **g)** nSH3 or cSH3 + RP1 + SH2/pYP
- **h)** ΔPHP-EQV + RP1/pYP
- **i)** ΔPHP or ΔEQV + RP1/pYP
- **j)** 6allo or RP1/pYP
- **k)** nSH3 (3allo) or cSH3 (3allo) + RP1

**Figure Legends:**

- SOS1 reference peptide (RP1)
- W to K mutation
- ΔPHP: PH-domain deletion in Linker 1
- 6allo: M19/K196/R199/T202R
- 3allo: M19/R196/Y199/T202R
- Numbers: K values in μM
- n.b.: no binding
- RP1, YP, or pYP: fluorescent peptides

**Notes:**

- HER2 Y-peptide (YP)
- HER2 pY-peptide (pYP)
- W to D mutation
- ΔEQV: EQV deletion in Linker 2
- W to A mutation
Figure 5

![Diagram of molecular interactions](https://example.com/diagram.png)

- **a)** RP1 + FL
- **b)** RP1 + nSH3
- **c)** RP1 + cSH3
- **d)** nSH3 + SH2
- **e)** nSH3 + SH2/YP
- **f)** cSH3 + SH2
- **g)** cSH3 + SH2/YP
- **h)** SH2 + nSH3/RP1

- **i)** SH2 + cSH3/RP1
- **j)** RP1 + nSH3/SH2
- **k)** RP1 + nSH3/SH2/YP
- **l)** RP1 + cSH3/SH2
- **m)** RP1 + cSH3/SH2/YP
- **n)** nSH3 + cSH3
- **o)** nSH3 (allo) + cSH3 (allo)

- **nSH3**
- **SH2**
- **cSH3**

- **L1, L2**
- **SOS1 reference peptide (RP1)**
- **HER2 pY-peptide (pYP)**

- **Kd** values in μM
- **n.b.** no binding

**nallo** or **callo**: M1R/Y37DK50D or M198R/K199D/T202R

Numbers: Kd values in μM

- **200**
- **120**
- **>200**
- **100**
- **>200**
- **5.3**
- **6.6**
- **5.0**
- **>200**
- **n.b.**

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