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High-Affinity Interactions of the nSH3/cSH3 Domains of Grb2 with the C-Terminal Proline-Rich Domain of SOS1

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Abstract

Grb2 is an adaptor protein that recruits Ras-specific guanine nucleotide exchange factor, Son of Sevenless 1 (SOS1), to the plasma membrane. SOS1 exchanges GDP by GTP, activating Ras. Grb2 consists of an SH2 domain flanked by N- and C-terminal SH3 domains (nSH3/cSH3). Grb2 nSH3/cSH3 domains have strong binding affinity for the SOS1 proline-rich (PR) domain that mediates the Grb2–SOS1 interaction. The nSH3/cSH3 domains have distinct preferred binding motifs: PxxPxR for nSH3 and PxxxRxxKP for cSH3 (x represents any natural amino acid). Several nSH3-binding motifs have been identified in the SOS1 PR domain but none specific for cSH3 binding. Even though both nSH3 and cSH3 exhibit the strongest binding to the SOS1 peptide PVPPPVPPIRRRP, this mutually exclusive binding combined with other potential nSH3/

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Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.9b10710>.

Full details of NMR titration experiments, including titration curves, overlaid spectra, and chemical shift perturbations mapped on the modeled structures of nSH3/cSH3–SOS1 peptides, as well as modeled complexes of full-length Grb2 with an SOS1 PR domain (PDF)

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cSH3 binding regions in SOS1 makes understanding the Grb2–SOS1 interaction challenging. To identify the SOS1–cSH3 binding sites, we selected seven potential binding segments in SOS1. The synthesized peptides were tested for their binding to nSH3/cSH3. Our NMR data reveal that the PKLPPKTYKREH peptide has strong binding affinity for cSH3, but very weak for nSH3. The binding specificity suggests that the most likely Grb2–SOS1 binding mode is through nSH3–PVPPPVPVPPRRP and cSH3–PKLPPKTYKREH interactions, which is supported by replica-exchange simulations for the Grb2–SOS1 complex models. We propose that nSH3/cSH3 binding peptides, which effectively interrupt Grb2–SOS1 association, can serve as tumor suppressors. The Grb2–SOS1 mechanism outlined here offers new venues for future therapeutic strategies for upstream mutations in cancer, such as in EGFR.

Graphical Abstract

INTRODUCTION

Growth factor receptor bound protein 2 (Grb2) is an adaptor protein that transmits downward growth factor signals. In response to epidermal growth factor receptor (EGFR) activation, Grb2 establishes a link between an active receptor tyrosine kinase (RTK) and Ras-specific guanine nucleotide exchange factor (GEF) such as Son of Sevenless 1 (SOS1) at the plasma membrane.^{1–5} SOS1 exchanges GDP by GTP, activating small GTPase Ras.^{6,7} Ras regulates cell proliferation through the mitogen-activated protein kinase (MAPK, Raf/MEK/ERK) and phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR pathways.^{8–11} Ras oncogenes frequently result in human somatic malignancies. However, therapeutic targeting of oncogenic Ras remains unsuccessful. Most efforts focus on blocking Ras regulators and effectors, such as SOS1, B-Raf, and PI3K α .^{12–14} The Grb2–SOS1 interaction has attracted increasing attention in the clinic, and recent targeted therapy has shown that synthesized peptide inhibitors can interrupt the Grb2–SOS1 interaction, effectively preventing tumor development.^{15–20}

Grb2 (residues 1–217) contains three successive Src homology (SH) domains: N-terminal SH3 (nSH3, residues 1–58), SH2 (residues 60–152), and C-terminal SH3 (cSH3, residues 156–215) domains (Figure 1A). X-ray crystal structure analyses illustrated that the adaptor

protein forms an autoinhibited homodimer.²¹ Specific, high-affinity interactions of the SH2 domain with the phosphorylated tyrosine (pY) containing motif pYxNx (x represents any natural amino acid) of the RTK^{22,23} suggest that Grb2 can release the autoinhibition upon binding to the pY-containing motif, liberating its “arms”, the nSH3/cSH3 domains. The released domains recruit an SOS1 C-terminal proline-rich (PR) domain, relocating SOS1 to the plasma membrane.²⁴ GTP-bound Ras binding to the exposed SOS1 allosteric site at the Ras exchange motif (REM) leads to Ras GDP/GTP exchange at the catalytic site of CDC25.²⁵ SOS1 activates Ras at the plasma membrane, which activates MAPK signaling in the cytoplasm. Thus, the Grb2–SOS1 interaction activating SOS1 is important for the Ras activation.

SH3-binding ligands typically contain the canonical PxxP motif, which tends to fold into a polyproline type II (PPII) helix.^{26,27} Ligands binding to Grb2 nSH3 have the consensus PxxPxR motif with an arginine abutting the PxxP core. By contrast, cSH3-binding ligands do not have the PxxP motif. Instead, they contain the core sequence RxxK, which stabilizes the cSH3 negatively charged pockets.²⁸ The cSH3 binding motif PxxxRxxKP, which contains a proline adjoining the RxxK core, enhances the binding affinity for cSH3 even though the exact function of the proline has been unclear.²⁹ Grb2-associated-binding protein 2 (Gab2), with an APPPRPPKP motif with a proline at the last position, has a strong binding affinity ($K_D \approx 50 \mu\text{M}$) for cSH3.³⁰ This motif contains a nonproline residue at the first position and folds into a PPII helix. Unlike the nSH3 binding motif PxxP, the cSH3 binding motif RxxK has not been defined only as a PPII helix; rather, upon binding cSH3, the PPVNRNLKP motif (RxxK) of Gab2 forms a 3_{10} helix,^{30,31} suggesting that the cSH3 binding motif has distinct binding modes, rendering the puzzle still unsolved.

The Grb2 interaction with the SOS1 PR domain is at least 100-fold stronger than the interactions of the individual nSH3/cSH3 domains with the truncated SOS1 PR peptides.³² This suggests that Grb2–SOS1 association likely involves simultaneous binding of nSH3 and cSH3 domains to the SOS1 PR domain. However, the SOS1 PR domain lacks the cSH3 PxxxRxxKP binding motif. It was reported that cSH3 may interact with the SOS1 PR domain through the nSH3 PxxPxR binding motif.³³ A sequence search for PxxPxR shows four distinct binding sites on SOS1: PVPPPVPPIRRRP, DSPPAIPPRQPT, ESPPLLPPREPV, and IAGPPVPPIRQST (Figure 1B). Both nSH3/cSH3 have the strongest binding affinity for PVPPPVPPIRRRP, but nSH3 outcompetes cSH3. Besides, cSH3 has an extremely weak binding affinity for the rest of the segments ($K_D > 1000 \mu\text{M}$). The mutually exclusive nSH3/cSH3 binding to PVPPPVPPIRRRP suggests that cSH3 may use an additional, still undiscovered, binding site.

To clarify how Grb2 interacts with SOS1, here we examine the binding affinity of nSH3/cSH3 for selected SOS1 PR peptides by NMR measurements. The analysis reveals a previously undiscovered strong cSH3 binding motif in SOS1. We suggest likely binding modes of Grb2–SOS1 based on the analysis of binding affinities. Since structural data of the Grb2–SOS1 complex are unavailable, to verify the concurrent nSH3/cSH3–SOS1 associations, we construct the four most probable Grb2–SOS1 complex models and sample the conformational ensembles of complexes by using replica-exchange molecular dynamics (REMD) simulations. The sampling shows that concurrent nSH3/cSH3 binding is influenced

by steric constraints. Clarifying how Grb2 nSH3/cSH3 domains associate with SOS1 may facilitate the development of peptide inhibitors to prevent Ras activation.

The various SOS1 PR peptides are selected by their unique motifs and denoted S1 to S10. The segments are aligned by the critical “R/K” residues.

RESULTS

Candidate nSH3/cSH3 Binding Sites on the SOS1 PR Domain.

In order to identify potential nSH3/cSH3 binding motifs, we analyzed the amino acid sequence of SOS1 PR and used NMR to confirm and characterize the binding interactions. The strong interaction between nSH3/cSH3 and SOS1 is due to electrostatics. Unlike the basic SH2 with an isoelectric point, $pI = 9.11$, nSH3/cSH3 are acidic with $pI = 4.96$. The nSH3/cSH3 binding motifs always involve at least an arginine or lysine. For example, structural data show that nSH3 associates with SOS1 VPPPVPPIRRR and cSH3 with Gab2 APPPRPPKP with high affinity (Figure 1A). Four nSH3 binding peptides, PVPPPVPPIRRR, DSPPAIPPRQPT, ESPLLPPREPV, and IAGPPVPPIRQST, were characterized. APNSPRTPLTPPPAYS was selected due to its PxxPR, which is similar to the nSH3 binding PxxPxR motif (with an extra tyrosine present between alanine and serine). To examine the cSH3 binding sites, we selected the three R/KxxK-embedding peptides LEIEPRNPKP, PKPLPRFPKK, and PKLPPKTYKREH. Since the continuous arginines are critical for nSH3/cSH3 binding, we also tested the peptide PSPHGTRRHLPSP. A recent study indicates Grb2 cSH3 binding to Vav nSH3 via the PPPPG motif.^{34,35} Thus, the PSPFTPPPPQTPSP sequence, which contains four successive Pro, was also selected. These SOS1 peptides are designated S1–S10 following the sequential residue numbers (Table 1).

Binding interface on nSH3/cSH3 Domains.

Structural analysis uncovered the binding surface on nSH3/cSH3 domains (Figures 2A and 3A). SH3 binding motifs usually form a PPII helix and require positively charged residues. The PPII helix lacks internal hydrogen bonds, and the backbone adopts the SH3 hydrophobic pockets. The nSH3 hydrophobic pocket is composed of residues Tyr7, Phe9, Trp36, Phe47, Pro49, and Tyr52.³⁶ The nSH3–VPPPVPPIRRR peptide interaction shows that Asp15 and Asp33 of nSH3 form salt bridges with the peptide's arginines (Figure 2A). Noticeably, Asp15 and Glu16 are surrounded by nonpolar residues Phe9, Ala11, Ala13, Trp36, and Phe47. Thus, the VPPPVPPIRRR arginines particularly target the isolated Asp15 and Glu16. The high specificity and strong interaction of nSH3 with VPPPVPPIRRR results in a high binding affinity. By contrast, the cSH3 hydrophobic pocket consists of residues Phe165, Phe167, Trp193, Phe205, Pro206, and Tyr209.³⁷ For the cSH3–APPPRPPKP complex, Glu171 and Glu174 of cSH3 form salt bridges with the arginine and lysine from the peptide (Figure 3A).³⁸ Unlike the isolated Asp15 and Glu16 of nSH3, Glu171 and Glu174 together with Asp168, Gln170, and Asp172 form a large negatively charged cluster. The arginine and lysine of APPPRPPKP stabilize the interaction with cSH3.

Sequence alignment of nSH3 and cSH3 shows an agreement in the binding interface for the hydrophobic residues but not for the negatively charged residues (Figure S1A). The hydrophobic residues Tyr7, Phe9, Trp36, Phe47, Pro50, and Tyr52 of nSH3 are aligned with Phe165, Phe167, Trp193, Phe205, Pro206, and Tyr209 of cSH3. However, the critical residues Asp15 and Glu171 for the salt bridge interaction are not aligned, suggesting that nSH3 and cSH3 have distinct binding motifs.

The ^1H – ^{15}N NMR spectra of free nSH3/cSH3 and the assigned amide signals are shown in Figures S2A and S3A. During the course of the titration, we observed strong signal attenuation in nSH3 residues Lys10, Lys26, and Asn35. Apo-nSH3 Lys10 and Lys26 exhibited slow exchange on the chemical shift time scale. When SOS1 peptides were titrated into nSH3 approximately to the 2:1 molar ratio, the signals of these residues exhibited a switch to the fast exchange regime. Conversely, Asn35 exhibited significant signal attenuation upon addition of the peptides.

nSH3 Shows Stronger Affinity for the PxxPxR Motif.

S1 (LEIEPRNP KP) displays weak affinity for nSH3 (Table 2). S1 is missing the nSH3 binding PxxPxR motif but involves two Glu followed by a PxxP motif embedding “RxxK”. Small amide signal shifts were observed when the titrant reached the end point of titration (Figures S1B and S5A). Similar to S1, S2 (PKPLPRFPKK) does not follow the PxxPxR motif. S2 has a “PxPxP” motif followed by “RxxK”. The “PxPxP” motif less likely forms a regular PPII helix. Surprisingly, S2 showed fairly strong binding to nSH3, with $K_D \approx 250 \mu\text{M}$ (Table 2), and induced large chemical shift perturbations (CSPs) in Glu2, Phe9, Asp15, Asn35, Tyr37, Phe47, and Tyr52 (Figures 2 and 4A), suggesting that nSH3 may alternatively bind to S2 through the PxxPxK motif. Thus, a substituted lysine for arginine reduces the binding affinity but retains the nSH3 binding motif.

The S4 (PVPPPVP RRRR) peptide has the strongest affinity for nSH3 (Table 2). With several prolines, S4 forms a well-structured PPII helix, and the three successive arginines “RRR” form salt bridges with Asp15 and Glu16 (Figure 2A). Asp15 pairs the first arginine, and Asp33 interacts with the last arginine. The middle arginine has the least binding effect. In addition to Asp15, residues Tyr7, Phe9, Trp35, Phe47, and Tyr52 exhibited large CSPs likely due to the hydrophobic interactions (Figures 2 and 4C). Similarly, S5 (DSPPAIPPRQPT), S6 (ESPPLL PREPV), and S9 (IAGPPVP PRQST) peptides also follow the nSH3 binding motif PxxPxR, showing a small K_D for nSH3 (Table 2).³³ The K_D values of nSH3–S5/S6/S9 are 56, 117, and 82 μM . Unlike the strong binding between nSH3 and S4, S5, S6, and S9, S3 (APNSPRTPLTPPPAYS) contains a critical arginine adjacent to multiple prolines but still shows weak binding to nSH3, judging by the overall small CSPs (Figure S5C, Table 2). The amino acid sequence of S3 contains the PxxPR motif instead of PxxPxR. A missing amino acid between PxxP and “R” results in the mismatch to the peptide binding pocket on nSH3. The weak affinity of nSH3–S3 suggests nSH3 does not favor the PxxPR motif (Figures S2D and S5C).

Without any positively charged residues, S7 (PSPFTPPPPQTPSP) weakly interacts with nSH3 and induces small chemical shifts (Table 2, Figure S2F, and Figure S5E), suggesting that to trigger the association the nSH3-binding motif requires a critical arginine or

lysine. Since the positively charged residues are essential for interaction with nSH3, S8 (PSPHGTRRHLPSP) involving “RR” may associate with nSH3; however, unlike the other SH3 binding candidates, S8 contains no prolines proximal to “RR”. Nonetheless, even though S8 does not follow the nSH3 binding PxxPxR motifs, it displays a fair affinity for nSH3 ($K_D \approx 210 \mu\text{M}$) (Table 2). Interestingly, Asp15, Phe47, and Tyr52 still form strong interactions (inferred from large CSPs); however, Tyr7 and Phe9 show smaller CSPs. Instead, Glu2, Ile4, and Thr12 exhibit large CSPs (Figures S2G and S5G), which suggests that the nSH3–S8 interaction may have an atypical binding interface, resulting in a binding affinity not as strong as for the typical nSH3 binding motif.

S10 (PKLPPKTYKREH) has weak affinity for nSH3, with $K_D \approx 660 \mu\text{M}$ (Table 2). Even though this peptide contains a PxxPxK motif, which is similar to S2, the nSH3–S10 interaction is much weaker, and the overall signal shifts are smaller than for S2 (Figures 2 and 4E). Except for Asp15, typical interface residues Tyr7, Phe9, Phe47, and Tyr52 exhibit relatively smaller CSPs. Instead, Glu2, Ile4, Thr12, and Ser18 have larger CSPs. Noticeably, S10 and S8 induce similar CSP patterns in nSH3, implying a similar binding interface to nSH3–S8, but weaker binding affinity.

S4 and S10 Display Strong and Comparable Affinity for cSH3.

S1 (LEIEPRNPKP) has very weak affinity for cSH3 (Table 2) and induces small CSPs (Figures S3B and S5B). The observed CSPs are too small to determine the K_D value. Comparison of the S1 sequence with the cSH3 binding PxxxRxxKP motif indicates that S1 lacks the first proline and has a glutamate instead. Even though cSH3 binding favors the RxxKP motif, the S1 glutamate may repulsively interact with Asp166, resulting in weak association. Similar to S1, the S2 (PKPLPRFPKK) peptide also has the core “RxxK” motif. However, the front proline is not at the right position. The “PxPxP” motif is not favored to form a stable PPII helix. At the rear of S2 there are two consecutive lysines instead of “KP”. Even though S2 barely follows the cSH3 binding PxxxRxxKP motif, it binds cSH3 with a $K_D \approx 400 \mu\text{M}$, much stronger than in the case of S1 (Table 2). Interestingly, the S2 RxxK interaction with cSH3 induces small CSPs around Glu171, but the PxPxP region results in larger CSPs at Trp193 and Phe205 (Figures 3 and 4B).

Of all the peptides, S4 (PVPPPVPPIRRR) displays the highest affinity for cSH3 with $K_D \approx 142 \mu\text{M}$ (Table 2). Unlike the “RxxK” motif interacting with Glu171 and Glu174, the “RRR” motif strengthens the interaction with Glu171, Asp187, and Asp190.³³ In addition, Trp193, Phe205, and Tyr209 also have large CSPs due to hydrophobic interactions (Figures 3 and 4D). Our S4 results are consistent with a previous study.³³ However, without the “RRR” motif, the single salt bridge³³ formed by cSH3’s Glu171 with the arginines in S5 (DSPPAIPPRQPT), S6 (ESPPLLPPREP), S9 (IAGPPVPPRQST), and possibly S3 (APNSPRTPLTPPPAYS) does not strengthen the cSH3 binding. Our NMR data indicate that the cSH3–S3 interaction is very weak (Table 2), as inferred from the practically negligible signal shifts (Figures S3D and S5D). This is consistent with the result of the previous study, which found that cSH3 also has very weak affinity for S5, S6, and S9 ($K_D > 1000 \mu\text{M}$). Since “RRR” is so important for nSH3/cSH3 binding, an alanine scan was performed for S3, showing that PVPPPVPPIRRAP has $K_D = 72 \mu\text{M}$ for nSH3 and $K_D = 318 \mu\text{M}$ for cSH3.³³

Even though the alanine mutation decreases the binding affinity, the “RR” still results in S3 binding to cSH3 stronger than S5, S6, or S9. Apart from S4, another “RR” segment also exists in the SOS1 PR domain, and thus S8 (PSPHGTRRHLPSP) was selected for the test.

S8 (PSPHGTRRHLPSP) contains an “RR” motif but exhibits weak binding affinity for cSH3 (Table 2). Since the “RR” motif lacks the proximal prolines, S8 cannot form a PPII helix and fit into the cSH3 hydrophobic pocket, resulting in the overall small CSPs of cSH3 (Figures S3G and S5H). The weak affinity suggests that the proximal prolines in “RR” are important for cSH3 binding. By contrast, the S7 (PSPFTPPPPQTPSP) peptide contains multiple prolines and has the “PPPP” motif. Unlike the strong binding between cSH3 and the “PPPPG” motif of the Vav nSH3 domain,^{34,35} S7 induced only minor signal shifts in cSH3 (Figures S3F and S5F), indicative of very weak binding (Table 2). Even though S7 contains a PxxPxxPxxP motif, three continuous PxxP, most likely forming a robust PPII helix, without positively charged residues it is incapable of forming a stable complex with cSH3.

S10 (PKLPPKTYKREH) has strong binding affinity for cSH3 ($K_D \approx 154 \mu\text{M}$), which is a novel interaction unreported previously. The affinity of cSH3–S10 is as strong as the cSH3–S4 interaction (Table 2). Noticeably, the S10 peptide does not follow the cSH3 PxxxRxxKP binding motif. The first proline is not located in the right position. Like the RxxK motif, S10 contains a “KxxK” motif, which, however, is followed by an additional arginine instead of proline. For the KxxK motif, the substituted first lysine for arginine does not result in an expected low binding affinity of cSH3–S10. Surprisingly, S10 induces very large CSPs near Trp193, Phe205, and Tyr209 but relatively low CSPs around Glu171 (Figures 3 and 4F), which suggests that the cSH3–S10 complex is characterized by stronger hydrophobic interactions than the salt bridges.

Construction of Grb2–SOS1 Complex Models.

To probe the Grb2–SOS1 interaction, we constructed the models for Grb2–SOS1 complexes formed by concurrent binding of both nSH3 and cSH3 to SOS1, based on the (pairwise) affinities of nSH3/cSH3 binding to individual SOS1 peptides. The pairwise affinities suggest the most probable complex models might be nSH3–S4/cSH3–S10, followed by nSH3–S5/cSH3–S4, nSH3–S5/cSH3–S10, nSH3–S9/cSH3–S4, nSH3–S9/cSH3–S10, nSH3–S6/cSH3–S4, nSH3–S6/cSH3–S10, etc. The four most probable Grb2–SOS1 binding modes are nSH3–S4/cSH3–S10 (mode 1), nSH3–S5/cSH3–S4 (mode 2), nSH3–S5/cSH3–S10 (mode 3), and nSH3–S9/cSH3–S4 (mode 4). The corresponding models were initially constructed using the available NMR/crystal structures and sampled by replica exchange simulation (Figures S6 and 5). The contact frequencies between nSH3/cSH3 and SOS1 were analyzed over the conformational ensembles of each mode. The high contact frequency of modes 1, 3, and 4 suggests a stable Grb2–SOS1 interaction; for mode 2, nSH3 loses contact with S5 and tends to dissociate, while the cSH3–S4 contact remains stable.

The linkers between two distinct nSH3/cSH3 binding sites may play a critical role in stabilizing the Grb2–SOS1 interaction. Among the four binding modes, the linkers are 143 residues long for mode 1, 17 residues for mode 2, 114 residues for mode 3, and 127 residues for mode 4. The long linkers of modes 1, 3, and 4 allow the large structural

fluctuation needed to relocate nSH3/cSH3. By contrast, the short linker of mode 2 restricts the structural flexibility between S4 and S5. While S4 contains “RRR”, S5 has only a single “R”, hence weaker nSH3 binding. In addition, linker fluctuations unstructure S5, which further destabilizes the nSH3–S5 association. Thus the nSH3–S5 interaction is more likely affected by the structural relaxation of the short linker. The orientation of nSH3 retains the salt bridge with S5 but loses hydrophobic interactions. The simulations suggest that the Grb2–SOS1 interaction may rely on modes 1, 3, and 4 with a long linker more than on the less stable mode 2 with the short linker.

The contact frequency shows that the nSH3–SOS1 and cSH3–SOS1 interactions are interdependent. For modes 1 and 3, cSH3 binds to S10, while nSH3 binds to S4 or S5, respectively; nSH3–S4 has higher contact frequency than nSH3–S5 does, resulting in closer cSH3–S10 contact. Similarly, in the case of modes 2 and 4, unlike the nSH3–S9 complex, which shows stronger interaction in mode 4, the nSH3–S5 complex in mode 2 is weaker and loses contact, leading to the lower contact frequency of cSH3–S4. Thus, nSH3–SOS1 binding enhances cSH3–SOS1 interaction, which then strengthens the nSH3–SOS1 interaction. The mutual influence of nSH3–/cSH3–SOS1 interactions limits the probable binding modes of Grb2–SOS1.

DISCUSSION

Activation of Ras by hormone-activated EGFR is mediated by Grb2 adaptor protein, which recruits SOS1 to the plasma membrane. SOS1 exchanges GDP by GTP, activating Ras. Ras then activates its effectors, e.g., Raf, PI3K α , and RASSF5 (NORE1A), and their respective signaling pathways, MAPK, PI3K/Akt/mTOR, and Hippo.^{39,40} To obtain a mechanistic insight into how Grb2 recruits SOS1, we aim to elucidate their interaction, which to date is still not entirely understood. Exploiting NMR experiments and replica exchange computer simulations we examined seven potential SH3 binding sites on SOS1: S1, S2, S3, S4, S7, S8, and S10. Including the previously tested S5, S6, and S9, the results suggest that nSH3 has stronger binding affinity for SOS1 than cSH3 does. The SOS1 PR domain involves several occurrences of the nSH3 binding PxxPxR motif (S4, S5, S6, and S9) but lacks the cSH3 binding motif PxxxRxxKP. Even though cSH3 has weak binding affinity for the nSH3 binding motif, it displays strong binding to S4 (PVPPPVPpRRRP), which contains three successive arginines (“RRR”). However, the nSH3–S4 binding is still stronger than cSH3–S4. Therefore, nSH3–SOS1 association can outcompete cSH3–SOS1. Previous studies indicated that cSH3 has strong binding affinity for Gab1/2, which suggests that Grb2 is more likely to form a ternary structure with SOS1/2 and Gab1/2 where nSH3 binds to SOS1/2 and cSH3 associates with Gab1/2. SOS1/2 and Gab1/2 transduce MAPK and PI3K signaling separately.^{29,41} However, the Grb2–SOS1/2–Gab1/2 ternary structure does not explain the high affinity of the 1:1 Grb2–SOS1 interaction. We notice that the cSH3 core binding motif “R/KxxK” exists at both N- and C-termini of the SOS1 PR domain, which is different from the nSH3 binding motif PxxPxR, which locates in the middle. The “R/KxxK” motifs (S2 and S10) provide alternative binding sites for cSH3. We suggest that nSH3–SOS1 binding does not block the cSH3–SOS1 binding. The multiple alternative binding sites could allow simultaneous nSH3/cSH3 binding to SOS1 peptides in various binding modes.

Our NMR data (CSPs) point to distinct binding modes of nSH3/cSH3 with SOS1 peptides (Figures 4, S4, and S5). For nSH3, S4 (PVPPPVPPIRRP) follows PxxPxR, which forms a robust PPII helix and provides large positively charged residues, inducing high CSPs around Tyr7, Phe9, Asp15, Trp35, Tyr37, Phe47, and Tyr52. S2 (PKPLPRFPKK) shows weaker affinity albeit similarly high CSPs at the typical nSH3 binding interface. However, S10 (PKLPPKTYKREH) contains the PxxPxK motif, which resembles PxxPxR but induces relatively high CSPs at the nSH3 atypical binding interface Glu2, Ile4, Thr12, Asp15, and Ser18. The nSH3 atypical binding interface is also involved in the nSH3 interaction with S8 (PSPHGTRRHLPSP). Lacking the PxxPxR motif, S1 (LEIEPRNP KP), S3 (APNSPRTPLTPPPAYS), and S6 (PSPFTPPPPQTPSP) induce very small CSPs indicative of low binding affinity. Therefore, we conclude that the nSH3 binding motif strictly follows PxxPxR. The R → K mutation results in an atypical binding interface of nSH3 and lower binding affinity. For cSH3, even though the S4 region (PVPPPVPPIRRP) does not follow the cSH3 binding motif PxxxRxxKP, it induces large CSPs at the typical peptide-binding surface of cSH3 (Asp168, Gln170, Glu171, Trp193, Phe205, Arg209, and Tyr209) and displays strong binding affinity. By contrast, S1 (LEIEPRNP KP) contains the “RxxKP” motif but results in weak binding likely due to the repulsion between negatively charged residues of S1 and cSH3. S2 (PKPLPRFPKK) also has the “RxxK” motif and shows stronger affinity than S1. The “PxPxP” region of S2 induces higher CSPs in Trp193, Phe205, and Arg209 than in Asp168 and Glu171. Unlike S2, S10 (PKLPPKTYKREH) involves the “PxPxP” motif followed by “KxxK” and shows a surprisingly strong binding to cSH3. S10 does not induce the same large CSPs at the typical binding surface on cSH3. The “KxxK” motif forms weaker salt bridge interactions than the “RxxK” does, reflected in significantly smaller induced CSPs at Asp168 and Glu171. However, large CSPs are observed for Asn192, Trp193, Trp194, Phe205, and Arg207. This implies that KxxK triggers cSH3–S10 association, in which the weaker salt bridges are compensated by hydrophobic interactions. Thus, S10 interaction with cSH3 involves a binding mode distinct from cSH3–S4 but exhibits comparable and strong affinity. Notably, S10 is specific to cSH3, not nSH3. Therefore, unlike the S4-dependent inhibitors blocking both nSH3/cSH3–SOS1 interaction, we suggest that an S10-based inhibitor may only interrupt cSH3–SOS1 association, suppressing PI3K signaling.

Taken together, the binding affinities of individual nSH3/cSH3–SOS1 peptide interactions elucidate how Grb2 may associate with the SOS1 PR domain by engaging nSH3/cSH3 concurrently. Among S1–S10, S4 demonstrates the strongest binding affinity for nSH3. The similar affinities of nSH3 for S4, S5, S6, and S9 suggest that nSH3 has four alternative binding sites on the SOS1 PR domain. cSH3 shows the strongest binding to S4, which is an order of magnitude stronger than to S1, S3, S5, S6, S7, S8, or S9. Because of the exclusive cSH3 binding at S4, the previous study suggested that the interaction of Grb2–SOS1 requires cSH3 binding to S4 and nSH3 binding to one of the other alternative binding sites, most likely S5, which has the second strongest affinity. Here we identified S10 as another cSH3 binding site, comparable to S4 in affinity. Based on our results, cSH3 has three alternative binding sites: S2, S4, and S10. While cSH3 binding to S4 is about two times stronger than to S2, the cSH3–S10 binding is roughly similar. We thus propose that

there are multiple binding modes of Grb2–SOS1 interaction, and the most probable binding complex might be nSH3–S4/cSH3–S10.

A recent study suggested that PxxPxR motifs (S4, S5, S6, and S9) are not required for the in vivo interactions of Grb2 with SOS1,⁴² and SOS1 PR domain regions outside the conserved PxxPxR motif could drive high-affinity interactions with Grb2. The S2, S8, and S10 peptides, identified in our work as alternative binding regions for nSH3 and cSH3, may provide the additional interaction sites required for their concurrent (bidentate) binding to SOS1. In vivo, dimeric Grb2 has an inhibitory effect on cell viability but could be disrupted by association with phosphorylated tyrosine of RTK or the phosphorylated Tyr160 of Grb2, resulting in the monomeric, active, Grb2.⁴³ Grb2 mutants may shift the population from dimer to monomer, leading to malignant cancer. Therefore, mechanistic insights into Grb2–SOS1 interactions may enlighten future therapeutic strategies.

To conclude, the nSH3–SOS1 interaction is stronger than cSH3–SOS1.⁴⁴ nSH3 has four similarly strong binding sites, S4, S5, S6, and S9, in SOS1 PR. By contrast, cSH3 has three alternative binding sites, S2, S4, and S10, and cSH3–S2 is weaker than cSH3–S4/S10 interaction. nSH3/cSH3 binding to the various SOS1 peptides takes place through distinct binding modes. The cSH3–S4 interaction is arginine-dependent, inducing large CSPs at residues involved in salt bridges and hydrophobic interactions. By contrast, the CSPs of cSH3–S10 are small for salt bridges but much larger for hydrophobic interactions. The positively charged residues from S10 trigger the association with cSH3, and the proline segment complements the interaction, showing comparable binding affinity to that of cSH3–S4. Thus, when both nSH3 and cSH3 are present, nSH3–S4 association is expected to outcompete cSH3–S4, resulting in the cSH3–S10 complex. Based on the binding affinities for the isolated nSH3 and cSH3, the four most probable binding modes of Grb2–SOS1 were constructed. The simulation suggests that the long linkers of modes 1, 3, and 4 relocate nSH3/cSH3 and stabilize the Grb2–SOS1 interaction. The short linker of mode 2, however, exhibits spatial constraints and large fluctuations, resulting in nSH3–S5 dissociation. Even though nSH3 binds S5 strongly, the high affinity of individual nSH3/cSH3–SOS1 peptides may not favor Grb2–SOS1 association in mode 2. Our experimental and computational results suggest that the most probable binding mode, nSH3–S4/cSH3–S10, mediates Grb2–SOS1 association, thus potentially resolving the Grb2/SOS1 interaction enigma to better understand the mechanism of EGFR-induced Ras activation. Further experimental studies involving full-length Grb2 and SOS1 PR domain are required to test these predictions.

MATERIALS AND METHODS

Sample Preparation and NMR Measurements.

Wild-type human Grb2 nSH3/cSH3 plasmids and selected SOS1 PR peptides were commercially obtained from Biomatik Corporation. The plasmids encoding His₆-tagged constructs were transformed into BL21 competent *E. coli* cells. The cells were grown in M9 media and induced with 1 mM IPTG at 16 °C overnight. The cells were then collected and disrupted by sonication in 50 mM Tris, 100 mM NaCl, 50 mM imidazole, pH 7.6, and NaN₃ 0.02%. The ¹⁵N-labeled proteins were purified by Ni–NTA chromatography and eluted with 200 mM imidazole. The preliminarily purified proteins were buffer exchanged into 50 mM

Tris, 100 mM NaCl, 5 mM DTT, pH 7.6, and NaN₃ 0.02%, followed by size exclusion chromatography.

NMR experiments were performed at 298 K on a Bruker Avance III 600 MHz spectrometer equipped with a cryoprobe. All samples were prepared in 20 mM NaH₂PO₄/Na₂HPO₄, 50 mM NaCl, 5 mM DTT, 10% D₂O, and 0.02% NaN₃ at pH 7.6. The initial protein concentrations of nSH3 were 233, 196, 157, 196, 227, 122, and 122 μ M in binding assays with S1, S2, S3, S4, S7, S8, and S10, respectively; similarly for cSH3, the concentrations were 160, 106, 151, 106, 81, 76, and 81 μ M for binding to S1, S2, S3, S4, S7, S8, and S10. The stock solution concentrations of various SOS1 peptides were 10 times the corresponding initial nSH3/cSH3 concentrations. The data were processed in TopSpin 3.6 and analyzed using Sparky. NMR titrations were performed by adding unlabeled peptides to ¹⁵N-labeled proteins and monitored by ¹H-¹⁵N SOFAST-HMQC spectra recorded at each titration point. The signals of unbound nSH3 and cSH3 domains were assigned based on the published data.^{45,46} Starting protein concentrations were ~100–200 μ M; the titration continued until the peptide/protein molar ratio of 3:1 was reached. Signal shifts were quantitated as amide chemical shift perturbations calculated as follows: $\Delta\delta = \sqrt{(\Delta\delta_H)^2 + (\Delta\delta_N/5)^2}$, where δ_H and δ_N are signal shifts for the ¹H and ¹⁵N resonances, respectively.

The titration curves were fitted to a single-site binding model using the in-house Matlab program Kdfit.⁴⁷ The reported K_D values were determined by global fit for the selected residues, and the reported error was calculated as the standard deviation of the K_D values for the individual fits for the same residues. For the weak binders such as nSH3–S1/S3/S7 and cSH3–S8, the overall small magnitude of the observed CSPs (maximal $\delta < 0.05$ ppm) and the limited number of titration curves precluded statistically reliable determination of the K_D values. These K_D were denoted by the asterisks in Table 2, and both the globally fit K_D and the mean K_D are reported. The cases when the K_D could not be determined due to negligibly small CSPs are denoted by “no binding detected” (NBD).

Grb2–SOS1 Modeling and Simulation.

To construct the Grb2–SOS1 complex, the Grb2 structure was extracted from the Grb2 homodimer structure (PDB: 1GRI). However, the full-length SOS1 PR structure is unavailable. The structure of the SOS1 S4 peptide was obtained from the nSH3–SOS1 (VPPPVPPIRR) complex (PDB: 1AZE).³⁶ By contrast, the cSH3–SOS1 structure is unavailable. We used the backbone conformation of the Gab2 peptide (APPPRPPKP) from the cSH3–Gab2 complex (PDB: 2W0Z) to construct the cSH3–SOS1 structure.³⁰ We built four Grb2–SOS1 complexes: nSH3–S4/cSH3–S10 (mode 1), nSH3–S5/cSH3–S4 (mode 2), nSH3–S5/cSH3–S10 (mode 3), and nSH3–S9/cSH3–S4 (mode 4) (Figure S6). The side chains of Gab2 APPPRPPKP were mutated to the SOS1 peptides accordingly for the four complexes. The rest of the SOS1 disordered regions and Grb2 residues 28–33 were modeled using the MODELLER server.⁴⁸

The Grb2–SOS1 structures were sampled by the REMD simulation. The simulation was performed for 100 ns by using the generalized Born implicit solvent of NAMD2.^{12,49} and the CHARMM42 force field.⁵⁰ The temperature range was 300–320 K, divided into

four windows. The time step was 1 fs, and 1000 steps were used for the exchange attempt. During the simulation, the temperatures were exchanged between trajectories of the four windows. After the simulation, the trajectories were organized by combining the same-temperature frames into the same trajectories. The representative complex structures (Figure 5) were selected from the window with a temperature of 300–305 K. The analysis of contact frequency was performed by measuring the distances between α carbons ($C\alpha$) of the nSH3/cSH3–SOS1 interfaces. The contact was counted when the $C\alpha$ – $C\alpha$ distance was smaller than 12 Å. The protocol follows our previous work.⁴⁰

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Grb2 and SOS1 PR domain. (A) Full-length human Grb2 (PDB: 1GRI) contains 217 residues and is composed of nSH3 (blue, residues 1–58), SH2 (white, residues 60–153), and cSH3 (green, residues 156–215) domains. The polar, nonpolar, positively charged, and negatively charged residues are colored green, black/white, blue, and red, respectively, in the amino acid sequence of Grb2 (top). Available NMR/crystal structures of nSH3/cSH3 domains with the binding partners are nSH3–VPPPVPPRRR (PDB: 1AZE) and cSH3–APPPRPPK (PDB: 2W0Z). (B) Candidate nSH3/cSH3 binding sites on SOS1 are denoted as S1–S10 in the PR domain (residues 1014–1333) amino acid sequence, colored the same way as for Grb2.

Figure 2.

NMR spectra and titration curves of nSH3-S2/S4/S10 binding. (A) Solution structure of the nSH3-VPPPVPPIRRR complex (PDB: 1AZE) shows the typical nSH3-peptide binding interface. Residues colored green, gray, blue, and red represent the polar, nonpolar, positively charged, and negatively charged side chains, respectively. Others are colored white. Binding peptide VPPPVPPIRRR derived from the SOS1 PR domain is shown in orange, and the nitrogen atoms of backbone and side chains are colored blue. The nSH3 residues Y7, F9, W36, F47, P49, and Y52 form hydrophobic interactions with the peptide; D15, E16, and D33 form salt bridges. (B) Superposition of ^1H - ^{15}N NMR spectra of nSH3 in the unbound state (blue) and upon saturation with a 3-fold molar excess of S2 (yellow), S4 (red), and S10 (green). Signals belonging to typical interface residues are emphasized by

rectangles and are zoomed-in in (C) F9, D33, and F47; (D) D15; (E) E16, W36, and Y52; and (F) Y7. (G) Titration curves for selected residues of nSH3 ([P]) upon addition of S2, S4, or S10 ([L]) are plotted as a function of [L]/[P] ratio; the symbols depict experimental amide chemical shift perturbations; the curves are the results of fit to a single-site binding model. The insets show signal shifts during titration for residue D15: [L]/[P] = 0 (blue), 1 (red), 2 (green), and 3 (yellow). In K_D calculations, 14 residues (E2, F9, A11, D15, E16, L17, S18, Y37, A39, F47, K50, N51, Y52, and I53) were used for S2 binding, 15 residues (E2, K6, A11, A13, D15, E16, L17, S18, G22, D23, D33, Y37, F47, K50, and N51) for S4, and seven residues (E2, Y7, T12, D15, S18, N51, Y52) for S10. The buffer condition and the protein concentrations are detailed in the Materials and Methods section.

Figure 3.

NMR spectra and titration curves of cSH3-S2/S4/S10 binding. (A) Crystal structure of cSH3-Gab2 APPPRPPKP complex (PDB: 2W0Z) illustrates how cSH3 interacts with the RxxK motif, forming the binding interface. The coloring of residues follows that in Figure 2. The critical cSH3 residues at the interface, F165, F167, W193, F205, P206, and Y209, form hydrophobic interactions with the peptide; E171, E174, and D190 form salt bridges. (B) Superposition of ^1H - ^{15}N NMR spectra of cSH3 in the unbound state (blue) and at the end point of titration with S2 (yellow), S4 (red), and S10 (green). Signals belonging to the interface residues are emphasized by rectangles and zoomed-in in (C) F205, (D) F167, D190, W193, (E) E171, E174, F209, and (F) F165. (G) Titration curves for the selected residues of cSH3 ([P]) upon addition of S2, S4, or S10 ([L]) are plotted as a function of

[L]/[P] ratio; the symbols depict experimental chemical shift perturbations, the curves are the results of fit to a single-site binding model. The insets show signal shifts during titration for the W193 indole NH group (W193 ϵ): [L]/[P] = 0 (blue), 1 (red), 2 (green), and 3 (yellow). In K_D calculations, six residues (D168, W193 ϵ , W194, F205, R207, and N208) were used for S2 binding, 16 residues (Y160, F165, D166, F167, D168, Q170, E171, D172, G173, W193, W193 ϵ , G196, M204, F205, R207, N208, and Y209) for S4, and 15 residues (Y160, V161, F167, D168, E171, D172, G196, A197, N192, W193, W193 ϵ , W194, M204, F205, R207, and Y209) for S10. The buffer condition and the protein concentrations are detailed in the Materials and Methods section.

Figure 4.

NMR characterization of the peptide binding interface on nSH3 and cSH3. Amide chemical shift perturbations (CSPs, δ) at the titration end point are shown as a function of nSH3 or cSH3 residue number for the following interactions: (A) nSH3-S2, (B) cSH3-S2, (C) nSH3-S4, (D) cSH3-S4, (E) nSH3-S10, and (F) cSH3-S10. The CSPs for indole NH signals of W36 (nSH3) and W193 (cSH3) are shown as bars at positions 60 and 220, respectively, and marked as W36e and W193e. Structure images to the right of the plots show CSPs mapped onto surfaces of nSH3/cSH3 (PDB: 1GRI) in complexes with the corresponding modeled peptides, respectively. The surface coloring is based on the observed CSP values and ranges from white (0 ppm) to red (maximal $\delta = 0.15$ ppm); residues with δ greater than one standard deviation are indicated. The bound peptides are colored based on nonpolar (white), polar (green), negatively charged (red), and positively charged (blue) residues. N and C indicate the N and C termini of peptide.

Figure 5.

The four most probable structural models of the Grb2-SOS1 complex. Grb2-SOS1 interactions are suggested by the strong binding affinity of (A) nSH3-S4/cSH3-S10 (mode 1), (B) nSH3-S5/cSH3-S4 (mode 2), (C) nSH3-S5/cSH3-S10 (mode 3), and (D) nSH3-S9/cSH3-S4 (mode 4). The models of Grb2-SOS1 PR are obtained from replica exchange simulation for 100 ns. Grb2 nSH3, SH2, cSH3, and SOS1 PR domain are colored blue, white, green, and yellow, and the binding peptides (S4, S5, S9, and S10) are shown as red tubes. The heat maps of contact frequency show the interactions between nSH3/cSH3 residues (*y*-axis) and various SOS1 peptides residues (*x*-axis) during the simulation. The dark brown color indicates highly frequent contacts. The three main interaction regions of nSH3/cSH3 are around residues 10, 35, and 50 for nSH3 and 170, 195, and 205 for cSH3. These maps show correlation between nSH3-SOS1 and cSH3-SOS1 interactions. For mode 2, the unfolded S5 tends to dissociate from nSH3, resulting in unstable Grb2-SOS1 association.

Table 1.

Candidate of nSH3/cSH3 Binding Sites

peptide	residues	segment	motif
S1	1014–1023	LEIEPRNPKP	PRxPKP
S2	1021–1030	PKPLPRFPKK	PxPxPRxPKK
S3	1079–1093	APNSPRTPLTPPPAYS	PxxPRxPxxP
S4	1148–1159	PVPPVPVPRRRP	PxxPxR
S5	1177–1188	DSPPAIPPRQPT	PxxPxR
S6	1209–1220	ESPPLLPPREPV	PxxPxR
S7	1253–1266	PSPFTPPPPQTPSP	PxxPPPPxxP
S8	1264–1277	PSPHGTRRHLPSP	RR
S9	1287–1298	IAGPPVPPRQST	PxxPxR
S10	1303–1314	PKLPPKTYKREH	PxxPxKxxKR

Table 2.

Dissociation Constants for nSH3/cSH3–SOS1 Peptide Interaction^a

peptide	this work					ref 32	
	nSH3		cSH3			nSH3	cSH3
	K_D (μ M)	$\delta_{\max}$ (ppm)	K_D (μ M)	$\delta_{\max}$ (ppm)		K_D (μ M)	K_D (μ M)
S1	730 $\pm$ 890*	0.05	NBD	0.02			
S2	250 $\pm$ 96	0.16	440 $\pm$ 146	0.12			
S3	280 $\pm$ 103*	0.05	NBD	0.01			
S4	37 $\pm$ 22	0.38	142 $\pm$ 18	0.39		39 $\pm$ 1	125 $\pm$ 13
S5						56 $\pm$ 5	1396 $\pm$ 87
S6						117 $\pm$ 2	1718 $\pm$ 33
S7	580 $\pm$ 462*	0.04	NBD	0.02			
S8	208 $\pm$ 42	0.14	400 $\pm$ 447*	0.05			
S9						82 $\pm$ 1	1318 $\pm$ 44
S10	660 $\pm$ 320	0.11	156 $\pm$ 33	0.46			

^aThe dissociation constants (K_D , in μ M units) in this work (left columns) were obtained by globally fitting experimental NMR titration data for select residues to a single-site binding model. $\delta_{\max}$ is the largest CSP over all analyzed residues at the titration end point. The asterisks mark the K_D values determined from a limited number of titration curves (hence poor statistics and large standard deviations) due to the very small observed CSPs (maximal $\delta < 0.05$ ppm), reflecting weak binding. In these cases, the mean K_D values from the individual fits for the same residues are 1250 μ M (nSH3-S1), 270 μ M (nSH3-S3), 950 μ M (nSH3-S7), and 650 μ M (cSH3-S8). NBD stands for “no binding detected”. See further details in the Methods section. The K_D values in the right columns are published data from the previous work.³²