Grb2 SH3 binding to peptides from Sos: evaluation of a general model for SH3–ligand interactions

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Background: Grb2 acts as an adaptor protein in the transduction of signals from receptor tyrosine kinases to Ras. It binds to phosphotyrosine on the cytoplasmic tail of cell-surface receptors via its central SH2 domain, and to its immediate downstream target, Sos, via two SH3 domains. The basis of the Grb2–Sos interaction is not fully understood. We previously proposed a model for SH3 domain binding specificity, based on two solution structures of the Src SH3 domain complexed with high-affinity ligands, in which the ligands are bound in a polyproline type II conformation in two distinct orientations, class I and class II. Here, we have used this model to predict the identity and orientation of Grb2 SH3 ligands in the human Sos protein.
Results: Six contiguous fragments from the carboxy-terminal portion of hSos (amino acids 1000–1333), each containing a single potential SH3 binding site, were expressed in *E. coli* as GST fusion proteins. Four of these proteins were predicted to associate with SH3 domains. The amino-terminal Grb2 SH3 domain was shown to bind strongly to only these four fragments.
Conclusions: We have used a general model for SH3–ligand interactions to predict the nature of Grb2 SH3 interactions with the hSos protein. Comparison of the four hSos sequences that bind Grb2 revealed a preference for the PXXPXR motif, consistent with the predicted class II-type binding interaction. The interaction between Grb2 and hSos peptides is predominantly via the amino-terminal SH3 domain, although the carboxy-terminal SH3 domain may increase the overall stability of the Grb2–hSos complex.

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Introduction

Growth factor receptor-bound protein-2 (Grb2) is the most extensively characterized adaptor protein used in growth factor-stimulated signaling pathways [1–4]. It functions in receptor tyrosine kinase-initiated signaling pathways [5] by linking cell-surface receptors to Ras via the human Son-of-Sevenless (hSos) protein, a guanine nucleotide exchange factor [6]. Grb2 is a 25 kDa protein composed of 217 amino acids with a central Src homology 2 (SH2) domain flanked by two Src homology 3 (SH3) domains. Its upstream targets in the transduction of signals from the plasma membrane include the epidermal growth factor receptor (EGFR) and the activated, receptor-associated protein Shc (2,7,8). The human Grb2 gene was cloned on the basis of the association between the protein it encoded and the tyrosine-phosphorylated cytoplasmic domain of the activated EGFR [1]. This interaction was found to be mediated by the SH2 domain of Grb2. SH2 domains — structural modules found in many other proteins involved in signal transduction — bind phosphorylated tyrosine residues in specific primary sequence contexts [9,10]. The Grb2 SH2 domain binds to the sequence Tyr-X-Asn-X (where X can be a variety of amino acids) in a phosphorylation-dependent manner. GRB-3.3, an alternatively spliced variant that lacks part of the SH2 domain, was recently shown to be expressed in high levels in the developing thymus and has been implicated in the induction of apoptosis in thymocytes [11].

The Grb2 SH3 domains are necessary for the interaction between Grb2 and the guanine nucleotide exchange factor hSos [6,12]. SH3 domains are modular units found within a variety of proteins involved in either signal transduction or the cytoskeleton, or both. They are composed of ~60 amino acids and have been shown to effect protein–protein association by binding proline-rich sequences [9,13,14]. The structures of unliganded SH3 domains from a number of different proteins have been determined [15–23]. The solution structure of the SH3 domain of PI3K bound to a peptide ligand has been determined using NMR [24]. More recently, Abl and Fyn SH3 domain complexes have been solved by crystallographic means [25], and the solution structure of the SH3 domain from Src complexed to two proline-rich peptides in two different orientations has been solved by NMR [26]. Sequence similarity between the Src and Grb2 SH3 domains, as well as the similarity of their ligands, has been used to predict the orientation of the Grb2 amino-terminal SH3 domain complexed with a peptide derived from hSos [26].

hSos, the human homolog of the *Drosophila* son-of-sevenless guanine nucleotide exchange factor, contains a central domain that is highly conserved among members of the CDC25 family of guanine nucleotide exchange factors and a proline-rich carboxy-terminal domain [8]. The region of hSos necessary for Grb2 binding was mapped to the proline-rich portion of the protein and candidate

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sequences within this region were identified [3]. Although it is certain that Grb2 binds to proline-rich regions of hSos, it remains unclear whether both SH3 domains bind to the same or different sequences in this domain. Genetic studies on *Caenorhabditis elegans* and *Drosophila* homologs of Grb2 have identified mutations in each of the SH3 domains that render the protein inactive [27–29]. The studies described in this report were designed to determine the role of each SH3 domain in the formation and stability of the Grb2–hSos complex. To do this, we have used a general model for SH3–ligand interactions [26], which proposes that the two classes of SH3 ligands, class I and class II, have distinct, defined binding modes and recognizable motifs. This model correctly predicts the identity of the hSos peptides that bind to Grb2, all of which are class II-type peptides.

Results

Cloning and expression of SH3 domains

The cDNA encoding the Grb2 protein was amplified from a human brain stem cDNA library, using published cDNA sequences [1]. The PCR primers were designed with restriction sites to allow efficient subcloning of the amplified DNAs into the pGEX-2TK vector. In addition to the intact Grb2 protein, individual domains were also cloned into the expression vector. Grb2 (amino acids 1–217), the amino-terminal SH3 domain plus the SH2 domain (Grb2 1–160) and carboxy-terminal SH2–SH3 (Grb2 55–217) proteins were overexpressed in *E. coli* strain BL-21 as glutathione-S transferase (GST) fusion proteins (Fig. 1). The fusion proteins were purified by glutathione agarose chromatography. The carboxy-terminal SH3 domain was proteolytically unstable when expressed as a GST fusion protein. To overcome this problem, the carboxy-terminal SH3 domain was expressed as a fusion with its neighboring SH2 domain, resulting in increased stability. The SH2 domain was found to have no effect on

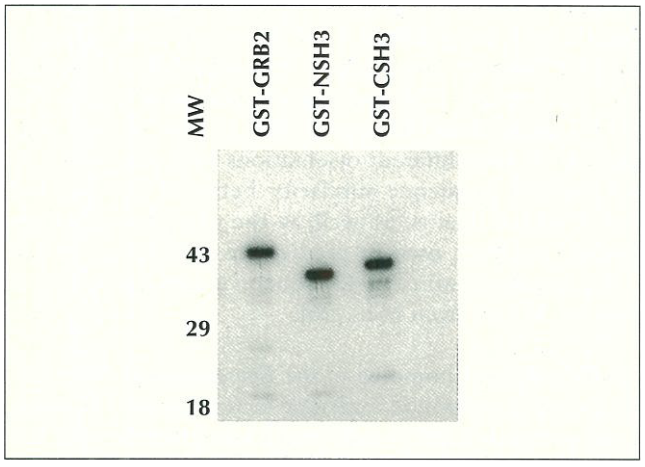

Fig. 1. Autoradiograph of ^{32}P-phosphorylated GST fusion proteins. Plasmids were constructed to encode GST fused to amino acids 1–217 of Grb2 (GST-GRB2) or to individual SH3 domains from Grb2. GST-NSH3 designates the fusion protein containing the amino-terminal SH3 domain (amino acids 1–160 of Grb2), and GST-CSH3 containing the carboxy-terminal SH3 domain (amino acids 55–217 of Grb2).

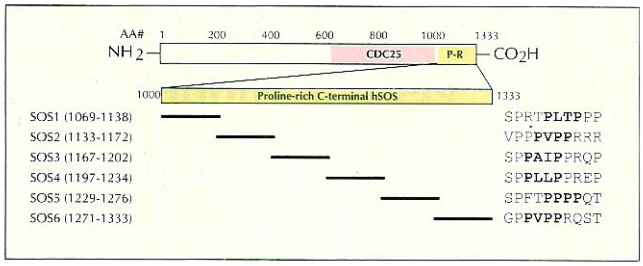

Fig. 2. The identification of potential Grb2 SH3 binding sites in the carboxy-terminal domain of hSos. Each peptide contains a **PXXP** motif. The CDC25 homology region contains the guanine nucleotide exchange factor catalytic domain.

the binding of the amino-terminal SH3 domain to a synthetic peptide ligand when the binding ability of the amino-SH3-SH2 polypeptide was compared to that of a fusion protein containing the SH3 domain alone (data not shown). The binding activities of the amino-terminal SH3-SH2 and the carboxy-terminal SH2-SH3 GST fusion proteins are therefore considered to be due solely to the SH3 domain in the remainder of this paper.

Small scale expression and ^{32}P-labeling of Grb2 proteins

The labeling and purification procedures were adapted from Kaelin *et al.* [30]. For small scale expression, 20-ml cultures of *E. coli* harboring the expression plasmids were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at 37 °C for 1 h. The bacterial lysates were incubated with glutathione agarose. The ^{32}P-labeling reaction was carried out while the recombinant GST fusion protein was bound to the resin. The kinase was removed by repeated washing and the labeled protein was eluted with reduced glutathione. The GST fusion protein was used for the binding assays (Fig. 1). The probe concentrations were adjusted to give the same specific activity (dpm) for each experiment. The levels of ^{32}P incorporation were similar for the five GST–SH3 fusion proteins.

Cloning and expression of hSos peptides

The cDNA encoding the hSos protein was cloned from a human T-cell (Jurkat) cDNA library using a cDNA probe generated by PCR [8]. Individual regions were amplified using PCR and subcloned into pGEX-2TK (Fig. 2). All constructs were fully sequenced in both directions. Cultures of *E. coli* strain BL21 transformed with the hSos expression plasmids were induced with IPTG at room temperature for 10 min prior to cell lysis. The induction was carried out for a short period of time, since at longer induction times the expression levels varied dramatically (data not shown). Bacterial lysates were separated by electrophoresis through a 12 % polyacrylamide gel and transferred to an Immobilon-P membrane. The level of protein expression was determined by Western blotting with polyclonal anti-GST antibodies (Fig. 3). In addition to the hSos proteins, two negative controls, GST alone and a GST fusion with FKBP25 (FK506 binding protein 25), were included.

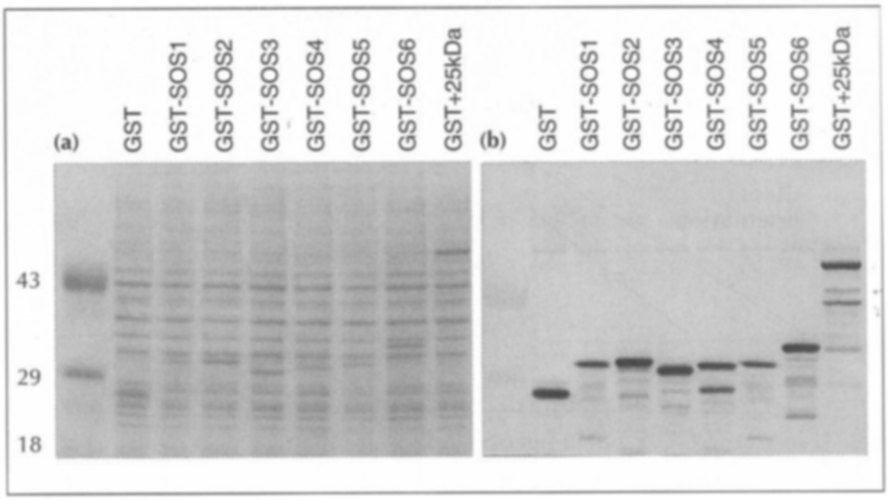

Fig. 3. Expression of hSos fragments. **(a)** Total bacterial lysates were separated by electrophoresis (12 % SDS-PAGE) and stained with Coomassie blue dye. **(b)** Proteins in (a) were transferred to Immobilon-P and analyzed with polyclonal anti-GST antibodies. GST+25kDa designates a control fusion protein containing FKBP25.

Binding studies using ^{32}P-labeled Grb2 fusion proteins

Proteins immobilized on identical Immobilon-P membranes were fully denatured with 6M guanidinium hydrochloride. The denaturant was removed by sequential dilution. The membranes were blocked with non-fat dry milk and incubated with the ^{32}P-labeled SH3 proteins. The membranes were washed to remove unbound probe and exposed to film.

The full length Grb2 protein bound to fragments SOS2, SOS3, SOS4 and SOS6 (Fig. 4a). There was no detectable binding to SOS1 and SOS5. The amino-terminal SH3 domain showed the same pattern of hSos fragment binding (Fig. 4b). The carboxy-terminal SH3 domain did not interact with any of the Sos peptides under these conditions (Fig. 4c).

Discussion

Proline-rich peptides have been shown to form stable complexes with SH3 domains. In each of the complexes reported to date, the ligands are bound in a polyproline type II (PPII) helix conformation. The PPII helix has three residues per turn with amino acids i and i+3 occupying equivalent positions along the edge of the helix, as illustrated in Fig. 5. The interface of the core PPII helix and its SH3 receptor is formed by two edges of the helix with residues i, i+2, i+3, i+5 and i+6 (where i is the residue bound to pocket P_I) interacting with three distinct sites within the binding domain [26]. The first site, designated P_I, contains a highly conserved acidic amino acid that can form a salt bridge with an Arg residue that is present at position i in many SH3 ligands. The Abl SH3 domain lacks the conserved acidic residue, however, and its ligands, 3BP-1 and 3BP-2, have hydrophobic groups in place of the Arg [25,31].

Recent structural studies using the Src SH3 domain have shown that two distinct binding orientations (class I and class II) are possible (Fig. 5). Amino acids i+2 and i+3 in a class I mode or i–2 and i–3 in a class II mode (Figs 5, 6 and discussion below) interact with pockets P_V and P_{II}, respectively; these pockets together constitute the second binding site. Amino acids i+5 and i+6 (or i–5 and i–6) interact with the third binding site, pockets P_{IV} and P_{III} (Fig. 6).

The class I orientation has been observed in complexes of PI3K, Fyn and Abl SH3 domains [24,25] as well as the Src complex with the RLP2 peptide [26]. The position of the Arg residue in class I ligands (with the exception of the Abl SH3 ligands, as mentioned above) is at the amino-terminus of the peptide. The interaction of the Arg with conserved acidic residues, therefore, orients the peptide so that the amino-terminus is located near the P_I

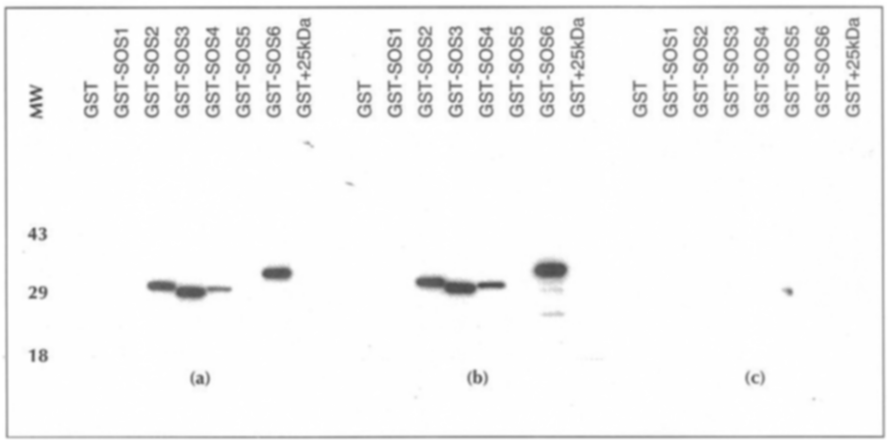

Fig. 4. Filter binding of GST fusion proteins to hSos fragments. **(a)** Binding of hSos fragments to GST fusion proteins containing the amino-terminal SH3 domain; **(b)** binding to GST fusion proteins containing full-length Grb2; **(c)** binding to GST fusion proteins containing the carboxy-terminal SH3 domain.

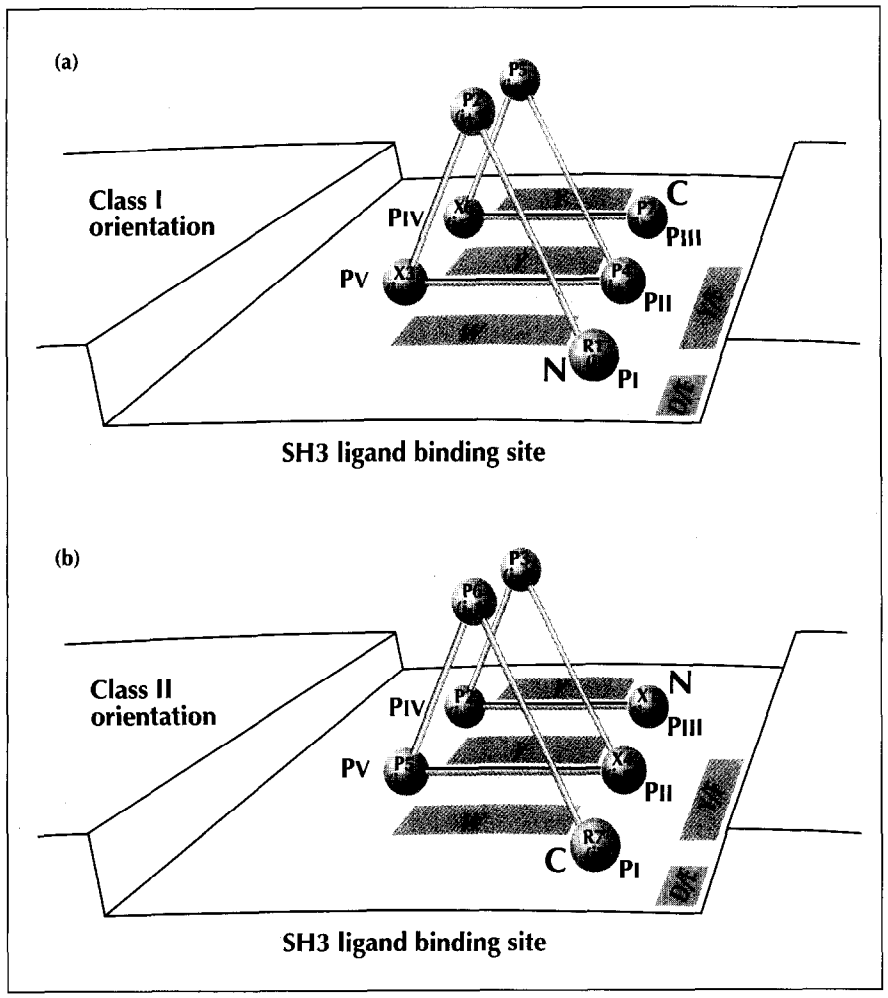

Fig. 5. Mnemonic device for analyzing SH3–ligand interactions. **(a)** Class I SH3–ligand interaction, showing conserved SH3 residues. **(b)** Class II SH3–ligand interaction. The two orientations of ligands are noted by the numbering system and the labels C and N, signifying the carboxy- and amino-terminal amino acids. Optimal residues for seven positions of the core binding element are depicted.

pocket. In class II ligands, the Arg residue is at the carboxyl terminus of the peptide, so the peptide binds in the opposite orientation. It is important to note that in both types of complexes the P_{II}/P_V and P_{III}/P_{IV} pockets are occupied by XP dipeptide fragments (where X is a hydrophobic amino acid and P is Pro) rather than PX dipeptides, which have been shown to have no measurable binding affinity [26]. The more compact nature of the XP dipeptide compared to the corresponding PX one results in topographical complementarity to the second and third pockets.

Recently, we proposed a general model for formation of a complex between an SH3 domain and a peptide ligand [26]. On the basis of this model, we predicted that a synthetic decapeptide derived from SOS2 (N-Ac-VPPPVP-PRRR-NH_2, hereafter called 'the SOS2 peptide') would bind to the amino-terminal SH3 domain of Grb2 in a class II orientation. Binding studies using mutated peptide ligands supported the prediction that the amino-terminal SH3 domain binds to SOS2 in a class II orientation.

Here, we have used the guidelines for structure prediction proposed in [26] to predict which of six hSos fragments will bind to Grb2, and in which orientation. The presence of a conserved glutamic acid residue in both Grb2 SH3 domains (Glu16 in the amino-terminal SH3 domain and Glu174 in the carboxy-terminal domain) suggests they should both be able to bind to Arg-containing ligands in either the class I or class II orientation. Alignment of the amino acid sequences of SH3 domains

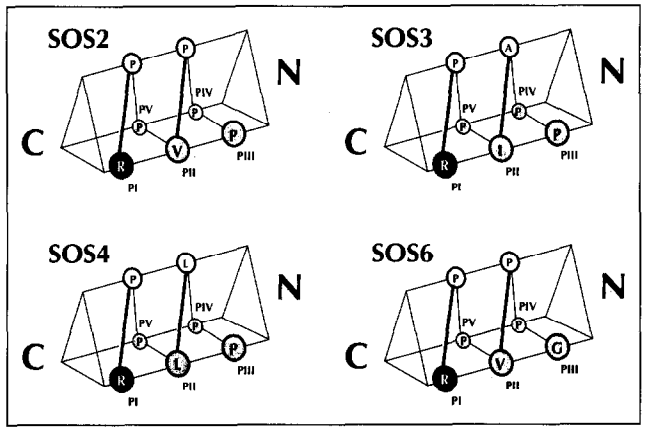

Fig. 6. A schematic representation of the four hSos peptides that bind to Grb2 amino-terminal SH3 in a proline type II helix conformation. The Arg residue (red) forms a salt bridge with Glu16 (amino-terminal SH3) or Glu174 (carboxy-terminal SH3). This interaction dictates the orientation of the complex and determines the identity of the XP dipeptides that occupy the P_{II}/P_V and P_{III}/P_{IV} binding pockets. Similar interactions are present in the lower-affinity complexes of the carboxy-terminal SH3.

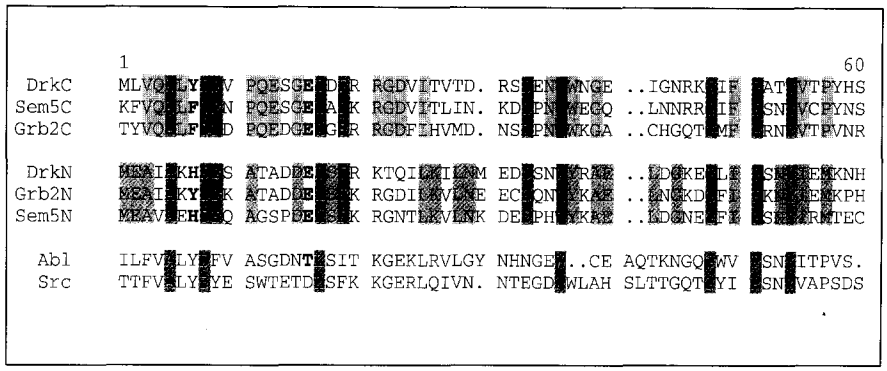

Fig. 7. Amino acid sequence alignment of amino- and carboxy-terminal SH3 domains from human (Grb2), *Drosophila* (Drk) and *C. elegans* (Sem5) as well as v-Src and c-Abl. Residues highlighted in blue are conserved among most SH3 domains. The residues in pink and green are conserved in the carboxy- and amino-terminal SH3 domains respectively. The conserved glutamic acid residue in the amino- (Glu16) and carboxyl- (Glu174) domains is highlighted in yellow. Bold designates the residues that line the ligand-binding site.

(Fig. 7) shows that the human Grb2 amino-terminal SH3 is more closely related to the *C. elegans* Sem-5 amino-terminal SH3 and *Drosophila* Drk amino-terminal SH3 than to the human Grb2 carboxy-terminal SH3 [32]. Thus the conservation of the glutamic acid residue may be functionally significant, as it is not part of an overall high degree of similarity between the amino- and carboxy-terminal SH3 domains.

Using the above considerations, the SOS1 and SOS5 peptides are predicted to be poor Grb2 ligands. Despite the presence of an amino-terminal arginine in Sos1, it is seen to be out of register for a class I binding mode (Fig. 8). However, four Sos-derived peptides — SOS2, SOS3, SOS4 and SOS6 — contain the class II consensus features: a carboxy-terminal arginine and two XP dipeptides at positions i–3, i–2 and i–6, i–5. We therefore predicted that both SH3 domains should bind to fragments SOS2, SOS3, SOS4 and SOS6 in a class II orientation, as shown in Fig. 6, and that SOS1 and SOS5 should not bind in either orientation. These predictions are consistent with the results of the filter binding studies. The proline-rich regions of the six hSos derived fragments are shown in Fig. 8. The four fragments that bind, unlike the two that do not, conform to

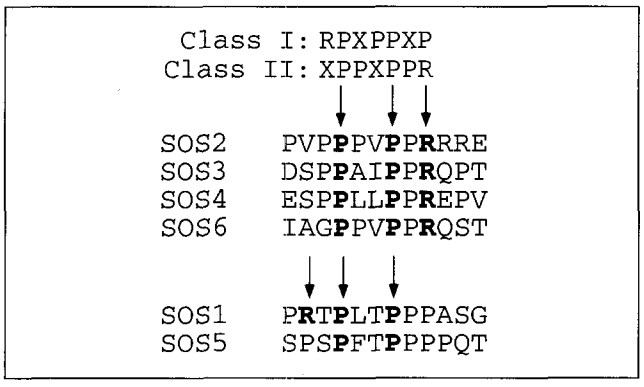

Fig. 8. Amino acid sequence alignment of proline rich peptides from hSos. Top, typical class I and class II motifs. SOS2, SOS3, SOS4 and SOS6 bind to the SH3 domains of Grb-2 in a class II orientation. Critical residues are indicated with arrows. SOS1 and SOS5 do not bind. The SOS1 peptide contains a potential class I sequence (Arg at the amino-terminus of the PXXP motif). Class I binding of this peptide would result in the placement of a PX dipeptide (TP) in the P_{II}/P_V binding site instead of the preferred XP dipeptide, and is therefore predicted to be disfavored.

the general model for SH3–ligand complexes. We propose that the carboxy-terminal arginine of the four binding fragments binds in the P_I pocket that contains the conserved acidic residue (Glu16 of the amino-terminal SH3 and Glu174 of carboxy-terminal SH3). This interaction orients the peptide in the binding site and it determines the register for the proline residues that fill the P_{II}/P_V and P_{III}/P_{IV} pockets. These are occupied by VP and PP in SOS2, IP and PP in SOS3, LP and PP in SOS4, and VP and GP in SOS6.

The filter binding assay also demonstrates differences between the two SH3 domains of Grb2 in binding to hSos peptides. We have previously shown [32] that the association between the amino-terminal SH3 domain of Grb2 and the SOS2 peptide has a K_d of 5.7 μM, whereas the carboxy-terminal SH3 domain binds more weakly, with a K_d of 39 μM. The filter binding assay was capable of detecting the stronger interaction, but not the weaker one. The affinity of the intact Grb2 protein for an immobilized peptide containing the PPPVPPR sequence derived from SOS2 has been determined, and found to have a K_d of 25 nM, using the BioSensor technology (BIAcore Pharmacia) [33]. In the BIAcore experiment, the ligand is immobilized on a surface. It is likely that the observed K_d is due to binding of both SH3 domains to different molecules of the same peptide. Similarly, when intact Grb2 binds to the intact hSos protein, it is likely that both SH3 domains bind to different peptides. We have attempted to measure the affinity of Grb2 for the SOS2 peptide using the fluorescence enhancement method. The data appear to reflect the independent association of the two SH3 domains with two independent binding constants (J.K. Chen and S.L.S., unpublished results), similar to the binding constants seen when the associations between individual SH3 domains and hSos-derived peptides are studied independently [32].

It is likely that the two Grb2 SH3 domains bind cooperatively to hSos [34], allowing two relatively weak interactions to give a high affinity complex. A functional model of the Grb2–hSos association is shown in Fig. 9. We predict that each of the SH3 domains forms a complex with one of the hSos peptides found here to be capable of binding to the amino-terminal SH3 domain (SOS2, SOS3, SOS4 or SOS6). The association of a single SH3 domain — possibly the amino-terminal domain, due to

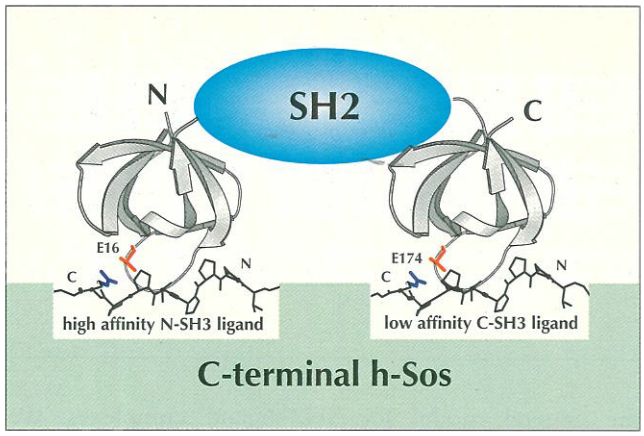

Fig. 9. A functional model for Grb2–hSos association. The conserved Glu residues (red) form salt bridges with the Arg residues (blue). This interaction determines the orientation of both binding interactions, which are both predicted to be class II-type interactions.

its higher affinity — reduces the entropic cost associated with the formation of the second SH3–ligand complex, enhancing the overall binding. Both of the Grb2–hSos interactions are predicted to have class II orientation. Indeed, recent structural investigations [35–38] have confirmed the class II orientation of Sos peptides bound to Grb2 amino- and carboxy-terminal SH3 domains.

Significance

Using a predictive model for SH3–ligand interactions we have examined the interaction of Grb2 with hSos. The general model for complexes between SH3 domains and proline-rich ligands was developed by analyzing the structures of the Src SH3 domain and two different high-affinity ligands. The two classes of complexes observed differ most significantly in their peptide orientation. Class I ligands contain an Arg residue at the amino terminus of a Pro-containing region, while in class II ligands the Arg is at the carboxyl terminus. The Arg residue binds, via a salt bridge, to a conserved acidic amino acid in the P_I pocket of the SH3 domain, thereby determining the orientation of the peptide. These results were generalized to provide a predictive tool to analyze SH3 ligand complexes.

The Grb2–hSos complex is the most biologically significant SH3-mediated association identified to date. It has a crucial role in linking growth factor receptors to the Ras-mediated signaling pathway, resulting in cellular growth and differentiation. The interaction between Grb2 and hSos is mediated by two Grb2 SH3 domains, and hSos contains several potential SH3 binding sequences. Here, we have used our predictive model to identify the SH3 ligands within hSos, and to predict the orientations of the resulting complexes. Indeed, of six possible SH3 ligands only four, as predicted, bound to Grb2 SH3 domains. Of the two Grb2 SH3 domains, the amino-terminal one appears to be the more important. The results presented here provide insight into the Grb2–hSos interaction, and demonstrate that it is possible to identify potential SH3 binding sites within proline-rich regions on the basis of the primary amino acid sequence of the ligand.

Materials and methods

Materials

All chemicals were purchased from Sigma Chemical Co. Restriction enzymes were purchased from Boehringer Mannheim. Oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer. The Immobilon-P membrane was purchased from Millipore. Heart muscle kinase was purchased from Sigma. The ^{32}P-γ-ATP (6000 Ci mmol^{-1}) was purchased from Amersham. Reduced glutathione, glutathione-agarose and polyclonal anti-GST antibodies were purchased from Molecular Probes Inc. All sequencing reactions were performed using the Sequenase kit from USBiochemical. The pGEX-2TK vector was kindly provided by Professor William G. Kaelin Jr. (Harvard Medical School).

Cloning of Grb2

The cDNA encoding the entire Grb2 protein was amplified using the polymerase chain reaction from a human brain stem cDNA library (Clontech) using nested primers based on the published nucleotide sequence. The forward (5'-AGG CTA GGA TCC ATG GAA GCC ATC AAA-3') and reverse (5'-GGA ATC TGA ATT CCC TTA GAC GTT CCG GTT CAC GGG-3') PCR primers contained engineered *Bam*HI and *Eco*RI sites, respectively. The amplified PCR product was digested with *Bam*HI and *Eco*RI and cloned directly into the *Bam*HI/*Eco*RI digested pGEX-2TK vector. The sequence was confirmed by dideoxy sequencing in both directions using vector-based sequencing primers as well as Grb2 specific primers. The pGEX-2TK:Grb2 plasmid was used to transform competent *E. coli* BL-21.

Large-scale overexpression of Grb2 protein and fragments

Overexpression of the GST-Grb2 fusion proteins was accomplished by inoculating 1.2 l of Luria broth containing ampicillin, 100 mg l^{-1}) with a 4 ml overnight culture. The bacteria were allowed to grow at 37 °C until OD_{600} = 0.8 and were treated with 1 mM IPTG. The culture was induced for 6 h at which time the cells were pelleted (4000 rpm, 30 min, 4 °C). The cells were resuspended in 50 ml lysis buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA and 1 mM phenylmethylsulphonyl fluoride (PMSF) and pulse sonicated (3 x 30 s). The lysates were clarified by centrifugation (15 000 rpm, 30 min, 4 °C) and the supernatant was applied directly to a column containing 10 ml glutathione-agarose. The column was washed with ten volumes of phosphate-buffered saline (PBS) and the fusion protein was eluted with 5 mM glutathione in 50 mM Tris-HCl pH 8.0. Glutathione was removed by dialysis against four changes of 150 mM NaCl, 20 mM Tris-HCl pH 8.0.

Small scale expression and ^{32}P labeling of GST–SH3 fusion proteins

The individual amino-terminal and carboxy-terminal SH3 domains from Grb2 were subcloned into the pGEX-2TK vector and confirmed by sequencing. The labeling procedure

was adapted from Kaelin *et al.* [30]. Overnight cultures (2 ml) of transformed *E. coli* were diluted 1:10 and grown for 2 h at 37 °C. The cultures were induced with 1 mM IPTG and grown for an additional 3 h at 37 °C. The cells were collected by centrifugation (4000 rpm, 5 min, 4 °C) and resuspended in 2 ml NETN (100 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, 0.5 % NP-40) and sonicated (3 x 30 sec) at 4 °C. The bacterial debris was pelleted (14,000 rpm, 5 min, 4 °C) and the supernatant was mixed with 100 µl glutathione–Sepharose (2 h, 4 °C). The mixture was incubated for 2 h and the resin was washed with 3 x 1 ml NETN and once in heart-muscle kinase (HMK) buffer (100 mM NaCl, 20 mM Tris-HCl pH = 7.5, 12 mM $MgCl_2$) and finally resuspended in 100 µl reaction mixture containing 10 µl 10x HMK buffer, 1 µl 1 M dithiothreitol (DTT), 4 µl resuspended HMK and 8 µl ^{32}P-γ ATP. The reaction was incubated at 4 °C for 1 h and stopped with the addition of 1 ml HMK stop buffer (10 mM sodium phosphate pH = 8.0, 10 mM sodium pyrophosphate, 10 mM EDTA, and 1 mg ml^{-1} BSA). The glutathione beads were washed with 5 x 1 ml NETN and the phosphorylated proteins were eluted with 1 ml glutathione solution (120 mM NaCl, 100 mM Tris-HCl pH = 8.0, 20 mM reduced glutathione). The proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (Fig. 2). The efficiency of labeling was determined by Bradford protein assay and liquid scintillation counting.

Cloning and expression of hSos fragments

The gene encoding the human Sos protein was cloned from a human T cell (Jurkat) library (Stratagene) using a PCR amplified cDNA probe. PCR primers used to generate the cDNA probe were designed using the published hSos sequence [8]. Additional PCR primers were designed to allow the amplification, subcloning and expression of the six individual hSos peptides as GST fusion proteins. GST fusion proteins were prepared by subcloning the PCR-amplified cDNAs into pGEX-2TK vector and confirmed by sequencing. Bacteria containing the appropriate plasmids were grown in 2 ml cultures to an OD_{600} = 0.6 and induced with 1 mM IPTG. After 10 min at 25 °C the bacteria were collected by centrifugation (10 000 x g, 4 °C, 10 min) and lysed by boiling in SDS-PAGE loading buffer. The proteins were separated on a 12 % SDS-PAGE gel and transferred to an Immobilon-P membrane.

Western blotting with ^{32}P-labeled GST-SH3 fusion proteins

The membranes were placed protein side up in the blocking buffer (25 mM Hepes–KOH pH = 7.7, 25 mM NaCl, 5 mM $MgCl_2$, 5 % non-fat dry milk, 1 mM DTT, 0.05 % NP-40) and incubated with shaking for 2 h at 4 °C. The filters were washed with two changes of the denaturing solution (6 M guanidinium HCl, 25 mM Hepes–KOH pH 7.7, 25 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT) at 4 °C. The denaturing solution was serially diluted (each time 1:1 with 25 mM Hepes–KOH pH 7.7, 25 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT), until a final guanidinium hydrochloride concentration of 0.187 M was reached. Each intervening wash was carried out for 20 min at 4 °C. The filters were washed with two changes of fresh 25 mM Hepes–KOH pH = 7.7, 25 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT (10 min washes) and 25 mM Hepes–KOH pH 7.7, 25 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 5 % non-fat dry milk, 0.05 % NP-40 (1 h wash) and finally with 25 mM Hepes–KOH pH 7.7, 25 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1 % non-fat dry milk 0.05 % NP-40 (1 h wash). The hybridization was carried out at 4 °C by placing the filters in 10 ml hybridization solution (20 mM Hepes–KOH pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 1 % non-fat dry milk, 0.05 % NP-40 and 5 mg ^{32}P-labeled GST-SH3 protein) for 6 h. The filters were washed with three changes of fresh hybridization solution (10 ml), covered with Saran wrap and exposed to film.

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