A Potential SH3 Domain-binding Site in the Crk SH2 Domain*

(Received for publication, January 19, 1996, and in revised form, April 16, 1996)

Mordechai Anafi‡§, Michael K. Rosen¶**, Gerald D. Gish‡, Lewis E. Kay‡¶, and Tony Pawson††§§§

From the ‡Programme in Molecular Biology and Cancer, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Ave., Toronto, Ontario, M5G 1X5 Canada, the §Department of Molecular and Medical Genetics, ¶Protein Engineering Network of Centres of Excellence and ||Departments of Biochemistry and Chemistry, University of Toronto, Toronto, Ontario, M5S 1A8 Canada

The Src homology 2 (SH2) domain of the mammalian adaptor protein Crk-II contains a proline-rich insert, predicted to lie within an extended DE loop, which is dispensable for phosphopeptide binding. Using the yeast two-hybrid system, this region of the Crk-II SH2 domain was found to interact with a subset of SH3 domains, notably the Abi SH3 domain. Furthermore, this proline-rich insert was found to modify the efficiency with which Crk-II was phosphorylated by the p140*, a tyrosine kinase. In vitro, the interaction of full-length non-phosphorylated Crk-II with a glutathione S-transferase-Abi SH3 domain fusion protein was very weak. However, phosphorylation of Crk-II on Tyr-221 which induces an intramolecular association with the SH2 domain, or addition of a phosphopeptide corresponding to the Crk-II Tyr-221 phosphorylation site, stimulated association of Crk-II with the Abi SH3 domain. NMR spectroscopic analysis showed that binding of the Tyr-221 phosphopeptide to the Crk SH2 domain induced a chemical shift change in Val-71, located in the proline-rich insert, indicative of a change in the structure of the proline-rich loop in response of Crk SH2-pTyr-221 interaction. These results suggest that the proline-rich insert in the Crk SH2 domain constitutes an SH3 domain-binding site that can be regulated by binding of a phosphopeptide ligand to the Crk SH2 domain.

Src homology 2 (SH2) and SH3 domains are distinct protein-binding modules found in a wide variety of intracellular signaling proteins. SH2 domains recognize specific phosphotyrosine (pTyr)-containing motifs, and their binding is therefore directly regulated by phosphorylation of their ligands. In contrast, SH3 domains bind phosphotyrosine phosphopeptides, comprised of a conserved pTyr-binding pocket and a more variable binding site for residues C-terminal to the pTyr (12, 13). In principle, SH2 domains might have other surfaces involved in protein-protein interactions. For example, the SH2 domains of tensin and Stat proteins are predicted to have an -20-amino acid insert in the CD loop relative to other SH2 domains (13), and the Crk SH2 domain apparently has an insert in the DE loop (see below). These sequences might provide contact sites for additional protein ligands. Furthermore, recent data have suggested that some SH2 domains can bind specific phospholipids, notably phosphatidylinositol 3,4,5-trisphosphate (14).

Adaptor proteins such as Grb2, Nck, and Crk are composed primarily of SH2 and SH3 domains, and are involved in the formation of signaling complexes downstream of receptor tyrosine kinases (for review, see Ref. 15). These phosphopeptides therefore provide a useful system to investigate possible SH2-SH3 interactions. We have pursued this issue using the mammalian Crk-II adaptor protein. Crk-II is a relative of the avian v-crk oncoprotein product (16-18). Two mammalian Crk isoforms have been identified which display distinct biological properties (18). One of these, Crk-I, is similar to v-Crk in possessing an N-terminal SH2 domain followed by a single SH3 domain, and in having relatively potent transforming activity when overexpressed. The second Crk isoform, Crk-II, contains an SH2 and SH3 domain identical to those found in Crk-I, and an addi-
A Potential SH3 Domain-binding Site in the Crk SH2 Domain

**Experimental Procedures**

**Generation of Recombinant Plasmids**

pET11d Crk-II Constructs—Wild type (wt) or mutant forms of the mouse Crk-II cDNA were cloned into the NcoI/BamHII sites of the pET11d vector (Novagen). Two variants of the Crk-II construct were prepared: Crk-IIPro, in which the sequence encoding the proline-rich SH2 insert (amino acids 67–85) is deleted; and Crk-IIIcpro, in which the codon for Arg-38 is mutated to Ala, resulting in a Crk-II variant with an SH2 domain that is incapable of binding pTyr-containing proteins.

pCDNA3-HA Tagged Vectors—The wild type and the above mutated forms of the mouse crk-II cDNA were cloned into a modified HA-tagged pCDNA3 expression vector (Invitrogen Inc.).

pGEX-2T Constructs—Sequences coding for the SH2 domain of Crk-II (amino acids 6–125) with or without the proline-rich insert (amino acids 67–85) were amplified by polymerase chain reaction from murine Crk and Crk-II pro, respectively, and cloned into the pGEX-2T vector at the BamHI/EcoRI sites of the preparation of bacterial glutathione-S-transferase (GST)-Abl SH2 and SH3 fusion proteins was as described previously (23).

Yeast pACTII Crk SH2 Constructs—Varying fragments of the Crk-II SH2 domain were cloned into the BamHI/EcoRI sites of the pACTII vector (24; see Fig. 3A).

Yeast pAS1 SH3 Domains Constructs—SH3 domains from different sources were cloned in the pAS1 vector (25). The mouse Ab3 SH domain (amino acids 65–123), human Grb2 N- or C-terminal domains (amino acids 1–59 and 158–217, respectively), and the mouse Crk C-terminal SH3 (amino acids 235–293) where cloned at the SalI/BamHII sites. The three Nck SH3 domains (amino acids 1–255) were cloned into the EcoRI/BamHII sites of pAS1.

**Yeast Two Hybrid Assays**

The two-hybrid fusion vectors, encoding fusions between the DNA-binding domain of Gal4 and the different SH3 domains, and pACTII vectors encoding the transcriptional activation domain of Gal4 fused to different fragments of the Crk-II SH2 domain, were transformed into Saccharomyces cerevisiae strains Y2H153 and Y2H187, respectively. Yeast transformation was performed by the lithium acetate method (25) except that 10% dimethyl sulfoxide was included during 42°C heat shock. Co-expression of pAS and pACTII vectors where achieved by mating Ty1-153 and Ty1-187 yeast strains, each containing the appropriate vector. β-Galactosidase activity was detected on 5-bromo-4-chloro-3-indoyl β-D-galactoside plates after permeabilizing the yeast by liquid nitrogen treatment.

**In Vivo Complex Formation**

COS-1 cells were transiently transfected with wild type and mutated forms of the HA-Crk-II pCDNA3 expression vector alone, or in combination with v-Src or v-Crk. After 48 h the cells were lysed, and the Crk proteins were immunoprecipitated by mouse anti-HA antibodies. pTyr containing proteins were analyzed by anti-pTyr immunoblotting.

**Antibody Preparation**

Anti-Crk-II antibodies were raised against bacterially expressed full-length Crk-II. The antibodies were absorbed on immobilized Ab1 SH3-GST in order to eliminate any residual cross-reactivity with this protein. These antibodies recognized Crk-II protein in Western blot analysis.

Anti-Abl SH2 antibodies were raised to a bacterial GST fusion protein containing Abl SH2 domain. Anti-Abl SH2 antisera specifically recognized p140, p210, and p160 of rat, human, and viral origin, respectively.

**Preparation and Purification of Proteins**

**GST Fusion Proteins**—Cells were grown in LB media to OD600 = 0.6, and protein synthesis was induced with 1 mM isopropylthiogalactopyranoside. The cell pellets were homogenized in phosphate-buffered saline containing 1% Triton X-100, 1% Tween 20, 2 mM diethyloletritol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, and 2 mM benzamidine. The lysates were subjected to centrifugation at 28,000 × g for 30 min. The supernatants were then mixed with glutathione-agarose (Sigma), and the fusion proteins were eluted with 5 mM reduced glutathione. The proteins were then dialyzed to remove the glutathione and used immediately in the binding assays.

**Full-length Crk Proteins**—Cells were grown in LB media to OD600 = 0.6, and protein synthesis was induced with 1 mM isopropylthiogalactopyranoside. After 4 h, cells were collected by centrifugation and lysed by sonication. Insoluble debris was removed by centrifugation, and the protein was purified by successive anion exchange (Pharmacia DEAE-CL6B resin) and hydrophobic exchange chromatography (Pharmacia phenyl-Sepharose CL-4B resin). An aliquot of purified Crk-II was incubated with bacterially expressed Abl tyrosine kinase (Stratogene Science Inc.) for over 5 days at room temperature. More than 95% of the Crk-II was phosphorylated specifically on tyrosine 221. Phosphorylated Crk was analyzed as described in Rosen et al. (21) using NMR spectroscopic analysis which directly demonstrated that phosphorylated Crk-II forms an intramolecular SH2-pTyr interaction. Specific details of the phosphorylation reaction and biochemical analysis of the protein were as described (21).

**Crk-II/Abl SH3 Binding Assays**

100 μM purified Crk-II protein, treated or not with 140 μM pTyr-221 peptide, was incubated with similar amounts (approximately 1 μM) of GST-Abl SH3 fusion protein, or GST alone. The binding reaction was carried out for 2 h at 4°C in 50 μM of phospholipase C lysis buffer (50 mM Hepes pH 7, 150 mM NaCl, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 mM sodium fluoride). The GST-containing proteins were collected with glutathione beads. The beads were washed 4 times with phospholipase C buffer and the proteins were eluted by boiling in SDS sample buffer and subjected to 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to nitrocellulose in a semidyblotting apparatus at 0.8 mA/m2 for 1 h. Filters were blocked overnight in 5% nonfat dry milk in TBS-T (20 mM Tris, pH 7.5, 150 mM sodium chloride, and 0.05% Triton X-100) and probed with 10 ml of 1:200 dilution of anti-Crk-II serum which has been previously absorbed on immobilized Abl SH3-GST. Anti-Crk-II blots were developed using 125I-labeled protein A (Amersham) or by protein A-horseradish peroxidase conjugate (Bio-Rad) followed by enhanced chemiluminescence (ECL, Amersham).

**NMR Spectroscopy**

NMR experiments were performed on Varian Unity+500 and Unity+600 spectrometers equipped with three channels, pulsed field gradient triple-resonance probes with actively shielded z gradients, and gradient amplifier units. All spectra were recorded on a 1.5 mM sample of 15N-labeled Crk-23, consisting of residues 2–198 of murine Crk, in 50 mM phosphate buffer, pH 6.8, 200 mM sodium chloride, 1 mM EDTA, 2 mM diethyloletritol, 2 mM benzamidine, 0.02% sodium azide, 90% H2O, 10% D2O, at 35°C. pTyr-221 peptide was added to 1.5 mM to record spectra on the protein-ligand complex. Sequential assignment was performed according to procedures outlined by Friedrichs et al. (26), using the following enhancements in sensitivity, pulsed field gradient spectra (27–29): HNCO (30), HNCCaB (29), HbCHbCa(CO)NH (29), HNCAHb (31), 2HbCHbCa(CO)NH (29). The identity of most residues preceding proline was confirmed through HbCHbCa(Co)H (32) and HbCHbCa(CO)Ha spectra. In the latter experiment, which correlates 1Hb and 13Ca of residue i with 15N of residue i + 1, peaks due to 1Hb and 15N are of opposite sign to those due to 13Ca or 15N. The

2 L. E. Kay, unpublished data.
unique $^{15}$N chemical shift range of proline amide nitrogen atoms (132–140 ppm), and the negative sign of peaks correlating to proline thus allowed definitive assignment of the majority of residues N-terminal to proline. This information proved critical to assignment of residues in the proline-rich loop of the SH2 domain. The backbone assignment of Val-71 was also confirmed by preliminary analysis of the HCCCH TOCSY spectrum (33) recorded on the mCrk23-peptide complex, which provided chemical shifts of all $^{13}$C and $^1$H atoms in the residue. Spectra were analyzed using the program mmv34 (33), which was kindly provided by Dr. Bruce A. Johnson.

Exogenous Substrate Phosphorylation Assays

p160$^\text{src}$ was extracted from a cell line of Abelson virus-transformed ANN-I (generously provided by T. Hunter, Salk Institute), p140$^\text{src}$ was extracted from Src-transformed rat cell line, 57a. Cell extraction and immunoprecipitation were performed essentially as described by Anafi et al. (35) under conditions optimized for the detection of the Abl kinase activity: the cells were washed three times with cold Hanks’ balanced salt solution and lysed by Dounce homogenization in ice-cold protein lysis buffer (1% Triton X-100, 50 mM NaCl, 10 mM Tris, pH 8, 5 mM EDTA, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mg/ml bovine serum albumin, 2 mM phenylmethylsulfonyl fluoride, 10 $\mu$g/ml aprotinin, and 5 $\mu$g/ml leupeptin). Five $\mu$l of anti-Abl SH2 antibody was added to each sample (approximately 10$^6$ and 5 x 10$^5$ of ANN-I and 57a, respectively). The immunoprecipitation was carried out overnight on ice. Immune complexes were collected by adding protein A-Sepharose beads (Sigma Inc.) using a 2-$\mu$l packed volume of beads for 1 $\mu$l of serum added and gentle agitation at 4°C for 2 h. The beads were then washed twice with cold buffer containing 1% Triton X-100, 0.5 M NaCl, 10 mM Tris, pH 7.5, and 1 mg/ml bovine serum albumin; twice more without bovine serum albumin; once with phosphate-buffered saline containing 1% Triton X-100; once with 20 mM Hepes, pH 7.5, and finally once with the kinase reaction buffer containing 20 mM Hepes, pH 7.5, and 20 mM MnCl$_2$. The immobilized Abl was used to phosphorylate purified Crk-II proteins with and without the proline-rich region. The packed beads were resuspended with the kinase reaction buffer containing 2 $\mu$Ci of [γ-32P]ATP and different concentrations of purified Crk-II proteins. The reaction was terminated by addition of sample buffer and heating the samples at 95°C for 5 min. The level of the phosphorylation of Crk was determined by separation on SDS-PAGE followed by autoradiography and densitometric analysis.

RESULTS

A Proline-rich Insert in the Crk SH2 Domain That Is Not Required for Binding pTyr Proteins—The SH2 domain of Crk contains a 19-amino acid insert (residues 67–85) relative to the SH2 domains of Crkl and other cytoplasmic signaling proteins (Fig. 1A). Based on a sequence alignment of number of SH2 domains (13), and by homology modelling of SH2 structures, it appears that this proline-rich sequence is located within the DE loop (Fig. 1A). As such, the Crk SH2 proline-rich insert should be located adjacent to, but not in the binding surface for pTyr-containing peptides, and might therefore be dispensable for binding of the Crk SH2 domain to tyrosine-phosphorylated proteins. To test this possibility, GST fusion proteins containing either the wild type Crk SH2 domain, or a mutant SH2 domain in which the proline-rich insert was deleted, were isolated and examined for their ability to bind pTyr-containing proteins from a lysate of v-src transformed Rat-2 cells. A similar spectrum of tyrosine-phosphorylated proteins was bound by both the wild type and mutant SH2 domains, indicating that the proline-rich insert is not required for association of the Crk SH2 domain with phosphorylated proteins in vitro (Fig. 2A).

To test the influence of the proline-rich insert on binding of the Crk SH2 domain to pTyr-containing proteins in vivo, wild type and mutant forms of hemagglutinin (HA)-tagged Crk-II were expressed in COS-I cells (Fig. 2b, panel A). To increase the tyrosine phosphorylation of cellular proteins, cells transfected with the various forms of HA-Crk-II were co-transfected with expression vectors encoding v-Crk or the v-Src tyrosine kinase (Fig. 2b, panel b and c). Cells were lysed, immunoprecipitated with anti-HA antibodies, and immunoblotted with antibodies to pTyr (Fig. 2b, panels a–c). As shown, deletion of residues 67–85 encompassing the SH2 proline-rich insert (ΔPro) had no obvious effect on the spectrum of pTyr-containing proteins that co-precipitated with Crk-II. In contrast, substitution of the SH2 β5 arginine (Arg-38) with alanine (R38A) abrogated association of Crk-II with pTyr-containing proteins in vivo. These results indicate that the proline-rich insert of the Crk SH2 domain does not play a significant role in binding pTyr-containing proteins in vivo, raising the possibility that it has some distinct function.

The Crk Proline-rich SH2 Insert Interacts with Specific SH3 Domains—SH3 domains have recently been shown to bind proline-rich peptides that adopt a polyproline type II helix, with three residues per turn (13, 36). Such SH3 domain-binding peptides have a consensus sequence X-P-p-X, where X is generally an aliphatic residue, P is a proline, and p is a scaffolding residue, frequently also a proline (37–40). Each X-P pair fits into a hydrophobic binding pocket in the SH3 surface (36, 41). Of interest, these proline-rich sequences can potentially bind in either orientation (36, 41). Several potential SH3 domain-binding sites (pXXP motifs) of the Crk SH2 proline-rich insert are shown. The orientation of the peptide (N to C or C to N-terminal) is indicated.

Fig. 1. A, alignment of the amino acid sequences of the SH2 domains of Crk-II, Crkl, Syk N-terminal SH2, and Src. The SH2 sequences of human Crk-II (H-CRK) and Crkl were aligned based on the defined secondary structures of Syk and Src (13). Gaps have been introduced to optimize the alignment and are indicated by dashes. The amino acid numbers of human Crk-II are indicated, as is the proline-rich region, and Val-71 is underlined. B, possible SH3 domain-binding core sequences in the Crk SH2 proline-rich insert. Ten potential SH3 domain-binding sites (pXXP motifs) of the Crk SH2 proline-rich insert are shown. The orientation of the peptide (N to C or C to N-terminal) is indicated.
co-expressed in the yeast S. cerevisiae with chimeric polypeptides containing different fragments of the Crk SH2 domain, fused to the Gal4 transcriptional activation domain. As negative controls we co-expressed each of the SH3 domains joined to the Gal4 DNA-binding domain with an unfused Gal4 transcriptional activation domain, and each of the chimeric Crk SH2 polypeptides with an unfused Gal4 DNA-binding domain (not shown). Expression of the fusion proteins was confirmed by Western blotting with anti-HA antibody, which recognizes an HA epitope contained within the various fusion proteins (not shown).

As shown in Fig. 3A, the Abl SH3 domain gave a strong positive signal when tested against a fragment of the Crk SH2 domain containing amino acid residues 10–105 (construct 1), that includes the proline insert. The C-terminal SH3 domains of Grb2 also interacted with the Crk-II(C) SH3 domains in the yeast two-hybrid system. The relative level of β-galactosidase activity during staining the yeast with 5-bromo-4-chloro-3-indoly β-o-galactoside is indicated. ++++, relatively very strong interaction (appearance of blue color in less than 20 min). ++, relatively strong interaction (appearance of blue color in less than 1 h). +, intermediate interaction (blue color in less than 3 h). +, weak interaction (appearance of blue color in less than 6 h). +/−, very weak interaction (appearance of blue color by 12 h or more). −, no detectable binding (no trace of blue color by 20 h). B, selected amino acids of fragment No. 8 were mutated to alanine, and the altered proteins were tested for their interactions with the Abl, Grb2(C), and Grb2(N) SH3 domains in the yeast two-hybrid system. Val-71 is underlined.
The DNA-binding subunit of the Gal-4 promoter was fused with wild type, W99R, P112A, and W99R + P112A Abi SH3 domains. The wild type and mutant SH3 domains were co-expressed with different fragments of the Crk SH2 domain fused to the Gal-4 transcriptional activator, or with the vector encode the Gal-4 transcription activator (pACTII) alone. β-Galactosidase activity is indicated by the staining of permeabilized yeast incubated with 5-bromo-4-chloro-3-indoyl β-D-galactoside.

To localize the region within the Crk SH2 domain that interacts with Abi SH3, a series of truncated Crk SH2 polypeptide were tested in the two-hybrid assay. The smallest of these fragments to give a strong signal encompassed 42 amino acids, from residues 63 to 105 (Fig. 3B), and indeed all the proteins containing this sequence showed SH3 domain binding activity. However, a smaller construct containing only the proline-rich region (fragment 9; residues 63–88) did not bind significantly to the Abi or Grb2 SH3 domains, and only weak binding was exhibited by polypeptides containing the proline-rich sequence and N-terminal residues (fragments 3, 5, 7, and 9). Site-directed mutagenesis of Crk SH2 fragment 8 (Fig. 3B) showed that substitutions of selected proline residues of Crk proline-rich region reduced or completely abolished its association with SH3 domains. Taken together, these results indicate that the proline-rich insert is required for SH3 domain-binding, but that additional C-terminal sequences also influence the interaction with SH3 domains. Substitution of Pro-70 with Ala moderately reduced the association of fragment 8 with the Abi SH3 domain, but abrogated binding to the Grb2 SH3 domains. These results suggest that the Abi and Grb2 SH3 domains may have distinct but overlapping binding sites within the Crk SH2 proline-rich insert. Interestingly, the substitution of Arg-68 with Ala abolished the association of fragment 8 with those SH3 domains tested. This result suggests that Arg-68 may play an important role in SH3 domain interactions with the Crk proline-rich region.

To investigate in more detail the possibility that the association of Crk SH2 sequences with the Abi SH3 domain represents a typical SH3-mediated interaction, substitutions were introduced into conserved residues in the Abi SH3 domain that are known to be important for efficient binding to proline-rich ligands (Trp-99 and Pro-112 of mouse Abi-type IV). As shown in Fig. 4 the W99R mutant of the Abi SH3 domain bound poorly to the Crk SH2 domain, while the P112A mutant and the W99R/P112A double mutant showed no binding activity.

Regulated Binding of the Crk SH2 Proline-rich Region to the Abi SH3 Domain—The yeast two-hybrid system provides only an indirect measurement of protein-protein interactions. In order to directly assess the ability of the Crk-II protein to interact with the Abi SH3 domain, we investigated the association of full-length Crk-II, purified from bacteria, with a GST-Abi SH3 fusion protein. Using purified Crk-II at the concentration of 50 μM we could barely detect any binding of Crk-II to the Abi SH3 domain. However, a strong signal was obtained using the same concentration of purified Crk-II which had been previously phosphorylated to a high stoichiometry at Tyr-221 using the Abi tyrosine kinase (Fig. 5A). We have previously shown that phosphorylation of Crk-II on Tyr-221 results in an intramolecular SH2-pTyr interaction (21). This result, therefore, led us to investigate the effect on SH3 domain-binding of adding a pTyr-containing peptide (pTyr-221 peptide), corresponding to Crk-II residues 217–229, to unphosphorylated Crk-II. A Crk-II mutant (R38A), with a substitution in the SH2 domain that abrogates pTyr-binding, and Crk-II A were also tested. As shown in Fig. 5B, adding the pTyr-221 peptide to unphosphorylated Crk-II caused an increase in its association with the Abi SH3 domain. This effect was not observed with the Crk-II R38A mutant. Deletion of the proline-rich region from Crk-II completely abolished its association with the Abi SH3 domain even in the presence of pTyr-221 peptide. These results indicate that both SH2 pTyr binding activity and the SH2 proline-rich insert are necessary to induce the association between the Abi SH3 domain and full-length Crk-II detected in this assay.

Structural Studies—As part of an ongoing effort to structurally characterize Crk-II by NMR spectroscopy, we have recently determined the virtually complete backbone HN, 15N, and 13C chemical shift assignment of the non-proline residues in a 197-residue N-terminal fragment of the protein (termed Crk23) both free and bound to the pTyr-221 peptide. These values are listed in Tables I and II.
### TABLE I

Chemical shifts of unligated mCrk23

| Residue | \(\delta^1H\) | \(\delta^{13}C\) | \(\delta^{15}N\) | \(\Delta\delta^{1H}\) |
|---------|-------------|---------------|---------------|-----------------|
| ASN 4   | 174.1       | 127.4         | 127.4         | 0.0             |
| PHE 5   | 174.1       | 127.4         | 127.4         | 0.0             |
| VAL 7   | 174.1       | 127.4         | 127.4         | 0.0             |
| GLU 9   | 174.1       | 127.4         | 127.4         | 0.0             |
| LYS 11  | 174.1       | 127.4         | 127.4         | 0.0             |
| ARG 12  | 174.1       | 127.4         | 127.4         | 0.0             |
| SER 13  | 174.1       | 127.4         | 127.4         | 0.0             |
| THR 16  | 174.1       | 127.4         | 127.4         | 0.0             |
| SER 18  | 174.1       | 127.4         | 127.4         | 0.0             |
| PHE 20  | 174.1       | 127.4         | 127.4         | 0.0             |
| HIS 23  | 174.1       | 127.4         | 127.4         | 0.0             |

Values for \(\delta^1H\), \(\delta^{13}C\), and \(\delta^{15}N\) are referenced to external TSP, liquid ammonia, and sodium acetate, at 0.0, 0.0, and 25.85 ppm, respectively. Residues marked with *exhibit conformational heterogeneity. Degen-erate \(\delta^1H\) shifts are listed only once. Shifts marked NA have not been assigned.

### TABLE I—continued

A Potential SH3 Domain-binding Site in the Crk SH2 Domain

| Residue | \(\delta^1H\) | \(\delta^{13}C\) | \(\delta^{15}N\) | \(\Delta\delta^{1H}\) |
|---------|-------------|---------------|---------------|-----------------|
| SER 96  | 174.1       | 127.4         | 127.4         | 0.0             |
| LEU 97  | 174.1       | 127.4         | 127.4         | 0.0             |
| VAL 98  | 174.1       | 127.4         | 127.4         | 0.0             |
| ARG 99  | 174.1       | 127.4         | 127.4         | 0.0             |
| GLU 100 | 174.1       | 127.4         | 127.4         | 0.0             |
| SER 101 | 174.1       | 127.4         | 127.4         | 0.0             |
| VAL 102 | 174.1       | 127.4         | 127.4         | 0.0             |
| HIS 103 | 174.1       | 127.4         | 127.4         | 0.0             |
| THR 104 | 174.1       | 127.4         | 127.4         | 0.0             |
| SER 105 | 174.1       | 127.4         | 127.4         | 0.0             |
| HIS 106 | 174.1       | 127.4         | 127.4         | 0.0             |
| SER 107 | 174.1       | 127.4         | 127.4         | 0.0             |
| SER 108 | 174.1       | 127.4         | 127.4         | 0.0             |
| SER 109 | 174.1       | 127.4         | 127.4         | 0.0             |
| SER 110 | 174.1       | 127.4         | 127.4         | 0.0             |
| SER 111 | 174.1       | 127.4         | 127.4         | 0.0             |
| SER 112 | 174.1       | 127.4         | 127.4         | 0.0             |
| SER 113 | 174.1       | 127.4         | 127.4         | 0.0             |
| SER 114 | 174.1       | 127.4         | 127.4         | 0.0             |
| SER 115 | 174.1       | 127.4         | 127.4         | 0.0             |
| SER 116 | 174.1       | 127.4         | 127.4         | 0.0             |

Values for \(\delta^1H\), \(\delta^{13}C\), and \(\delta^{15}N\) are referenced to external TSP, liquid ammonia, and sodium acetate, at 0.0, 0.0, and 25.85 ppm, respectively. Residues marked with *exhibit conformational heterogeneity. Degen-erate \(\delta^1H\) shifts are listed only once. Shifts marked NA have not been assigned.
TABLE II—continued

A Potential SH3 Domain-binding Site in the Crk SH2 Domain
terminal SH3 domain of Crk-II, is an effective model system for studying changes that occur to the SH2 domain on binding to phosphopeptides. The sequential assignment has revealed features of the SH2 domain that are relevant to the biochemical studies described above. First, many of the non-proline residues in and immediately surrounding the proline-rich loop in the SH2 domain appear to exist in multiple conformations, as evidenced by significant line-broadening and/or the presence of multiple weak peaks in 1H-15N HSQC and 1H-15N/13COHNCO spectra. In the large majority of cases, these peaks do not change when peptide is added to NMR samples of Crk23, indicating that the structure and dynamics of most of the proline-rich loop do not change significantly on ligation of the SH2 domain. One residue that does change, however, is Val-71. As illustrated in Fig. 6, the backbone 1H and 15N chemical shifts of this residue change by 20 Hz and 30 Hz, respectively, upon addition of pTyr-221 peptide. Although many residues in the SH2 domain have amide chemical shifts that change on ligation, Val-71 is the only one of these that is not predicted by homology to lie in the established phosphopeptide binding pocket. Interestingly, as described above, mutation of Arg-68, the first non-proline residue N-terminal to Val-71, completely abrogated the interaction between the Crk proline-rich region and the Abl SH3 domain in the yeast two-hybrid system (Fig. 3B). The resonances of Arg-68 could not be definitively assigned in the spectra. Apparent exchange broadening (due to conformational averaging) of several candidate peaks precluded the observation of correlations to Pro-69 in HbCβ-CaCO(N(CO)Ca)Ha spectra that would be necessary to assign Arg-68 (see "Experimental Procedures").

Ability of Abl-kinases to Phosphorylate Crk-II Is Affected by the Crk Proline-rich Insert—To investigate if the Crk proline-rich insert is potentially involved in the interaction of full-length Crk-II with the Abl tyrosine kinase, we tested two Abl proteins, the normal p140 abl and its transforming counterpart p160 gag-ABL, for their abilities to phosphorylate different concentrations of wild type Crk-II or Crk-II3Pro, which lacks the proline-rich region. The amino acid sequences of the kinase domain and the tails of both cellular and viral Abl proteins are very similar; however, p140 abl contains an SH3 domain, which is replaced in p160 gag-ABL with viral Gag polypeptide sequences. In vitro p160 gag-ABL phosphorylated both wild type and mutant Crk-II proteins to a similar level with an apparent Km of approximately 25 μM. However, p140 abl phosphorylated the Crk-II3Pro up to 20-fold more efficiently than the wild type Crk-II (Fig. 7). Since a critical difference between p140 abl and p160 gag-ABL is the presence or absence of the Abl SH3 domain, these results are consistent with a model in which the proline-rich motif in the Crk-II SH2 domain interacts with the Abl SH3 domain, and thereby modifies Abl kinase activity.

**DISCUSSION**

Identification of an SH3 Domain-binding Site in the Crk SH2 Domain—Using the yeast two-hybrid assay and direct binding analysis, we have found that a proline-rich region located within the Crk-II SH2 domain can potentially form a binding site for SH3 domains, notably that from the Abl tyrosine kinase. This SH3 domain-binding site is apparently not required for association of the Crk SH2 domain with pTyr-containing ligands, consistent with modelling data suggesting that it may be located within an extended DE loop. Indeed phosphorylation of the Crk-II protein at Tyr-221, which has been shown to induce an intramolecular pTyr-SH2 interaction (19, 21), stimulates the binding of the Crk SH2 domain to the Abl SH3 domain.

The SH3 domain-binding site within the Crk SH2 domain is apparently located primarily in the proline-rich insert, as deletions and amino acid substitutions in this element abrogate the SH3 domain-binding properties of the Crk SH2 domain both in the yeast two-hybrid assay and in vitro binding experiments. However, it appears that residues C-terminal to the proline-rich insert are also required for SH3 domain-binding, possibly through an effect on the conformation of the proline insert. A related observation has been made for the Nef protein of HIV-1 which binds to the Hck SH3 domain through a primary proline-rich motif, but requires additional sequences for high affinity binding (42, 43).

Full-length unphosphorylated Crk-II bound only weakly to SH3 domains in a solution-binding assay (Kd > 100 μM, Fig. 5B). However, the same concentration of Crk-II phosphorylated on Tyr-221 bound more strongly to the Abl SH3 domain. In addition, incubation of unphosphorylated Crk-II with phosphopeptide corresponding to the Crk-II Tyr-221 phosphorylation site...
enhanced its binding to the Abl SH3 domain. The finding that a mutant Crk-II protein, containing a substitution in the SH2 domain which inhibits pTyr binding, fails to show phosphopeptide-induced SH3 domain-binding, suggests that the association of a pTyr ligand with the SH2 domain is required to stimulate SH3 domain binding activity. The observation that the backbone amide chemical shifts of Val-71 in mCrk23 change on binding of the pTyr-221 peptide is consistent with the demonstrated importance of residues at the N terminus of the loop in mediating interactions with the Abl SH3 domain. Taken together, these results suggest that the Crk SH2 domain has a weak but detectable SH3 domain binding activity in the absence of a phosphopeptide ligand. However, binding of a pTyr site to the Crk SH2 domain increases the affinity of the proline-rich insert for SH3 domains, possibly through induction of a conformational change involving the N-terminal region of the proline-rich insert.

Direct interactions between SH2 and SH3 domains have been previously reported for Fyn, Lck, and Grb2 (6, 10, 11). In the case of Fyn this is unlikely to be a typical SH3-mediated interaction, as association could be observed in the presence of proline-rich peptides or when the SH3 domain was mutated such that it lost the ability to bind proline-rich ligands. Likewise, in Grb2 the SH2-SH3 interaction observed between the two molecules in the crystallographic dimer does not involve the standard peptide-binding pocket of the SH3 domain. The intermolecular binding of the Lck SH3 domain to the Lck SH2 domain, observed in crystal structures, does involve the recognition of a Pro at the EF2 position of the SH2 domain by the SH3 domain. This Pro residue is an integral part of the SH2 domain, and is located within the EF loop which forms one of the jaws for the +3-binding pocket of the Lck SH2 domain.

In contrast, we have identified a proline-rich SH3 domain-binding site within the Crk SH2 domain that represents an insert relative to other SH2 domains, and does not appear to be directly involved in the recognition of pTyr-containing proteins. These observations make two points of more general significance for SH2- and SH3-mediated interactions. First, the proline-rich sequence in the Crk SH2 domain represents a protein-binding site quite distinct from the conventional binding surface for pTyr-containing proteins. Hence, individual SH2 domains may contain specific sequences that expand their potential range of binding partners. In addition, the ability of the Crk SH2 proline-rich insert to bind SH3 domains is enhanced by binding of a pTyr peptide to the pTyr-binding site. Although the molecular mechanism by which the binding of a pTyr peptide to the SH2 domain modifies the accessibility or conformation of the proline-rich insert remains to be tested, these results provide evidence for an SH3 domain-binding site that is regulated, albeit indirectly, by phosphorylation and consequent changes in protein-protein interactions.

Possible Functions of the Crk SH2 Proline-rich Insert—Several potential functions can be envisaged for the SH3 domain-binding site in the Crk SH2 domain. One possibility is that the intramolecular interaction between the Crk pTyr-221 site and the SH2 domain elicits an interaction between the proline-rich insert and one or other of the two Crk SH3 domains, thereby influencing their binding properties. However, we have not been able to demonstrate an intermolecular interaction between the proline-rich insert and the Crk SH3 domains using either the yeast two-hybrid system or direct binding assays (data not shown). Furthermore, no changes in backbone chemical shift of residues in the N-terminal SH3 domain are observed in NMR spectra either on adding of pTyr-221 peptide to Crk23 (21; Tables I and II) or on phosphorylation of Crk-II (21). However, it is possible that such an interaction with the C-terminal SH3 domain could be favored if it occurs in an intramolecular fashion.

Among tested SH3 domains the Crk proline-rich insert binds most efficiently to that from the Abl tyrosine kinase. Despite the relatively low affinity for binding of the Abl SH3 domain to the proline-rich motif in the Crk SH2 domain, this interaction might be stabilized in the context of full-length Abl and Crk-II proteins through a proximity effect. The N-terminal Crk SH3 domain associates with a proline-rich motif in the C-terminal Abl tail (19, 20); this interaction might facilitate association of the proline-rich insert to interact with the Abl SH3 domain, and thereby modify Abl activity. The Abl SH3 domain is known to be a negative regulator of Abl kinase and transforming activities, and has been postulated to bind an exogeneous inhibitor (44, 45). Tyrosine phosphorylation of Crk-II by Abl might therefore act as a feedback inhibitor of Abl by driving a bidentate association of Crk-II with Abl, in which the N-terminal Crk SH3 domain binds the Abl tail, and the proline-rich loop of Crk SH2 insert binds the Abl SH3 domain. In this respect Crk-II may bind Abl in a similar way as proposed for Abi-2, which modulates c-Abl kinase and transforming activity (46). Consistent with this possibility, we find that purified tyrosine-phosphorylated Crk-II retains its ability to bind to vitro to the Abl C-terminal tail (data not shown). Furthermore, we have observed that deletion of the Crk SH2 proline-rich motif makes Crk-II phosphoepitope a better substrate for p140{sup}crk{sub} but not for p160{sup}crk{sub}—both of which lack an SH3 domain. It is of interest that Crkl, which is very similar to Crk-II but lacks the SH2 proline-rich insert (Fig. 1), is a preferential substrate for the Grb-2 Abl tyrosine kinase in bcr-abl-transformed cells (47, 48). Arguing against this hypothesis is the finding that phosphorylated Crk-II is reported to be primarily in an uncomplexed state in vivo (15). It is also quite possible that the physiological binding partner for the Crk SH2 proline-rich insert is an unknown or untested SH3 domain, or a distinct module such as a WW domain that also recognizes proline-rich motifs (49–51).

In summary, we have identified an insert in the Crk-II SH2 domain which is dispensable for binding of pTyr-containing proteins, but can act as a specific SH3 domain-binding site. Within the context of the intact Crk-II protein, the SH3 domain binding activity of this element is stimulated by the association of a pTyr peptide with the conventional SH2-binding site, either through an intramolecular interaction with the Crk-I pTyr-221 site or through an intermolecular association with a phosphopeptide. Although the functional significance of such SH2-SH3 interactions remains to be established, they may serve to regulate signaling either by contributing to an intramolecular interaction that locks Crk-II in an inactive conformation, or by down-regulating the Abl tyrosine kinase.

REFERENCES

1. Pawson, T. (1995) Nature 373, 573–579
2. Cohen, G. B., Ren, R., and Baltimore, D. (1995) Cell 80, 237–248
3. McPhail, C. L. (1994) J. Exp. Med. 180, 1111–1115
4. Watters, S. B., Yamauchi, K., and Pessin, J. E. (1995) Mol. Cell. Biol. 15, 2791–2799
5. Rozakis-Adcock, M., van der Geer, P., Mbamalu, G., and Pawson, T. (1995) Oncogene 14, 1417–1426
6. Superti-Furga, G., Fumagalli, S., Koegl, M., Courtnidge, S. A., and Draetta, G. (1993) EMBO J. 12, 2625–2634
7. Flynin, D. C., Liu, T. H., Reynolds, A. B., and Parsons, J. T. (1993) Mol. Cell. Biol. 13, 7892–7900
8. Fumagalli, S., Totty, N. F., Hsuan, J. J., and Courtnidge, S. A. (1994) Nature 368, 871–874
9. Taylor, S. J., and Shalloway, D. (1994) Nature 368, 867–871
10. Maignan, S., Guilhotouc, J.-P., Fromage, N., Arnoux, B., Bequart, J., and Ducruix, A. (1995) Science 268, 291–293
11. Eds, M. J., Atwell, S. K., Shoelson, S. E., and Harrison, S. C. (1994) Nature 368, 764–769
12. Pascal, S. M., Singer, A. U., Gish, G., Yamazaki, T., Shoelson, S. E., Pawson, T., Kay, L. E., and Forman-Kay, J. (1994) Cell 77, 461–472
A Potential SH3 Domain-binding Site in the Crk SH2 Domain

13. Lee, C.-H., Kominos, D., Jacques, S., Margolis, B., Schlesinger, J., Shoelson, S. E., and Kuriyan, J. (1994) Structure 2, 423–436.
14. Ramshak, L. E., Chen, C.-S., and Cantley, L. C. (1995) Cell 81, 821–830.
15. Feller, S. M., Ren, R., Hanafusa, H., and Baltimore, D. (1994) Trends Biochem. Sci. 19, 453–458.
16. Tsuchie, H., Chang, C. H. W., Yoshida, M., and Vogt, P. K. (1989) Oncogene 4, 1281–1284.
17. Mayer, B. J., Hamaguchi, M., and Hanafusa, H. (1988) Nature 332, 272–275.
18. Matsuda, M., Tanaka, S., Nagata, S., Kojima, A., Kurata, T., and Shibuya, M. (1992) Mol. Cell. Biol. 12, 3482–3489.
19. Feller, S. M., Knudsen, B., and Hanafusa, H. (1994) EMBO J. 13, 2341–2351.
20. Ren, R., Ye, Z-S., and Baltimore, D. (1994) Gene Dev. 8, 783–795.
21. Rosen, M., Yamazaki, T., Gish, G. D., Kay, C. M., Pawson, T., and Kay, L. E. (1995) Nature 374, 477–479.
22. ten Hoeve, J., Morris, C., Heisterkamp, N., and Groffen, J. (1993) Oncogene 8, 2469–2474.
23. Pull, L., Liu, J., Gish, G., Mbamulu, G., Bowtell, D., Pelicci, P. G., Arilghaus, R., and Pawson, T. (1994) EMBO J. 13, 764–773.
24. Durfee, T., Becherer, K., Chen, P.-L., Yeh, S.-H., Yang, Y., Kilburn, A. E., Lee, W.-H., and Elledge, S. J. (1993) Gene Develop. 7, 555–569.
25. Ausubel, F., Brent, M. R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1990) Current Protocols in Molecular Biology, John Wiley and Sons, New York.
26. Friedrichs, M. S., Mueller, L., and Wittekind, M. (1994) J. Biomol. NMR 4, 703–726.
27. Kay, L. E., Kiefer, P., and Saarinen, T. (1992) J. Am. Chem. Soc. 114, 1063–1065.
28. Schleucher, J., Sattler, M., and Griesinger, C. (1993) Angew. Chem. Int. Ed. Engl. 32, 1489–1491.
29. Muhandiram, D. R., and Kay, L. E. (1994) J. Magn. Reson. B 103, 203–216.
30. Kay, L. E., Ikura, M., Tschudin, R., and Bax, A. (1990) J. Magn. Reson. 89, 496–514.
31. Clubb, R. T., Tanabal, V., and Wagner, G. (1992) J. Biol. Chem. NMR 2, 203–210.
32. Kay, L. E. (1993) J. Am. Chem. Soc. 115, 2053–2057.
33. Kay, L. E., Xu, G. Y., Singer, A. U., Muhandiram, D. R., and Forman-Kay, J. D. (1993) J. Magn. Reson. B 101, 333–337.
34. Johnson, B. A., and Blewitt, R. A. (1994) J. Biol. Chem. NMR 4, 603–614.
35. Anafi, M., Gazit, A., Gilon, C., Ben-Neriah, Y., and Levitzki, A. (1992) J. Biol. Chem. 267, 4518–4523.
36. Lim, W. A., Richards, F. M., and Fox, R. O. (1994) Nature 372, 375–379.
37. Cicchetti, P., Mayer, B., Thiedl, G., and Baltimore, D. (1992) Science 257, 803–806.
38. Ren, R., Mayer, B., Cicchetti, P., and Baltimore, D. (1993) Science 259, 1157–1161.
39. Liu, X., Marengere, L. E. M., Koch, C. A., and Pawson, T. (1993) Mol. Cell. Biol. 13, 5225–5232.
40. Yu, H., Chen, J. K., Feng, S., Dalgarno, D. C., Brauer, A. W., and Schreiber, S. L. (1994) Cell 75, 933–945.
41. Feng, S., Chen, J. K., Yu, H., Simon, J. A., and Schreiber, S. L. (1994) Science 266, 1241–1247.
42. Saksela, K., Cheng, G., and Baltimore, D. (1995) EMBO J. 14, 484–491.
43. Lee, C.-H., Leung, B., Lemmon, M. A., Zheng, J., Cowburn, D., Kuriyan, J., and Saksela, K. (1995) EMBO J. 14, 5006–5015.
44. Jackson, P., and Baltimore, D. (1989) EMBO J. 8, 449–456.
45. Franz, W. M., Berger, P., and Wang, Y. J. (1989) EMBO J. 8, 137–147.
46. Zonghan, D., and Pendergast, A. M. (1995) Gene Dev. 9, 2569–2582.
47. ten Hoeve, J., Arlinghaus, R. B., Guo, J. Q., Heisterkamp, N., and Groffen, J. (1994) Blood 84, 1731–1736.
48. Oda, T., Heaney, C., Hagogian, J. R., Okuda, K., Griffin, J. D., and Drucker, B. J. (1994) J. Biol. Chem. 269, 22925–22928.
49. Andre, B., and Springael, J.-Y. (1994) Biochem. Biophys. Res. Commun. 205, 1202–1205.
50. Bork, P., and Sudol, M. (1994) Trends Biochem Sci. 19, 531–533.
51. Chen H.-I., and Sudol M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7819–7823.
A Potential SH3 Domain-binding Site in the Crk SH2 Domain
Mordechai Anafi, Michael K. Rosen, Gerald D. Gish, Lewis E. Kay and Tony Pawson

J. Biol. Chem. 1996, 271:21365-21374.
doi: 10.1074/jbc.271.35.21365

Access the most updated version of this article at http://www.jbc.org/content/271/35/21365

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 50 references, 15 of which can be accessed free at http://www.jbc.org/content/271/35/21365.full.html#ref-list-1