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

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**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 Abl SH3 domain. Furthermore, this proline-rich insert was found to modify the efficiency with which Crk-II was phosphorylated by the p140$^{c\text{-}abl}$ tyrosine kinase. *In vitro*, the interaction of full-length non-phosphorylated Crk-II with a glutathione *S*-transferase-Abl 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 Abl 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)[1] 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 proline-rich sequences (for reviews, see Refs. 1 and 2). Although SH3 domain-binding does not directly depend on post-translational modification, there is evidence that some SH3-mediated protein-protein interactions are inducible, as noted for the assembly of a functional cytochrome oxidase complex in activated neutrophils (3). Serine phosphorylation of mammalian Sos1 may under some circumstances inhibit binding of the Grb2 SH3 domains (4, 5). Here, we have investigated the possibility that specific SH2 and SH3 domains might interact with one another, and have pursued a mechanism by which SH3 domain-binding to a proline-rich sequence might be regulated.

While SH2 and SH3 domains are separate elements, capable of functioning independently, they are frequently found within the same polypeptide chains. The Src protein-tyrosine kinase possesses neighboring SH2 and SH3 domains, that act synergistically to repress the catalytic activity of Src phosphorylated at Tyr-527 within the C-terminal tail (6). These domains also act in concert to bind exogenous Src substrates such as AFAP-110 and Sam68 (7–9). Crystal structures of linked SH3/SH2 domains from Grb2 (10) and from the Src-family kinase Lck have shown intermolecular interactions between the SH2 and SH3 domains of these polypeptides (11). Although the physiological relevance of such complexes remains uncertain, these observations raise the possibility that SH2 and SH3 domains might under some circumstances directly associate with one another.

SH2 domains have a well defined ligand-binding site for 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 polypeptides 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* oncogene 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-

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[1] The abbreviations used are: SH2 domain, Src homology 2 domain; GST, glutathione *S*-transferase; HA, hemagglutinin; pTyr, phosphotyrosine; SH3 domain, Src homology 2 domain; PAGE, polyacrylamide gel electrophoresis.

tional SH3 domain at the C terminus (18). *In vivo* the N-terminal SH3 domain of Crk-II associates with a proline-rich sequence in the tail of the Abl tyrosine kinase leading to phosphorylation of a residue (Tyr-221) located between the two SH3 domains (19, 20). Phosphorylation of Crk-II induces an intramolecular interaction between pTyr-221 and the Crk SH2 domain, which may serve to repress Crk-mediated signaling, since Tyr-221 is absent from Crk-I and v-Crk (20, 21).

A distinct gene product closely related to Crk in sequence, termed Crkl (for Crk-like), has been recently identified (22). A unique feature of the Crk SH2 domain is the presence of a proline-rich insert, which is absent from the SH2 domains of Crkl and other signaling proteins (see Fig. 1). We have investigated the possibility that this element within the Crk SH2 domain might bind SH3 domains.

## 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 *Nco*I/*Bam*HI sites of the pET11d vector (Novagen). Two variants of the Crk-II construct were prepared: Crk-II$^{\Delta Pro}$, in which the sequence encoding the proline-rich SH2 insert (amino acids 67–85) is deleted; Crk-II$^{R38A}$, 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 Crk-II$^{wt}$ and Crk-II$^{\Delta Pro}$, respectively, and cloned into the pGEX-2T vector at the *Bam*HI/*Eco*RI sites. 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 *Bam*HI/*Eco*RI sites of the PACTII vector (24; see Fig. 3*A*).

*Yeast pASI SH3 Domains Constructs*—SH3 domains from different sources were cloned in the pASI vector (25). The mouse Abl SH3 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 *Sma*I/*Bam*HI sites. The three Nck SH3 domains (amino acids 1–255) were cloned into the *Eco*RI/*Bam*HI sites of pASI.

### *Yeast Two Hybrid Assays*

The two-hybrid assay was performed as described (24). pASI 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 Tyr-153 and Tyr-187, 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 pASI and pACTII vectors where achieved by matting Tyr-153 and Tyr-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-I 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 Abl 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$^{c\text{-}abl}$, p210$^{bcr\text{-}abl}$, and p160$^{gag\text{-}abl}$ from rat, human, and viral origin, respectively.

### *Preparation and Purification of Proteins*

*GST Fusion Proteins*—Cells were grown in LB media to $OD_{600}$ = 0.6, and protein synthesis was induced with 1 mM isopropylthiogalactopyranoside. The cell pellets were homogenized by sonication in phosphate-buffered saline containing 1% Triton X-100, 1% Tween 20, 2 mM dithiothreitol, 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 $OD_{600}$ = 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 v-Abl tyrosine kinase (Oncogene 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).

*$^{15}N/^{13}C$-mCrk23*—$^{15}N/^{13}C$-mCrk23, comprising residues 2–198 of murine Crk, was expressed as described previously (21) in *Escherichia coli* strain BL21 (DE3) grown in M9 minimal media containing $^{15}NH_4Cl$ and [$^{13}C_6$]glucose as the sole nitrogen and carbon sources, respectively. Purification was exactly as described in Rosen *et al.* (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 μl of phospholipase C lysis buffer (50 mM Hepes pH 7, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 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 semidry blotting apparatus at 0.8 mA/cm$^2$ 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 blots were developed using $^{125}$I-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 $^{15}N/^{13}C$-labeled Crk23, 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 dithiothreitol, 2 mM benzamidine, 0.02% sodium azide, 90% $H_2O$, 10 %$D_2O$, 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 enhanced sensitivity, pulsed field gradient spectra (27–29): HNCO (30), HNCaCb (29), HbCbCa(CO)NNH (29), HNCAHA (31),[2] HaHbCbCa(CO)NNH (29).[2] The identity of most residues preceding proline was confirmed through HbCbCaCO(Ca)Ha (32) and HbCbCa(CO)N(COCa)Ha spectra.[2] In the latter experiment, which correlates $^1$Ha and $^{13}$Ca/$^{13}$Cb of residue i with $^{15}$N of residue i+1, peaks due to H$^{15}$N are of opposite sign to those due to $^{15}$N or $H_2{}^{15}$N. 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 an HCCH 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 nmrview (34), which was kindly provided by Dr. Bruce A. Johnson.

### *Exogenous Substrate Phosphorylation Assays*

$p160^{gag\text{-}abl}$ was extracted from a cell line of Abelson virus-transformed ANN-I (generously provided by T. Hunter, Salk Institute). $p140^{c\text{-}abl}$ was extracted from Src-transformed rat cell line, S7a. 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 antiserum was added to each sample (approximately $10^6$ and $5 \times 10^6$ of ANN-I and S7a, 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 [$\gamma$-$^{32}$P]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 densitometeric 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. 1*A*). 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. 1*A*). 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. 2*a*).

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. 2*b, 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. 2*b, panels b* and *c*). Cells were lysed, immunoprecipitated with anti-HA antibodies, and immunoblotted with antibodies

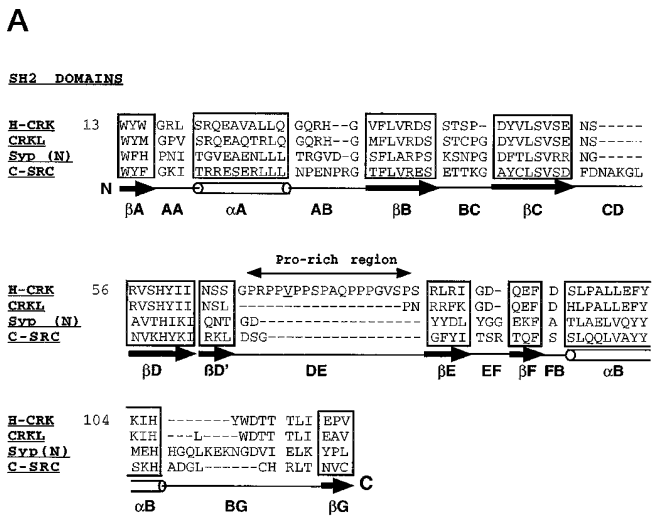


FIG. 1. *A,* alignment of the amino acids sequences of the SH2 domains of Crk-II, Crkl, Syp N-terminal SH2, and Src. The SH2 sequences of human Crk-II (H-CRK) and Crkl were aligned based on the defined secondary structures of Syp and Src (13). Gaps have been introduced to optimize the alignment and are 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 (P*XX*P 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.

to pTyr (Fig. 2*b, 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 $\beta$B5 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*-P, 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 core sequences can be identified in the Crk SH2 proline-rich insert, depending on the orientation of the peptide and the register that is chosen (Fig. 1*B*). To test the possibility that the Crk SH2 domain might have functional SH3 domain-binding sites, the yeast two-hybrid system was used to assess its ability to interact with specific SH3 domains. The pASI expression vector was used to generate a series of fusion proteins each containing a specific SH3 domain joined to the Gal4 DNA-binding domain. These constructs were then

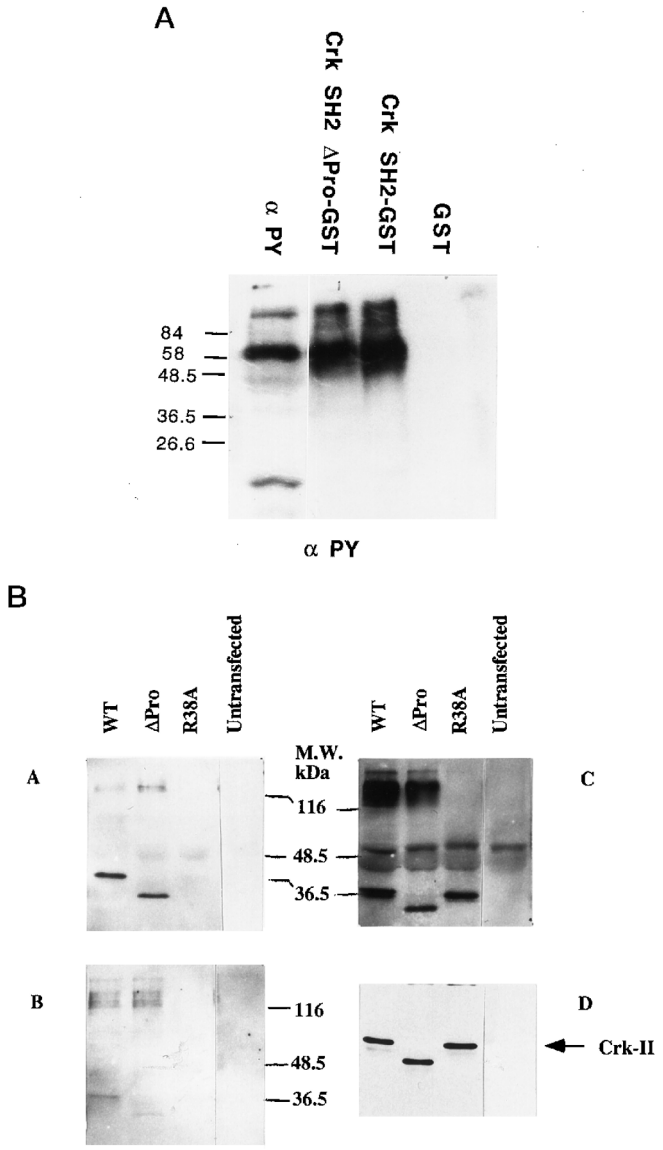


FIG. 2. **The Crk SH2 proline-rich insert is not required for binding to pTyr proteins.** *A,* lysates of v-*src* transformed Rat-2 cells were incubated with glutathione-Sepharose beads coupled to GST or GST-Crk SH2, or to GST-Crk SH2$^{\Delta Pro}$ that lacks the proline-rich insert. The resulting complexes were resolved by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis with anti-pTyr antibodies. A Rat-2 v-*src* lysate was immunoprecipitated with anti-pTyr antibodies as a positive control. *B,* HA-tagged wild type (WT) Crk-II and mutated forms of HA-Crk-II which lack the proline-rich region (ΔPro) or contain an inactivating substitution in the SH2 domain (R38A), were transiently expressed in COS-I cells (*panel A*). The same HA-Crk-II vectors were also expressed in combination with v-Crk (*panel B*) or v-Src (*panel C*) expression vectors. Each of the Crk-II proteins were immunoprecipitated with monoclonal anti-HA antibodies, and associated cellular pTyr-containing proteins were detected by Western blot analysis with anti-pTyr antibodies. *Panel D* show an anti-HA Western blot of total cell lysate of COS-I cells expressing each of the Crk-II proteins.

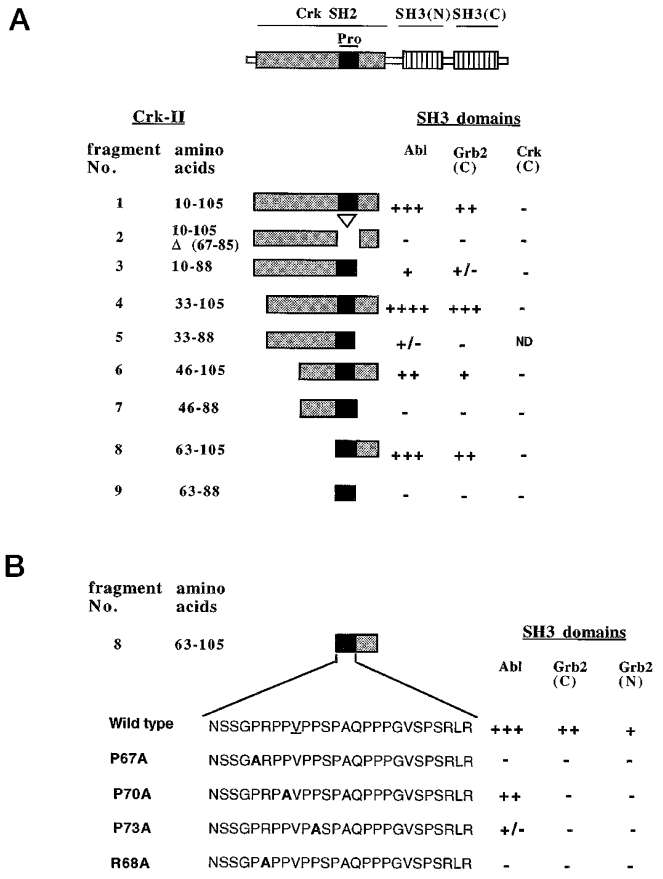


FIG. 3. **Identification of a minimal region of the Crk SH2 domain that interacts with SH3 domains.** *A,* varying fragments of the Crk SH2 domain were tested for their interaction with the Abl, Grb2(C), and Crk-II(C) SH3 domains in the yeast two-hybrid system. The relative level of β-galactosidase activity during staining the yeast with 5-bromo-4-chloro-3-indoyl β-D-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*.

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. 3*A*, 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 domain of Grb2 also interacted with the Crk SH2 fusion protein, although it gave a weaker signal than the Abl SH3 domain. Finally, a low signal was also detected with the N-terminal Grb2 SH3 domain. No interaction was observed with the SH3 domains of Crk (C-terminal), Nck, or phospholipase C-γ (data not shown). We have not been able to test the SH3 domains of v-Src, c-Src, p85 phosphatidylinositol 3-kinase, and Crk (N-terminal) in this system, as they independently stimulate transcriptional activation in the two-hybrid assay. To investigate the involvement of the Crk proline-rich insert in binding to SH3 domains, a deletion was introduced into the construct encoding Crk residues 10–105 that removes the insert (residues 67–85). Deletion of the proline-rich insert abrogated interaction of the Crk SH2 domain with Abl and Grb2 SH3 domains in the two-hybrid assay, indicating that the proline-rich insert is critical for as-

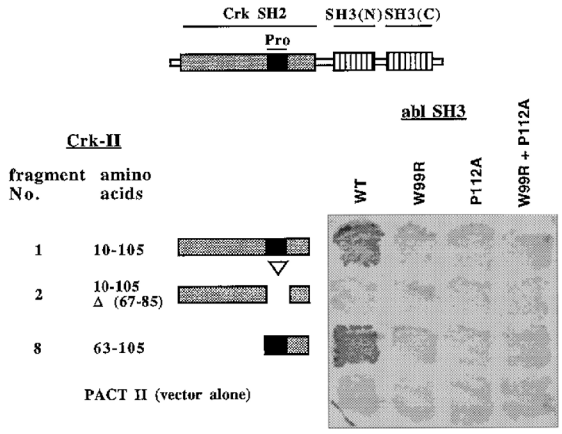


FIG. 4. **Substitutions of conserved residues in the Abl SH3 domain abolishes binding to the Crk SH2 proline-rich insert.** The DNA-binding subunit of the Gal-4 promoter was fused with wild type, W99R, P112A, and W99R+P112A Abl SH3 domains. The wild type and mutant SH3 domains were co-expressed with 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.

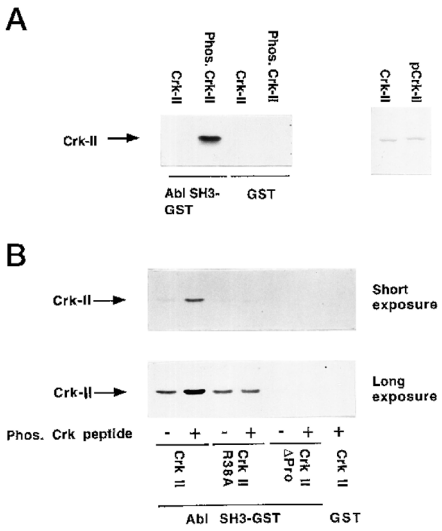


FIG. 5. ***In vitro*** **binding of the Crk SH2 proline-rich insert to the Abl SH3 domain is potentiated by an intramolecular interaction between Crk SH2 and pTyr-221.** *A*, 50 μM non-phosphorylated Crk-II or Crk-II specially phosphorylated on Tyr-221 were incubated with GST-Abl SH3 or GST alone. The GST containing proteins were collected with glutathione beads and the complexes were subjected to Western blot analysis with anti-Crk-II antibodies. *B,* unphosphorylated Crk-II or Crk-II preincubated with 140 μM pTyr-221 peptide were incubated at 100 μM concentration with GST-Abl SH3 or GST alone. The complexes were collected and analyzed as described for *A*. Three different Crk-II proteins were used: wild type Crk-II, Crk-II in which an invariant arginine crucial for binding to pTyr was mutated to alanine ($Crk\text{-}II^{R38A}$), or Crk-II in which the proline-rich region was deleted ($Crk\text{-}II^{\Delta Pro}$).

sociation with the Abl and Grb2 SH3 domains.

To localize the region within the Crk SH2 domain that interacts with Abl 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 (fragment 8, Fig. 3*A*), 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 Abl 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. 3*B*) 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 Abl SH3 domain, but abrogated binding to the Grb2 SH3 domains. These results suggest that the Abl 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 Abl SH3 domain represents a typical SH3-mediated interaction, substitutions were introduced into conserved residues in the Abl SH3 domain that are known to be important for efficient binding to proline-rich ligands (Trp-99 and Pro-112 of mouse Abl-type IV). As shown in Fig. 4 the W99R mutant of the Abl 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 Abl 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 Abl SH3 domain, we investigated the association of full-length Crk-II, purified from bacteria, with a GST-Abl SH3 fusion protein. Using purified Crk-II at the concentration of 50 μM we could barely detect any binding of Crk-II to the Abl 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 Abl tyrosine kinase (Fig. 5*A*). 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 $Crk\text{-}II^{\Delta Pro}$, that lacks the proline-rich SH2 insert, were also tested. As shown in Fig. 5*B*, adding the pTyr-221 peptide to unphosphorylated Crk-II caused an increase in its association with the Abl SH3 domain. This effect was not observed with the $Crk\text{-}II^{R38A}$ mutant. Deletion of the proline-rich region from Crk-II completely abolished its association with the Abl 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 Abl 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, $^{15}N$, and $^{13}C$ 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. Previous studies have shown that Crk23, which contains the SH2 domain and N-

TABLE I

*Chemical shifts of unligated mCrk23*

Values for $^{1}H$, $^{15}N$, and $^{13}C$ 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. Degenerate $^{1}Hb$ shifts are listed only once. Shifts marked NA have not been assigned.

| Residue | $^{1}HN$ | $^{15}N$ | $^{13}CO$ | $^{13}Ca$ | $^{13}Cb$ | $^{1}Ha$ | $^{1}Hb$ |
|---|---|---|---|---|---|---|---|
| ASN 4 | NA | NA | 174 | 52.8 | 39.0 | 5.01 | 2.99, 2.87 |
| PHE 5 | 7.74 | 116.0 | 172.3 | 56.4 | 39.8 | 4.88 | 3.45, 3.33 |
| ASP 6 | 9.01 | 122.2 | 177.8 | 52.7 | 41.1 | 4.93 | 2.73, 3.14 |
| SER 7 | 8.96 | 123.9 | 174.8 | 61.1 | 62.7 | 4.23 | 3.88 |
| GLU 8 | 8.56 | 117.6 | 177.6 | 56.9 | 29.8 | 4.43 | 2.31, 2.14 |
| GLU 9 | 7.75 | 122.1 | 174.9 | 54.8 | 28.4 | 4.50 | 2.47, 2.00 |
| ARG 10 | 7.97 | 128.6 | 176.3 | 57.8 | 30.2 | 2.29 | 1.48 |
| SER 11 | 8.40 | 112.1 | 175.3 | 60.1 | 62.3 | 4.12 | 3.82 |
| SER 12 | 7.93 | 116.0 | 174.9 | 59.9 | 64.1 | 4.44 | 4.10 |
| TRP 13 | 6.65 | 114.5 | 175.1 | 55.6 | 32.3 | 5.23 | NA |
| TYR 14 | 8.21 | 123.0 | 176.3 | 56.8 | 38.2 | 5.84 | 2.78 |
| TRP 15 | 9.42 | 129.0 | 175.5 | 57.6 | 29.7 | 4.30 | 2.68 |
| GLY 16 | 5.37 | 105.4 | 174.1 | 46.9 | ------ | 3.39 | 3.67 |
| ARG 17 | 8.53 | 128.2 | 174.2 | 56.7 | 28.5 | 4.37 | 1.95 |
| LEU 18 | 7.86 | 130.2 | 175.4 | 54.5 | 45.1 | 4.53 | 1.59 |
| SER 19 | 8.99 | 124.4 | 174.2 | 57.9 | 65.4 | 4.47 | NA |
| ARG 20 | 9.18 | 123.4 | 177.8 | 60.2 | 30.2 | 3.57 | NA |
| GLN 21 | 8.61 | 116.2 | 179.1 | 59.0 | 27.7 | 4.02 | 2.08 |
| GLU 22 | 7.87 | 121.1 | 178.5 | 58.9 | 30.0 | 4.00 | NA |
| ALA 23 | 8.32 | 121.7 | 178.8 | 55.0 | 17.7 | 3.81 | 1.44 |
| VAL 24 | 8.36 | 117.5 | 176.8 | 67.0 | 31.2 | 3.56 | 2.28 |
| ALA 25 | 7.66 | 120.6 | 180.2 | 54.6 | 17.6 | 4.12 | 1.50 |
| LEU 26 | 7.46 | 116.5 | 177.9 | 56.7 | 43.5 | 4.08 | 1.63, 1.02 |
| LEU 27 | 7.50 | 116.1 | 177.3 | 55.1 | 44.9 | 4.22 | 1.77, 1.12 |
| GLN 28 | 8.67 | 121.5 | 177.6 | 57.5 | 26.8 | 3.96 | 2.22, 2.01 |
| GLY 29 | 9.02 | 116.1 | 175.1 | 45.0 | ------ | 3.80, 4.24 | |
| GLN 30 | 7.91 | 118.4 | 176.9 | 54.4 | 28.6 | 4.33 | 2.14 |
| ARG 31 | 8.86 | 124.5 | 174.8 | 56.5 | 30.1 | 4.03 | 1.87 |
| HIS 32 | 8.16 | 120.4 | 176.6 | 55.5 | 28.5 | 4.67 | 3.19 |
| GLY 33 | 9.48 | 109.4 | 176.1 | 45.9 | ------ | 3.67, 4.77 | |
| VAL 34 | 8.12 | 126.7 | 176.2 | 62.5 | 31.0 | 5.47 | 2.16 |
| PHE 35 | 7.90 | 123.8 | 172.1 | 55.7 | 45.4 | 5.97 | 3.26, 2.67 |
| LEU 36 | 9.29 | 114.5 | 174.4 | 55.2 | 44.0 | 4.69 | 1.81 |
| VAL 37 | 9.27 | 120.3 | 173.4 | 61.1 | 34.0 | 5.62 | 2.75 |
| ARG 38 | 9.64 | 125.2 | 173.4 | 52.5 | 34.5 | 5.45 | 1.47 |
| ASP 39 | 9.01 | 123.4 | 176.4 | 54.7 | 40.4 | 5.00 | 2.84, 2.44 |
| SER 40 | 7.93 | 114.5 | NA | 57.5 | 63.3 | 4.53 | NA |
| SER 41 | NA | NA | 175.5 | 60.2 | 63.5 | 4.46 | 4.04 |
| THR 42 | 8.22 | 114.1 | 174.7 | 63.3 | 69.6 | 4.38 | NA |
| SER 43 | 7.86 | 118.7 | 170.9 | 54.0 | 62.7 | 4.92 | NA |
| PRO 44 | ----- | 137.8 | 177.4 | 64.0 | 31.6 | | |
| *GLY 45 | 8.75 | 114.1 | 173.1 | 45.1 | ------ | 4.36, 3.61 | |
| *ASP 46 | 7.90 | 120.2 | 176.4 | 53.4 | 41.9 | 5.19 | 3.02, 3.02 |
| TYR 47 | 8.90 | 119.0 | 173.6 | 56.5 | 42.9 | 5.54 | 2.85, 3.33 |
| VAL 48 | 9.84 | 119.7 | 174.2 | 61.4 | 35.3 | 4.89 | 1.82 |
| LEU 49 | 9.32 | 130.1 | 174.9 | 53.2 | 44.1 | 5.16 | 2.12, 1.34 |
| *SER 50 | 9.01 | 127.2 | 172.5 | 58.6 | 64.7 | 5.75 | NA |
| VAL 51 | 8.81 | 121.9 | 173.9 | 59.3 | 36.3 | 5.01 | 1.88 |
| *SER 52 | 8.78 | 121.0 | 173.8 | 57.2 | 62.4 | 4.89 | NA |
| GLU 53 | 8.59 | 132.1 | 175.1 | 56.8 | 32.2 | 4.30 | NA |
| ASN 54 | 8.89 | 126.4 | 174.3 | 54.5 | 37.1 | 4.29 | 3.07, 2.77 |
| *SER 55 | 10.23 | 112.0 | 173.0 | 60.0 | 62.1 | 4.35 | 4.23 |
| ARG 56 | 8.13 | 122.0 | 174.5 | 54.2 | 31.6 | 5.17 | 2.10, 1.91 |
| VAL 57 | 7.98 | 121.6 | 175.2 | 61.5 | 33.9 | 4.81 | 1.99 |
| SER 58 | 9.15 | 125.2 | 171.9 | 56.3 | 66.0 | 4.75 | 4.55 |
| HIS 59 | 8.27 | 120.7 | 174.1 | 54.5 | 32.1 | 4.99 | 2.82 |
| TYR 60 | 9.43 | 125.3 | 174.6 | 57.2 | 39.4 | 4.69 | 2.94 |
| ILE 61 | 8.64 | 126.2 | 174.5 | 61.5 | 38.0 | 4.40 | 2.00 |
| ILE 62 | 9.17 | 129.3 | 175.5 | 59.5 | 39.2 | 4.73 | 1.95 |
| ASN 63 | 9.17 | 124.4 | 175.1 | 53.1 | 41.3 | 4.98 | 2.97, 2.65 |
| *SER 64 | 9.10 | 120.0 | 174.8 | 57.3 | 64.3 | 4.02 | NA |
| *SER 65 | 8.72 | 120.6 | 174.3 | 58.2 | 64.1 | 4.69 | 3.94 |
| *GLY 66 | 8.31 | 111.0 | NA | 44.4 | ------ | 4.70 | |
| PRO 67 | ----- | NA | NA | NA | NA | | |
| ARG 68 | NA | NA | NA | NA | NA | NA | NA |
| PRO 69 | ----- | NA | NA | NA | NA | | |
| PRO 70 | ----- | NA | NA | 63.2 | 31.6 | | |
| VAL 71 | 7.79 | 118.6 | 173.7 | 59.0 | 32.9 | 4.51 | NA |
| PRO 72 | ----- | 139.5 | NA | NA | NA | | |
| PRO 73 | ----- | NA | 176.3 | 63.0 | 31.9 | | |
| *SER 74 | 8.15 | 116.4 | 173.2 | 55.8 | 63.8 | 4.87 | NA |
| PRO 75 | ----- | 136.8 | 176.2 | 63.5 | 31.6 | | |
| *ALA 76 | 8.04 | 121.3 | 176.8 | 52.0 | 19.1 | 4.39 | 1.43 |
| *GLN 77 | 7.90 | 119.6 | 173.1 | 53.3 | 29.2 | 4.69 | NA |
| PRO 78 | ----- | 138.9 | NA | NA | NA | | |
| PRO 79 | ----- | NA | NA | NA | NA | | |
| PRO 80 | ----- | NA | 177.4 | 63.0 | 31.8 | | |
| *GLY 81 | 8.42 | 109.2 | 176.3 | 45.1 | ------ | 4.00 | |
| *VAL 82 | 7.87 | 119.3 | 175.7 | 61.7 | 32.7 | 4.25 | 2.13 |
| *SER 83 | 8.49 | 121.5 | 172.4 | 56.1 | 63.3 | 4.86 | NA |
| PRO 84 | ----- | 137.7 | 176.4 | 63.0 | 32.1 | | |
| *SER 85 | 8.37 | 116.8 | 173.5 | 58.1 | 64.1 | 4.59 | 3.92 |
| *ARG 86 | 8.29 | 122.4 | 174.9 | 55.8 | 32.1 | 4.64 | 1.78 |
| *LEU 87 | 8.80 | 122.9 | 175.7 | 53.3 | 44.4 | 5.34 | 1.41, 1.57 |
| *ARG 88 | 8.97 | 121.6 | 174.3 | 54.8 | 33.8 | 5.48 | 1.91, 1.73 |
| ILE 89 | 8.66 | 123.8 | 175.6 | 59.1 | 40.3 | 4.91 | 1.88 |
| GLY 90 | 9.27 | 118.6 | 174.4 | 47.2 | ------ | 4.09, 3.79 | |
| ASP 91 | 8.73 | 126.6 | 175.4 | 53.5 | 40.8 | 4.76 | 2.78 |
| *GLN 92 | 8.34 | 120.6 | 173.7 | 54.4 | 30.8 | 4.27 | 2.09, 1.73 |
| GLU 93 | 7.75 | 121.2 | 174.7 | 54.3 | 33.1 | 5.22 | 1.74 |
| *PHE 94 | 9.10 | 118.7 | 175.1 | 56.7 | 45.3 | 4.82 | 3.31 |
| ASP 95 | 9.19 | 118.6 | 175.4 | 55.0 | 41.3 | 4.78 | 2.81 |

TABLE I—*continued*

| Residue | $^{1}HN$ | $^{15}N$ | $^{13}CO$ | $^{13}Ca$ | $^{13}Cb$ | $^{1}Ha$ | $^{1}Hb$ |
|---|---|---|---|---|---|---|---|
| SER 96 | 7.49 | 109.2 | 172.9 | 57.0 | 65.3 | 4.67 | 4.12 |
| LEU 97 | 9.35 | 122.6 | 175.7 | 58.3 | 40.1 | 4.15 | NA |
| PRO 98 | ----- | 134.1 | 177.3 | 66.5 | 31.7 | | |
| ALA 99 | 7.39 | 117.6 | 179.4 | 54.8 | 18.5 | 4.08 | 1.67 |
| LEU 100 | 7.21 | 123.2 | 177.5 | 58.2 | 42.3 | 2.16 | NA |
| LEU 101 | 8.04 | 118.5 | 178.5 | 57.4 | 41.0 | 3.56 | NA |
| GLU 102 | 8.23 | 116.1 | 179.9 | 58.6 | 29.3 | 3.65 | NA |
| *PHE103 | 7.73 | 121.3 | 179.3 | 61.9 | 39.9 | 4.22 | 2.97 |
| TYR 104 | 7.64 | 117.2 | 174.4 | 61.2 | 36.3 | 4.97 | 3.43, 2.33 |
| LYS 105 | 7.56 | 118.4 | 178.1 | 59.2 | 34.2 | 4.55 | 1.92 |
| ILE 106 | 6.80 | 110.9 | 174.3 | 60.2 | 39.7 | 4.22 | 1.66 |
| HIS 107 | 7.49 | 119.7 | 172.4 | 55.0 | 29.7 | 4.44 | 3.09, 2.05 |
| TYR 108 | 8.18 | 118.1 | 177.2 | 58.7 | 39.3 | 3.94 | 2.81, 2.61 |
| *LEU109 | 7.99 | 123.8 | 175.4 | 55.8 | 38.7 | 4.20 | 1.65, 1.14 |
| ASP 110 | 8.67 | 119.2 | 176.2 | 56.2 | 41.7 | 4.98 | 2.91 |
| *THR111 | 8.62 | 105.3 | 174.4 | 60.6 | 70.8 | 4.46 | NA |
| THR 112 | 8.12 | 116.9 | 172.3 | 60.5 | 71.1 | 5.15 | 4.07 |
| THR 113 | 7.24 | 114.8 | 175.2 | 60.7 | 70.1 | 4.76 | 4.30 |
| *LEU114 | 8.84 | 117.1 | 177 | 54.5 | 40.8 | NA | NA |
| *ILE 115 | 9.19 | 123.5 | 175.2 | 61.6 | 39.2 | 4.54 | 1.73 |
| GLU 116 | 7.25 | 114.1 | 171 | 54.0 | 31.4 | 4.86 | NA |
| PRO 117 | ----- | 135.5 | NA | 60.5 | 31.5 | | |
| VAL 118 | 8.17 | 123.7 | 173.9 | 61.6 | 32.0 | 3.92 | 1.67 |
| ALA 119 | 8.21 | 130.0 | 177.6 | 52.6 | 19.2 | 4.39 | 1.52 |
| *ARG120 | 8.44 | 121.2 | 176.5 | 55.9 | 30.3 | 4.48 | 1.82 |
| SER 121 | 8.11 | 115.9 | NA | 57.8 | 63.5 | 4.76 | NA |
| ARG 122 | NA | NA | 176 | 56.1 | 30.4 | 4.39 | 1.90 |
| GLN 123 | 8.40 | 121.4 | 176.2 | 56.0 | 29.3 | 4.39 | 2.12 |
| GLY 124 | 8.47 | 110.8 | NA | 45.2 | ------ | NA | |
| SER 125 | NA | NA | 174.9 | 58.5 | 63.8 | 4.52 | 3.96 |
| GLY 126 | 8.52 | 111.2 | 173.7 | 45.2 | ------ | 4.03 | |
| VAL 127 | 7.91 | 119.7 | 175.6 | 62.2 | 32.6 | 4.14 | 2.07 |
| ILE 128 | 8.20 | 125.3 | 175.6 | 60.6 | 38.3 | 4.20 | 1.87 |
| LEU 129 | 8.33 | 127.3 | 176.6 | 54.6 | 42.2 | 4.43 | 1.63 |
| ARG 130 | 8.35 | 122.7 | 175.8 | 55.7 | 30.7 | 4.39 | 1.85 |
| GLN 131 | 8.48 | 122.5 | 175.6 | 55.8 | 29.3 | 4.39 | 2.09 |
| GLU 132 | 8.58 | 122.6 | 176 | 56.4 | 30.2 | 4.35 | 2.05 |
| GLU 133 | 8.42 | 122.7 | 175.5 | 56.1 | 30.3 | 4.35 | 2.03 |
| ALA 134 | 8.25 | 125.8 | 175.2 | 51.8 | 19.7 | 4.27 | 1.25 |
| GLU 135 | 8.46 | 123.2 | 173.9 | 55.4 | 31.7 | 4.53 | 1.88 |
| TYR 136 | 8.96 | 122.8 | 175.7 | 56.4 | 42.2 | 5.71 | 3.15 |
| VAL 137 | 9.33 | 112.5 | 172.9 | 58.3 | 35.9 | 5.31 | 2.14 |
| ARG 138 | 9.37 | 121.3 | 175.5 | 53.1 | 33.9 | 5.30 | 1.81, 1.18 |
| ALA 139 | 8.98 | 128.3 | 178.8 | 52.6 | 21.9 | 4.41 | 1.66 |
| LEU 140 | 9.66 | 126.1 | 174.9 | 55.3 | 43.5 | 4.07 | 1.22, 0.72 |
| PHE 141 | 7.37 | 113.3 | 173.4 | 54.3 | 43.8 | 4.88 | 3.36, 2.45 |
| ASP 142 | 8.45 | 118.0 | 175.5 | 54.6 | 41.5 | 4.79 | 2.81 |
| PHE 143 | 8.46 | 121.1 | NA | 55.8 | 41.6 | 4.94 | NA |
| ASN 144 | NA | NA | 175 | 53.0 | 39.1 | 4.56 | 2.58, 2.77 |
| GLY 145 | 5.42 | 104.2 | 171.7 | 45.2 | ------ | 3.03, 3.79 | |
| ASN 146 | 9.65 | 120.1 | 174.3 | 53.7 | 40.7 | 4.83 | 2.81 |
| ASP 147 | 8.32 | 121.1 | 177.0 | 53.3 | 43.1 | 4.79 | 2.71 |
| GLU 148 | 8.87 | 121.8 | 176.9 | 59.0 | 29.6 | 4.16 | 2.12 |
| GLU 149 | 8.67 | 116.7 | 176.5 | 56.4 | 29.4 | 4.47 | 2.24, 2.02 |
| ASP 150 | 7.65 | 122.0 | 175.4 | 54.7 | 42.0 | 5.19 | 2.85 |
| LEU 151 | 9.33 | 127.7 | 171.6 | 51.0 | 43.8 | 5.02 | NA |
| PRO 152 | ----- | 133.8 | 176.5 | 61.9 | 32.7 | | |
| PHE 153 | 8.58 | 113.6 | 174.1 | 56.5 | 40.4 | 4.95 | 3.50 |
| LYS 154 | 9.20 | 121.8 | 175.5 | 53.4 | 34.9 | 5.11 | 1.90 |
| LYS 155 | 9.17 | 121.5 | 176.6 | 58.6 | 32.4 | 3.38 | 1.63 |
| GLY 156 | 8.87 | 115.8 | 173.8 | 44.6 | ------ | 3.45, 4.51 | |
| ASP 157 | 8.51 | 123.0 | 174.5 | 55.4 | 41.2 | 4.55 | 3.03, 2.62 |
| ILE 158 | 8.44 | 120.3 | 176.5 | 58.9 | 37.7 | 4.92 | 1.82 |
| LEU 159 | 9.12 | 128.8 | 174.4 | 53.1 | 44.7 | 5.06 | 1.40, 1.29 |
| ARG 160 | 8.93 | 123.1 | 175.8 | 54.3 | 32.6 | 4.67 | 1.79 |
| ILE 161 | 8.93 | 125.8 | 175.9 | 59.1 | 35.0 | 4.25 | 1.96 |
| ARG 162 | 9.38 | 127.7 | 175.4 | 55.6 | 31.1 | 4.48 | 1.82, 1.82 |
| ASP 163 | 8.10 | 116.0 | 173.9 | 53.9 | 43.7 | 4.75 | 2.77, 2.59 |
| LYS 164 | 8.26 | 120.7 | NA | 53.9 | 32.5 | 4.73 | NA |
| PRO 165 | ----- | NA | 175.9 | 64.5 | 32.2 | | |
| GLU 166 | 7.67 | 113.7 | 174.9 | 53.9 | 33.3 | 4.90 | 2.69, 2.44 |
| GLU 167 | 8.77 | 117.9 | 178.3 | 59.3 | 30.3 | 4.40 | 2.19 |
| GLN 168 | 8.55 | 111.2 | 175.2 | 55.2 | 29.2 | 4.22 | 2.05, 1.91 |
| TRP 169 | 6.89 | 120.2 | 173.3 | 56.2 | 32.4 | 5.36 | 3.11, 2.81 |
| TRP 170 | 8.59 | 120.8 | 174.3 | 52.0 | 33.0 | 5.68 | 3.46, 2.89 |
| ASN 171 | 8.95 | 121.2 | 174.1 | 53.4 | 41.9 | 5.48 | 2.76 |
| ALA 172 | 9.72 | 131.0 | 173.5 | 50.2 | 25.9 | 5.57 | 1.42 |
| GLU 173 | 9.12 | 119.0 | 175.9 | 53.5 | 35.4 | 5.75 | 1.92 |
| ASP 174 | 9.05 | 126.4 | 178.6 | 52.0 | 42.2 | 5.09 | 3.57 |
| SER 175 | 9.11 | 114.3 | 175.2 | 61.2 | 63.1 | 4.28 | 4.22 |
| GLU 176 | 8.29 | 121.3 | 176.6 | 55.5 | 30.3 | 4.58 | 2.37, 2.16 |
| GLY 177 | 8.29 | 109.3 | 174.6 | 45.0 | ------ | 3.88, 4.36 | |
| LYS 178 | 8.82 | 125.2 | 175.1 | 56.8 | 31.3 | 4.35 | 2.14, 1.87 |
| ARG 179 | 8.28 | 119.6 | 176.6 | 53.7 | 34.5 | 5.99 | 2.02, 1.78 |
| GLY 180 | 8.72 | 108.0 | 171.0 | 45.4 | ------ | 4.23 | |
| MET 181 | 9.05 | 118.2 | 177.3 | 54.0 | 31.9 | 5.67 | NA |
| ILE 182 | 9.63 | 113.2 | NA | 56.4 | 41.3 | 5.29 | NA |
| PRO 183 | ----- | NA | 176.2 | 59.6 | 29.7 | | |
| VAL 184 | 7.43 | 128.3 | 174.9 | 66.5 | 29.5 | 2.86 | NA |
| PRO 185 | ----- | 132.4 | 176.1 | 64.7 | 31.0 | | |
| TYR 186 | 7.80 | 116.2 | 175.0 | 57.4 | 38.4 | 4.85 | 3.69, 3.48 |
| VAL 187 | 7.88 | 111.5 | 173.0 | 58.6 | 35.8 | 5.31 | 1.87 |
| GLU 188 | 8.73 | 117.3 | 175.0 | 52.6 | 33.3 | 5.04 | 2.21, 1.96 |
| LYS 189 | 8.80 | 123.1 | 175.4 | 58.5 | 32.2 | 4.35 | 1.97 |
| TYR 190 | 8.27 | 125.6 | 172.8 | 56.9 | 41.2 | 4.78 | 2.86, 2.52 |
| ARG 191 | 7.91 | 128.2 | 172.4 | 52.3 | 30.9 | 4.68 | NA |
| PRO 192 | ----- | 136.5 | 176.3 | NA | NA | | |

TABLE II

*Chemical shifts of the mCrk23-pTyr$^{221}$ peptide complex*

Values for $^{1}H$, $^{15}N$, and $^{13}C$ 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. Shifts marked NA have not been assigned.

| Residue | $^{1}$HN | $^{15}$N | $^{13}$CO | $^{13}$Ca | $^{13}$Cb |
|---|---|---|---|---|---|
| ASN 4 | NA | NA | 174.0 | 53.0 | 39.2 |
| PHE 5 | 7.76 | 116.3 | 172.4 | 56.7 | 40.0 |
| ASP 6 | 8.94 | 122.1 | 177.7 | 53.1 | 41.3 |
| SER 7 | 8.93 | 123.7 | 174.8 | 61.3 | 62.9 |
| GLU 8 | 8.54 | 117.9 | 177.5 | 57.2 | 29.9 |
| GLU 9 | 7.76 | 122.0 | 175.0 | 55.2 | 28.6 |
| ARG 10 | 7.98 | 128.3 | 176.2 | 58.0 | 30.4 |
| SER 11 | 8.38 | 112.3 | 175.3 | 60.1 | 62.5 |
| SER 12 | 7.95 | 116.0 | 174.9 | 60.0 | 64.3 |
| TRP 13 | 6.67 | 114.5 | 175.0 | 55.9 | 32.4 |
| TYR 14 | 9.40 | 128.9 | 176.2 | 57.1 | 38.3 |
| TRP 15 | 9.40 | 128.8 | 175.4 | 57.8 | 29.8 |
| GLY 16 | 5.34 | 105.3 | 174.2 | 47.2 | ------ |
| ARG 17 | 8.56 | 128.3 | 174.2 | 56.8 | 28.6 |
| LEU 18 | 7.79 | 129.8 | 175.5 | 54.7 | 45.3 |
| SER 19 | 8.95 | 124.3 | 174.1 | 58.1 | 65.5 |
| ARG 20 | 9.27 | 123.0 | 177.7 | 60.4 | 30.3 |
| GLN 21 | 8.60 | 116.1 | 179.1 | 59.3 | 27.9 |
| GLU 22 | 7.90 | 121.1 | 178.4 | 59.1 | 30.2 |
| ALA 23 | 8.28 | 121.6 | 178.7 | 55.1 | 17.9 |
| VAL 24 | 8.40 | 117.5 | 176.8 | 67.3 | 31.5 |
| ALA 25 | 7.70 | 120.7 | 180.2 | 54.9 | 17.8 |
| LEU 26 | 7.45 | 116.4 | 177.9 | 56.9 | 43.7 |
| LEU 27 | 7.46 | 116.0 | 177.2 | 55.4 | 45.2 |
| GLN 28 | 8.70 | 121.5 | 177.6 | 57.7 | 27.3 |
| GLY 29 | 9.00 | 116.2 | 175.1 | 45.2 | ------ |
| GLN 30 | 7.91 | 118.4 | 176.9 | 54.7 | 28.8 |
| ARG 31 | 8.87 | 124.7 | 174.8 | 56.8 | 30.3 |
| HIS 32 | 8.18 | 120.4 | 176.4 | 55.8 | 28.6 |
| GLY 33 | 9.47 | 109.3 | 176.1 | 46.1 | ------ |
| VAL 34 | 8.14 | 126.8 | 176.2 | 62.9 | 31.2 |
| PHE 35 | 7.93 | 123.9 | 172.0 | 55.9 | 45.5 |
| LEU 36 | 9.33 | 114.7 | 174.5 | 55.4 | 44.3 |
| VAL 37 | 9.31 | 120.4 | 173.5 | 61.0 | 34.1 |
| ARG 38 | 9.65 | 124.6 | 173.3 | 52.6 | 34.8 |
| ASP 39 | 9.03 | 123.8 | 176.6 | 54.8 | 40.4 |
| SER 40 | 8.10 | 114.6 | NA | 58.3 | 63.4 |
| SER 41 | NA | NA | NA | NA | NA |
| THR 42 | NA | NA | 174.5 | 62.5 | 70.3 |
| SER 43 | 7.7 | 119.1 | NA | 54.1 | 62.2 |
| PRO 44 | ----- | 137.8 | 177.5 | 64.3 | 31.8 |
| GLY 45 | 8.79 | 114.1 | 173.1 | 45.0 | ------ |
| ASP 46 | 7.85 | 120.1 | 176.3 | 53.8 | 42.5 |
| TYR 47 | 8.78 | 119.0 | 173.1 | 56.9 | 43.1 |
| VAL 48 | 9.84 | 118.8 | 174.2 | 60.9 | 35.8 |
| LEU 49 | 9.21 | 129.8 | 175.0 | 53.1 | 44.4 |
| SER 50 | 9.05 | 127.4 | 172.5 | 59.0 | 64.8 |
| VAL 51 | 8.87 | 121.9 | 173.8 | 59.4 | 36.6 |
| SER 52 | 8.76 | 121.1 | 173.7 | 57.4 | 62.6 |
| GLU 53 | 8.61 | 132.6 | 175.1 | 57.1 | 32.6 |
| ASN 54 | 8.94 | 126.9 | 174.2 | 54.8 | 37.3 |
| SER 55 | 10.28 | 111.8 | 173.0 | 60.0 | 62.2 |
| ARG 56 | 8.12 | 122.1 | 174.5 | 54.5 | 31.8 |
| VAL 57 | 7.98 | 121.7 | 175.1 | 61.7 | 34.0 |
| SER 58 | 9.09 | 124.7 | 172.3 | 56.8 | 66.4 |
| HIS 59 | 8.51 | 121.4 | 173.3 | 54.7 | 32.4 |
| TYR 60 | 9.39 | 125.3 | NA | 57.6 | 39.2 |
| ILE 61 | NA | NA | 172.4 | 53.6 | 42.3 |
| ILE 62 | 8.92 | 128.3 | 175.5 | 59.7 | 39.8 |
| ASN 63 | 9.12 | 124.4 | 175.1 | 53.6 | 41.2 |
| SER 64 | 8.89 | 120.5 | 174.7 | 57.9 | 64.4 |
| *SER 65 | 8.62 | 120.3 | 174.2 | 58.3 | 64.4 |
| *GLY 66 | 8.33 | 111 | NA | 44.6 | ------ |
| PRO 67 | ----- | NA | NA | NA | NA |
| ARG 68 | NA | NA | NA | NA | NA |
| PRO 69 | ----- | NA | NA | NA | NA |
| PRO 70 | ----- | NA | NA | 63.2 | 31.7 |
| VAL 71 | 7.83 | 119.2 | NA | 59.3 | 33.0 |
| PRO 72 | ----- | 140 | NA | NA | NA |
| PRO 73 | ----- | NA | 176.3 | 63.2 | 32.0 |
| *SER 74 | 8.12 | 116.3 | NA | 56.0 | 64.0 |
| PRO 75 | ----- | 137.0 | 176.3 | 64.0 | 31.8 |
| *ALA 76 | 8.03 | 121.3 | 176.7 | 52.3 | 19.3 |
| *GLN 77 | 7.88 | 119.6 | NA | 53.4 | 29.5 |
| PRO 78 | ----- | 138.8 | NA | NA | NA |
| PRO 79 | ----- | NA | NA | NA | NA |
| PRO 80 | ----- | NA | 177.3 | 63.2 | 32.0 |
| *GLY 81 | 8.39 | 109.1 | 173.7 | 45.3 | ------ |
| *VAL 82 | 7.87 | 119.3 | 175.6 | 61.9 | 32.9 |
| *SER 83 | 8.47 | 121.5 | NA | 61.8 | 63.5 |
| PRO 84 | ----- | 137.5 | 176.4 | 63.2 | 32.2 |
| *SER 85 | 8.35 | 116.8 | 173.4 | 58.3 | 64.3 |
| *ARG 86 | 8.28 | 122.3 | 174.7 | 55.9 | 32.3 |
| *LEU 87 | 8.81 | 123.1 | 175.5 | 53.3 | 44.4 |
| *ARG 88 | 9.01 | 122.3 | 174.1 | 55.0 | 33.8 |
| ILE 89 | 8.67 | 124.4 | 174.9 | 59.0 | 40.5 |
| GLY 90 | 9.38 | 117.6 | 174.1 | 47.4 | ------ |
| ASP 91 | 8.64 | 126.5 | 175.2 | 53.6 | 40.8 |
| GLN 92 | 8.29 | 119.8 | 174.0 | 54.4 | 31.3 |
| GLU 93 | 7.71 | 120.6 | 174.6 | 54.5 | 33.2 |
| PHE 94 | 9.07 | 119.0 | 175.0 | 56.8 | 45.4 |
| ASP 95 | 9.20 | 118.7 | 175.3 | 55.0 | 41.4 |
| SER 96 | 7.48 | 109.4 | 172.9 | 57.2 | 65.6 |

TABLE II—*continued*

| Residue | $^{1}$HN | $^{15}$N | $^{13}$CO | $^{13}$Ca | $^{13}$Cb |
|---|---|---|---|---|---|
| LEU 97 | 9.31 | 122.5 | NA | 58.4 | 40.5 |
| PRO 98 | ----- | 134.0 | 177.2 | 66.8 | 31.8 |
| ALA 99 | 7.33 | 117.6 | 179.2 | 55.0 | 18.8 |
| LEU 100 | 7.11 | 123.3 | 177.6 | 58.4 | 42.6 |
| LEU 101 | 8.04 | 118.4 | 178.5 | 57.5 | 41.1 |
| GLU 102 | 8.18 | 116.1 | 179.7 | 58.8 | 29.5 |
| PHE 103 | 7.64 | 121.2 | 179.3 | 62.0 | 40.1 |
| TYR 104 | 7.62 | 117.2 | 174.3 | 61.6 | 36.5 |
| LYS 105 | 7.54 | 118.4 | 178.0 | 59.4 | 34.3 |
| ILE 106 | 6.77 | 110.8 | 174.3 | 60.4 | 39.8 |
| HIS 107 | 7.46 | 119.6 | 172.3 | 55.2 | 29.8 |
| TYR 108 | 8.20 | 118.4 | 176.5 | 58.7 | 39.6 |
| *LEU109 | 7.97 | 123.7 | NA | 56.2 | 39.5 |
| ASP 110 | NA | NA | 175.6 | 56.3 | 42.2 |
| THR 111 | 8.61 | 105.5 | 174.6 | 60.7 | 70.9 |
| THR 112 | 8.31 | 117.3 | 172.3 | 60.8 | 71.6 |
| THR 113 | 7.27 | 114.8 | 175.2 | 60.8 | 70.4 |
| LEU 114 | 8.84 | 117.0 | 176.9 | 54.8 | 41.2 |
| ILE 115 | 9.20 | 123.4 | 175.1 | 61.8 | 39.4 |
| GLU 116 | 7.25 | 114.1 | NA | 54.3 | 31.6 |
| PRO 117 | ----- | 135.7 | 176.8 | 60.7 | 31.6 |
| VAL 118 | 8.22 | 123.9 | 173.9 | 61.8 | 32.2 |
| ALA 119 | 8.20 | 130.0 | 177.5 | 52.9 | 19.4 |
| ARG 120 | 8.43 | 121.1 | 176.4 | 56.2 | 30.6 |
| SER 121 | 8.12 | 116.0 | 174.4 | 58.1 | 63.8 |
| ARG 122 | 8.41 | 123.0 | 176.0 | 56.3 | 30.6 |
| GLN 123 | 8.39 | 121.4 | 176.1 | 56.3 | 29.5 |
| GLY 124 | 8.47 | 110.7 | 173.9 | 45.4 | ------ |
| SER 125 | 8.29 | 116.0 | 174.8 | 58.7 | 63.9 |
| GLY 126 | 8.52 | 111.2 | 173.6 | 45.4 | ------ |
| VAL 127 | 7.90 | 119.7 | 175.6 | 62.4 | 32.8 |
| ILE 128 | 8.19 | 125.3 | 175.6 | 60.8 | 38.5 |
| LEU 129 | 8.32 | 127.3 | 176.6 | 54.9 | 42.4 |
| ARG 130 | 8.34 | 122.7 | 175.8 | 55.9 | 30.8 |
| GLN 131 | 8.47 | 122.5 | 175.6 | 56.0 | 29.5 |
| GLU 132 | 8.57 | 122.5 | 176.0 | 56.6 | 30.4 |
| GLU 133 | 8.41 | 122.5 | 175.5 | 56.3 | 30.5 |
| ALA 134 | 8.24 | 125.8 | 175.2 | 52.0 | 20.0 |
| GLU 135 | 8.45 | 123.1 | 173.9 | 55.6 | 31.9 |
| TYR 136 | 8.95 | 122.7 | 175.7 | 56.7 | 42.4 |
| VAL 137 | 9.32 | 112.5 | 172.8 | 58.5 | 36.0 |
| ARG 138 | 9.36 | 121.3 | 175.5 | 53.3 | 34.1 |
| ALA 139 | 8.97 | 128.3 | 178.7 | 52.9 | 22.1 |
| LEU 140 | 9.65 | 126.1 | 174.8 | 55.6 | 43.7 |
| PHE 141 | 7.37 | 113.3 | 173.3 | 54.5 | 44.0 |
| ASP 142 | 8.45 | 118.0 | 175.4 | 54.9 | 41.7 |
| PHE 143 | 8.46 | 121.0 | NA | 55.7 | 41.8 |
| ASN 144 | NA | NA | 175.0 | 53.2 | 39.3 |
| GLY 145 | 5.41 | 104.2 | 171.7 | 45.4 | ------ |
| ASN 146 | 9.61 | 120.0 | 174.3 | 54.0 | 40.9 |
| ASP 147 | 8.30 | 121.1 | 176.9 | 53.5 | 43.3 |
| GLU 148 | 8.87 | 121.9 | 176.8 | 59.2 | 29.7 |
| GLU 149 | 8.67 | 116.7 | 176.5 | 56.6 | 29.6 |
| ASP 150 | 7.64 | 122.1 | 175.3 | 54.9 | 42.1 |
| LEU 151 | 9.32 | 127.7 | NA | 51.1 | 44.0 |
| PRO 152 | ----- | 133.7 | 176.4 | 62.1 | 32.8 |
| PHE 153 | 8.57 | 113.5 | 174.0 | 56.8 | 40.6 |
| LYS 154 | 9.20 | 121.8 | 175.5 | 53.5 | 35.0 |
| LYS 155 | 9.16 | 121.5 | 176.6 | 58.8 | 32.5 |
| GLY 156 | 8.86 | 115.8 | 173.8 | 44.8 | ------ |
| ASP 157 | 8.50 | 123.1 | 174.5 | 55.5 | 41.4 |
| ILE 158 | 8.43 | 120.3 | 176.5 | 59.1 | 37.8 |
| LEU 159 | 9.12 | 128.8 | 174.4 | 53.4 | 45.0 |
| ARG 160 | 8.92 | 123.1 | 175.7 | 54.6 | 32.8 |
| ILE 161 | 8.92 | 125.8 | 175.9 | 59.3 | 35.2 |
| ARG 162 | 9.37 | 127.7 | 175.4 | 55.9 | 31.6 |
| ASP 163 | 8.09 | 116.0 | 173.9 | 54.2 | 43.9 |
| LYS 164 | 8.25 | 120.7 | NA | 53.8 | 33.3 |
| PRO 165 | ----- | NA | 175.9 | 64.8 | 32.4 |
| GLU 166 | 7.67 | 113.7 | 174.9 | 54.1 | 33.5 |
| GLU 167 | 8.76 | 117.9 | 178.3 | 59.6 | 30.5 |
| GLN 168 | 8.53 | 111.2 | 175.2 | 55.4 | 29.4 |
| TRP 169 | 6.88 | 120.2 | 173.3 | 56.5 | 32.5 |
| TRP 170 | 8.59 | 120.8 | 174.2 | 52.3 | 33.1 |
| ASN 171 | 8.94 | 121.2 | 174.0 | 53.6 | 42.1 |
| ALA 172 | 9.71 | 131.1 | 173.4 | 50.4 | 26.1 |
| GLU 173 | 9.11 | 119.0 | 175.8 | 53.8 | 35.6 |
| ASP 174 | 9.05 | 126.4 | 178.5 | 52.3 | 42.4 |
| SER 175 | 9.11 | 114.3 | 175.2 | 61.4 | 63.3 |
| GLU 176 | 8.28 | 121.2 | 176.6 | 55.7 | 30.5 |
| GLY 177 | 8.28 | 109.3 | 174.6 | 45.3 | ------ |
| LYS 178 | 8.81 | 125.2 | 175.1 | 56.9 | 31.5 |
| ARG 179 | 8.27 | 119.6 | 176.6 | 54.0 | 34.6 |
| GLY 180 | 8.72 | 108.0 | 170.9 | 45.6 | ------ |
| MET 181 | 9.04 | 118.2 | 177.2 | 54.3 | 32.1 |
| ILE 182 | 9.63 | 113.3 | NA | 56.5 | 41.4 |
| PRO 183 | ----- | 137.1 | 176.2 | 59.8 | 29.9 |
| VAL 184 | 7.41 | 128.2 | NA | 66.6 | 29.8 |
| PRO 185 | ----- | 132.3 | 176.1 | 64.9 | 31.1 |
| TYR 186 | 7.79 | 116.2 | 175.0 | 57.6 | 38.6 |
| VAL 187 | 7.87 | 111.4 | 173.0 | 58.7 | 35.9 |
| GLU 188 | 8.73 | 117.3 | 175.0 | 52.9 | 33.5 |
| LYS 189 | 8.79 | 123.2 | 175.3 | 58.6 | 32.5 |
| TYR 190 | 8.26 | 125.6 | 172.8 | 57.2 | 41.5 |
| ARG 191 | 7.90 | 128.2 | NA | 52.5 | 31.1 |
| PRO 192 | ----- | 136.4 | NA | NA | NA |

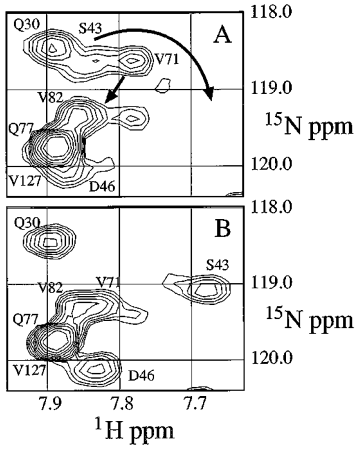


FIG. 6. **The chemical shift of Val-71 in the proline-rich loop changes on ligation of the SH2 domain.** $^{1}H/^{15}N$ HSQC spectra of free Crk23 (*top*) and Crk23 bound to the pTyr-221 peptide (*bottom*). Assignments of selected peaks are shown. The directions of movement of Ser-43 and Val-71 on binding peptide are indicated with *arrows*. Serine 43 should be located in the pTyr binding pocket, and likely shifts due to direct interaction with the bound peptide. Val-71 is located in the proline-rich loop (*underlined* in Figs. 1*A* and 3*B*).

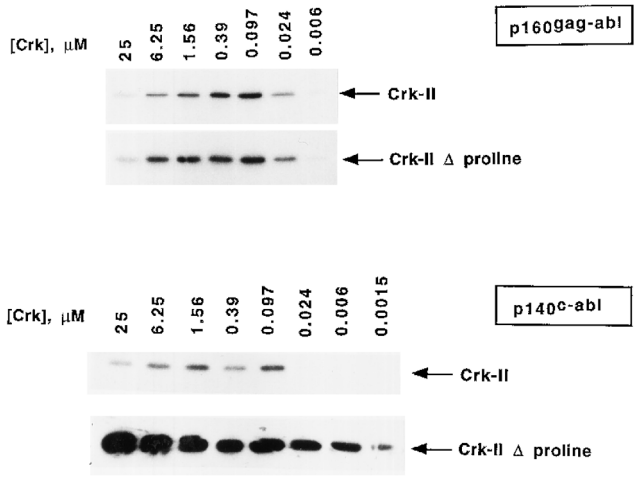


FIG. 7. **Exogenous substrate phosphorylation assays.** $p160^{gag\text{-}abl}$ was derived from a line of Abelson virus-transformed ANN-I. $p140^{c\text{-}abl}$ was extracted from Src transformed rat cell line, S7a. The cells lysates were immunoprecipitated with anti-Abl SH2 domain antibodies. The immobilized Abl proteins were subjected to kinase assays in which wild type Crk-II or Crk-II$^{\Delta Pro}$ were included as exogenous substrates. The level of the phosphorylation of Crk was determined by separation on SDS-PAGE followed by autoradiography.

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 $^{1}H/^{15}N$ HSQC and $^{1}H/^{15}N/^{13}CO$ HNCO 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 $^{1}H$ and $^{15}N$ 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. 3*B*). The resonances of Arg-68 could not be definitively assigned in the spectra. Apparent exchange broadening (due to conformational averaging) of several candidate peaks prevented the observation of correlations to Pro-69 in HbCb-Ca(CO)N(COCa)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 $p140^{c\text{-}abl}$ and its transforming counterpart $p160^{gag\text{-}abl}$, for their abilities to phosphorylate different concentrations of wild type Crk-II or Crk-II$^{\Delta Pro}$, 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, $p140^{c\text{-}abl}$ contains an SH3 domain, which is replaced in $p160^{gag\text{-}abl}$ with viral Gag polypeptide sequences. *In vitro* $p160^{gag\text{-}abl}$ phosphorylated both wild type and mutant Crk-II proteins to a similar level with an apparent $K_m$ of approximately 25 nM. However, $p140^{c\text{-}abl}$ phosphorylated the Crk-II$^{\Delta pro}$ up to 20-fold more efficiently than the wild type Crk-II (Fig. 7). Since a critical difference between $p140^{c\text{-}abl}$ and $p160^{gag\text{-}abl}$ 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 *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 ($K_d$>100 μM, Fig. 5*B*). 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 form 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 motif in the Crk SH2 domain with the Abl SH3 domain. Phosphorylation of Crk-II might further enhance the ability 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 exogenous 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 *in 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$^{\Delta Pro}$ a better substrate for p140$^{c\text{-}abl}$ but not for p160$^{gag\text{-}abl}$ which lacks 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 Bcr-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-II 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.

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