Characterization of the Interaction of Natural Proline-Rich Peptides with Five Different SH3 Domains

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Received April 25, 1994; Revised Manuscript Received June 29, 1994®

ABSTRACT: The interaction of six different proline-rich peptides with five SH3 domains has been investigated by using spectroscopic techniques. These peptides correspond to natural sequences and have been implicated in the interaction of some SH3 domains with other proteins. We have determined the K_d values for all of the possible combinations between the peptides and the SH3 domains. Low specificity and low affinity (>5 μM) are the most remarkable conclusions from these studies. None of the peptides tested here were found to bind with significant affinity to spectrin-SH3 or n-src-SH3. Abl-SH3 seems to be the most selective of the domains analyzed here, while Fyn-SH3 is the most promiscuous. CD and FTIR studies indicate that these peptides adopt to different extents a PPII-like structure in aqueous solution. However, analysis of the SH3 domain complexes with these peptides suggests that proline-rich peptides do not necessarily adopt an overall PPII structure over their entire length upon binding to the different SH3 domains.

The SH3[1] domains were first identified as homologous noncatalytic regions in cytoplasmic tyrosine kinases, phospholipase C, and the viral oncogene product Crk (Mayer *et al.*, 1988). These domains have no fixed topological position within their host proteins, and they are found in a large variety of functionally different proteins (Musacchio *et al.*, 1992a; Pawson, 1992; Pawson & Gish, 1992; Pawson & Schlessinger, 1993). SH3 domains are discrete protein modules which can be expressed, purified, and crystallized independently of the rest of the protein [see, e.g., Musacchio *et al.* (1992b)].

The presence of SH3 domains in a wide variety of unrelated proteins, many of which are involved in signal transduction, led to the speculation that this motif could play an important role in protein–protein interactions which are essential to cellular functions and/or subcellular localization (Fry *et al.*, 1993; Musacchio *et al.*, 1992a; Pawson, 1992; Pawson & Gish, 1992; Pawson & Schlessinger, 1993; Mayer & Baltimore, 1993). This possibility prompted many groups to attempt to find the *in vivo* partners of SH3 domains.

Two proteins, called 3BP1 and 3BP2, were first identified for their ability to interact with Abl-SH3. 3BP1 contains a region homologous to the GTPase-activating proteins of small G-proteins, while 3BP2 function remains unknown (Cicchetti *et al.*, 1992). Mutagenic analysis of these proteins has identified short proline-rich sequences as binding sites for the Abl-SH3 domain. These regions share the consensus sequence XPXXPPPΨXP where Ψ is a hydrophobic residue and X is any amino acid; prolines at positions 2, 7, and 10 have been shown to be essential for the binding to this domain (Ren *et al.*, 1993).

Peptide competition experiments have also indicated that proline-rich regions may be involved in the binding of mSos1 (a nucleotide-releasing factor of Ras) to the SH3 domain of the adaptor protein Grb2 (Rozakis-Adcock *et al.*, 1993), and similar motifs are involved in the interaction between the regulatory subunit of PI3K and the SH3 domains of tyrosine kinases Src, Lyn, and Fyn (Liu *et al.*, 1993; Pleiman *et al.*, 1994). In some cases, the binding of an SH3 domain has been shown to increase the enzymatic activity of the target protein (Gout *et al.*, 1993; Pleiman *et al.*, 1994).

Schreiber and co-workers have found proline-rich peptides which have micromolar affinity for the PI3K- and Src-SH3 domains by screening a biased combinatorial peptide library (Chen *et al.*, 1993; Yu *et al.*, 1994). These authors have shown by the combination of structural analysis using NMR and site-directed mutagenesis that the ligand peptide is bound in a polyproline II (PPII) conformation (Yu *et al.*, 1994). If this were the case for all SH3 domains, it could facilitate the search for the different SH3 ligands as well as the possible design of peptides with higher affinities that could be used as inhibitors, since the conformational space to be explored would be enormously diminished. However, this is still an open question. The large number of proteins containing SH3 domains or proline-rich regions in the cell indicates that the rules governing the specificities of these interactions cannot be very simple.

We have determined the K_d values for all the possible interactions between six different proline-rich peptides and five different SH3 domains (from Abl, Fyn, c-src, n-src, and spectrin proteins). The role of PPII secondary structure of the peptides for their interaction with SH3 domain as well as the physiological significance of these interactions is discussed in this paper.

EXPERIMENTAL PROCEDURES

Chemicals. The buffers used were citric acid and sodium phosphate from Merck (Darmstadt, Germany). All other reagents were of the highest purity available. Double-distilled deionized water was used throughout.

Expression and Purification of the Recombinant SH3 Domain. The pET3d plasmid coding for the different SH3

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® Abstract published in *Advance ACS Abstracts,* August 15, 1994.

[1] Abbreviations: OD, optical density; CD, circular dichroism; SH3, src-homology 3 domain; PI3K, phosphatidylinositol 3-kinase; p85α, regulatory subunit of PI3K; HCl·Gnd, guanidinium hydrochloride; IC_{50}, concentration of peptides that shows 50% inhibition; TFE, trifluoroethanol; FTIR, Fourier transform infrared; PPI, polyproline I structure; PPII, polyproline II structure; P10, homopeptide containing 10 proline residues; K_d, dissociation constant.

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Chart 1

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fyn     mgTGVTLFVALYDYEARTEDDLSFQKGEKFQILNSS-EGDWWEARSLTTGGTGYIPSNYVAPVDS

c-src   mGGVTTFVALYDYESRTETDLSFKKGERLQIVNNT-EGDWWLAHSLTTGQTGYIPSNYVAPSDS

                                        *

abl     meNDPNLFVALYDFVASGDNTLSITKGEKLRVLGYNHNGEWCEAQTK-NGQ-GWVPSNYITPVNS

spc     mDETGKELVLALYDYQEKSPREVTMKKGDILTLLNST-NKDWWKVEVN-DRQ-GFVPAAYVKKLD
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domains was used to express the individual domains in the *E. coli* BL21 strain (Musacchio *et al.*, 1992b). The expressed domains, except the spectrin SH3 domain, were precipitated from the supernatant of the broken cells between 40 and 75% saturation of ammonium sulfate, resuspended in 5 mM phosphate buffer, pH 7.0, and purified on a Superdex-75 column equilibrated and eluted with the same buffer. The spectrin-SH3 domain was purified as previously indicated (Musacchio *et al.*, 1992b).

The sequences of the expressed clones are indicated in Chart 1. The amino acids that were added for cloning purposes are in lowercase type. The neuronal form of c-src, n-src, has the same sequence as c-src with an insertion of hexapeptide RKVDVR in the position indicated by the asterisk (Martinez *et al.*, 1987).

Calculation of Solvent-Accesible Area. The solvent-exposed area of different aromatic amino acids in the SH3 domain of spectrin was calculated using the program of Shrake and Rupley (1973) as implemented by Miller *et al.* (1987).

Protein Concentration Determination. Protein concentration was determined by absorbance at 280 nm. The extinction coefficients for the protein denatured in 6 M HCl·Gnd were calculated by the method of Gill and von Hippel (1989). These values are 15 220 M^{-1} cm^{-1} for spectrin-SH3, 16 500 M^{-1} cm^{-1} for Fyn-SH3, 15 340 M^{-1} cm^{-1} for Abl-SH3, and 16 500 M^{-1} cm^{-1} for the c-src- and n-src-SH3 domains.

Peptide Synthesis. Peptides were provided by the EMBL peptide service. They were sysnthezised in solid phase in an MPS column. The molecular weights of the resulting products were confirmed by mass spectrometry. Peptide purity was assessed by analitycal HPLC. Peptide concentration was determined by amino acid analysis. A typical error of this procedure is about 10%, although in the case of proline-rich peptides the error could be larger.

Fluorescence Spectroscopy. The fluorescence emission spectra of the tryptophan residues of the SH3 domains were used to monitor changes in their environment upon peptide binding. Fluorescence was measured in an AMINCO Bowman Series 2 luminescence spectrometer. Excitation was at 298 nm with a 2-nm slit. Fluorescence was detected through a 4-nm slit. In these experiments, the protein concentration was kept at 10 μM. All experiments were performed at 25 °C.

Determination of the K_d by Fluorescence. Assuming a one-to-one complex between the SH3 domains and the peptides, it is possible to determine the K_d for different peptides by monitoring the changes in the fluorescence emission, using the following equation:

$$F = F_f + (F_b - F_f)[pep_f]/(K_d + [pep_f]) \quad (1)$$

where F_f is the fluorescence of the free domain (Dom_f), F_b is the fluorescence of the complex (Comp), $[pep_f]$ is the concentration of the free peptide in solution, and K_d is the dissociation constant in the equilibrium.

$$K_d = [Dom_f][pep_f]/[Comp] \quad (2)$$

The concentration of the free peptide can be calculated by substracting the concentration of the complex as estimated by the fluorescence change from the concentration of added peptide.

Circular Dichroism. CD spectra were recorded in a Jasco-710 instrument at 25 °C. Far-UV spectra were recorded in the range of 180–250 nm with a 0.01 cm path length cuvette. The near-UV spectra were recorded in the range 250–320 nm with a 1-cm path length cuvette. Protein and peptide concentrations were 200 μM in 5 mM phosphate buffer at pH 7.0.

Infrared Spectroscopy. Infrared spectra were acquired in a Nicolet 520 FTIR spectrometer equipped with an MCT detector. The samples, at ~5 mM concentration, were prepared in D_2O buffers and placed onto CaF_2 windows in a thermostated Harrick cell (Harrick, Ossining, NY) with 50-μm Teflon spacers. Five hundred spectra were taken in five cycles using a sample shuttle, and the buffer contribution was subtracted from the spectra of the samples. Resolution enhancement techniques and band decomposition were performed as previously described (Fernandez-Ballester *et al.*, 1994).

Cross-Linking Experiments. Cross-linking of the SH3 domains was done by incubating the different domains (100 μM) with or without the different proline-rich peptides with 1%, w/v, formaldehyde at room temperature for 20 min. The reaction was stopped by addition of 3× Laemmli buffer (Serrano & Avila, 1985; Filimonov *et al.*, 1993).

RESULTS

(A) Conformation of Peptides in Solution

Poly-L-proline is known to occur in two ordered structural forms: a right-handed helix of *cis*-imide groups with ten residues per three turns (polyproline I, or PPI) and a left-handed helix of *trans*-imide groups with three residues per one turn (polyproline II, or PPII). The PPII secondary structure has an axial translation of 3.12 Å per residue, (ω = 180°, ϕ = −78°, ψ = 149°). Polyproline adopts a PPII structure in aqueous solution and a PPI structure in some organic solvents (Rabanal *et al.*, 1993; Williamson, 1994).

(1) Circular Dichroic Spectra. In our experimental conditions, a polyproline with 10 residues (P10) shows a minimum at 204 nm (−83 000 mdeg cm^2 mol^{-1}) and a maximum at 228 nm (4,300 mdeg cm^2 mol^{-1}) (Figure 1A), which are typical of a PPII conformation (Manning & Woody, 1991; Rabanal *et al.*, 1993). The far-UV CD spectra of the seven peptides used in this study are shown in Figure 1. All peptides display a minimum centered at 201 nm, with the exception of 3BP-2 (205 nm) (Figure 1B), indicating that they are not completely unstructured in solution. However, the typical positive band

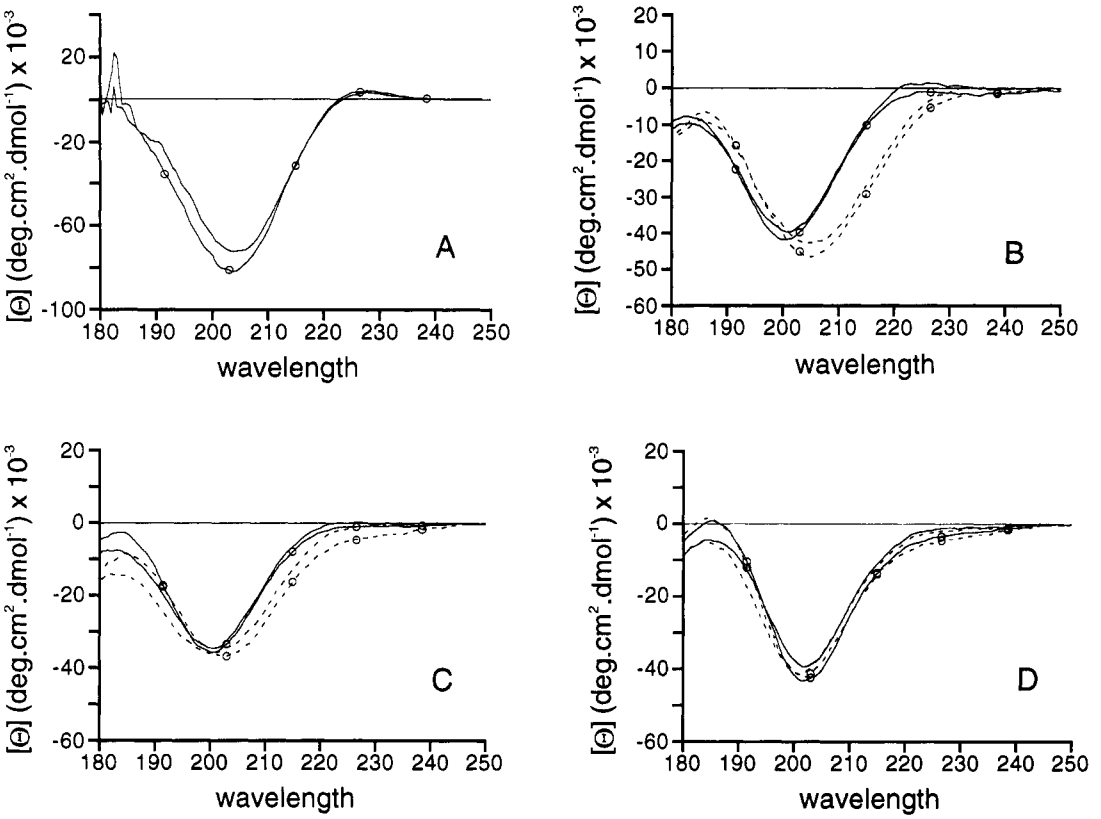

FIGURE 1: Far-UV CD analysis of the six different proline-rich peptides analyzed in this work and the homoproline peptide. All of the peptides were analyzed at 4 °C, in 5 mM phosphate buffer, pH 7.0. The peptide concentrations were 500 μM. (A) P10. (B) 3BP-1 (continuous lines) and 3BP-2 (broken lines). (C) p85α-1 (continuous lines) and p85α-2 (broken lines). (D) Sos1-3 (continuous lines) and Sos1-4 (broken lines). The spectra without circles represent the peptides in aqueous solution. The spectra with open circles represent the peptides in the presence of 30% TFE.

Table 1: Sequence of the Peptides Used in This Study

sequence	name	length	Pro[a]
1 2 3 4 5 6 7 8 9 10 11 12 13[b]			
P P P P P P P P P P	P10	10	10
R A P T M P P P L P P	3BP-1[c]	11	6
P P A Y P P P P V P	3BP-2[c]	10	7
N E R Q P A P A L P P K G	p85α-1[d]	13	4
K P R P P R P L P V A	p85α-2[d]	11	5
P P E S P P L L P P R	Sos1-3[e]	11	6
H S I A G P P V P P R	Sos1-4[e]	11	4
° ° °			

[a] Number of proline residues. [b] Sequence and numbering of peptides. [c] Sequences derived from regions of 3BP-1 and 3BP-2 that bind to the SH3 domain of Abl (Ren et al., 1993). [d] Sequences derived from a region of p85α (Liu et al., 1993). These peptide were shown to inhibit binding of p85α with the v-src SH3 domain. [e] Sequences derived from a region of mSos1 (Rozakis-Adcock et al., 1993; there named Pro3 and Pro4) shown to compete in the interaction of mSos1 with Grb2. ° Essential positions for the binding of 3BP-1 to Abl-SH3 (Ren *et al.*, 1993). Of the peptides used in this work only 3BP-1, 3BP-2, and sos1-3 fulfill this consensus requirement.

of PPII is only observed in 3BP-1 (Figure 1B) and p85α-1 (Figure 1C).

3BP-2 is the peptide with the largest proline content (Table 1), and consequently, it is expected to show the highest PPII conformation population. In principle, the absence of the typical positive PPII band at 228 nm could be due to the presence of a Tyr residue in the peptide (Vuilleumier et al., 1993). However, introduction of a Tyr residue at the equivalent position in 3BP-1 does not abolish its positive band at 228 nm (data not shown). This means that there is another secondary structure component in this peptide, resulting in a negative band at 228 nm. The presence of a negative band at 225 nm has been interpreted to be due to β-turn conformations (Woody, 1974).

Addition of trifluoroethanol (TFE) to the P10 peptide induces some small conformational changes as seen around the minimum at 204 nm, but it does not shift its conformation to PPI (Figure 1A). TFE seems to favor intramolecular hydrogen bond formation at the expense of solvent hydrogen bonding (Nelson & Kallenbach, 1986; Sönnichsen *et al.*, 1992). We have used TFE to reveal the intrinsic conformational variability of our peptides. If the peptide is in equilibrium between a PPII structure and another secondary structure containing main-chain hydrogen bonds, TFE should displace the equilibrium from PPII to the other conformation. Addition of 30% TFE to all peptides studied in this work only results in minor conformational changes (Figure 1), suggesting that they are conformationally restricted. Titration with TFE of 3BP-2 and p85α-2 indicates that although at 30% TFE the conformational changes are small, at higher concentrations the conformation of p85α-2 significantly changes, while 3BP-2 remains the same (data not shown). This result shows that although these peptides are conformationally restricted, in some peptides there is the possibility of adopting alternative conformations in which intramolecular hydrogen bonds are formed.

(2) FTIR Spectra. The main characteristic of the infrared spectrum of the P10 peptide (PPII) is a narrow band at 1620 cm^{-1} with a shoulder around 1643 cm^{-1} (Figure 2). Band assignment is not always unambiguous and can be complex, but in the light of previous work (Krimm & Bandekar, 1986; Byler & Susi, 1986; Arrondo et al., 1993), an approximation useful for comparing the spectra with the other data presented

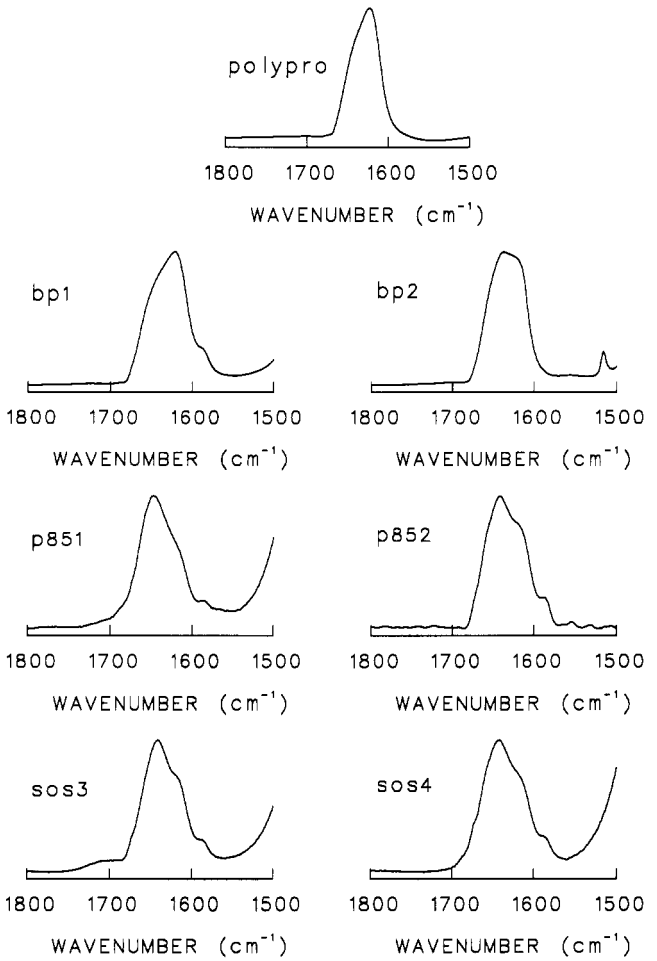

FIGURE 2: FTIR spectra of the six different proline-rich peptides analyzed in this work and the homoproline peptide. A band around 1670 cm^{-1} produced by trace amounts of trifluoroacetic acid (TFA) in the peptide preparation was mathematically subtracted in all cases.

Table 2: Infrared Analysis of the Conformation in Solution of the Different Peptides

band	1619/1643[a]	band	1619/1643[a]
PP10	100	p85α-2	21
3BP-1	44	Sos1-3	21
3BP-2	47	Sos1-4	20
p85α-1	17		

[a] We show the ratio between the intensities of the areas of the component band around 1619 cm^{-1} and the band at 1643 cm^{-1}. The values have been normalized, giving a value of 100 to PP10 (the real value is 0.90). It should be noted that other bands are also present.

can be made. The shoulder around 1643 cm^{-1} is typical of nonstructured conformations (Arrondo et al., 1993). Thus the band around 1620 cm^{-1} could be the most characteristic one of the PPII conformation. The FTIR spectra of the peptides used in this study indicate that 3BP-1 and 3BP-2 are similar to P10 (Figure 2). Band decomposition of these spectra (data not shown) shows that the ratios of the bands at 1620 and 1643 cm^{-1} in these two peptides are more similar to that found in the PP10 peptide than to those found in the other peptides (Table 2). In the other peptides the band at 1620 cm^{-1} is also present but to a lesser extent when compared to the band at 1643 cm^{-1}, suggesting that there is a smaller average population of PPII structure. This is in agreement with the TFE titration of the 3BP-2 and p85α-2 peptides. In all cases other bands are present, indicating the existence of alternative conformations (Figure 2).

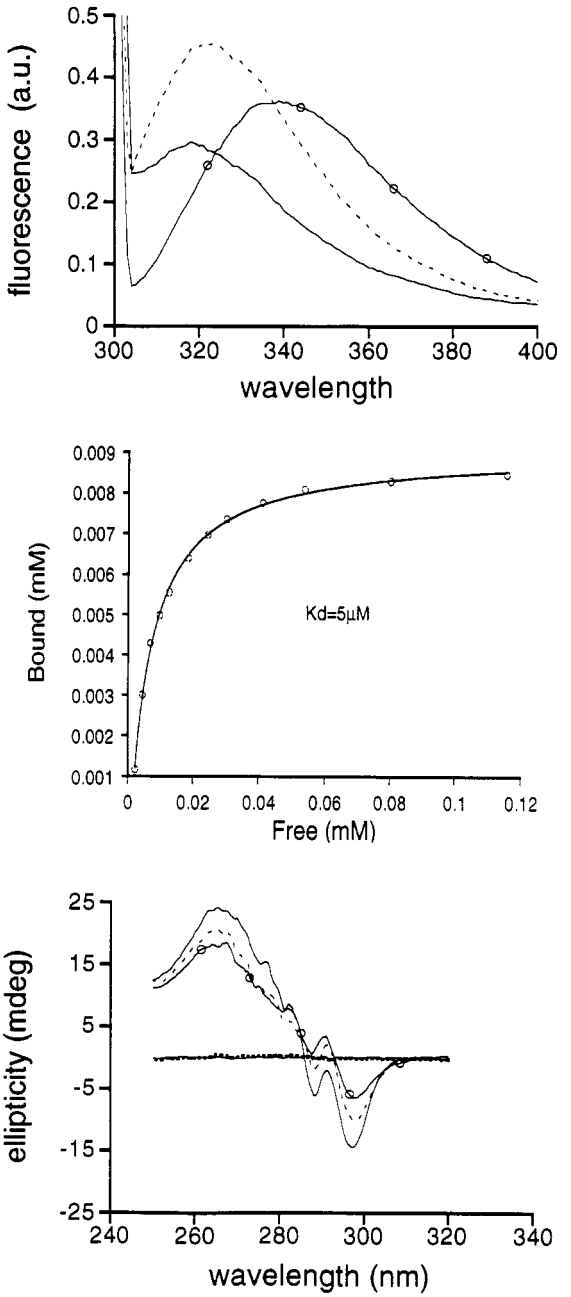

FIGURE 3: Fluorescence and near-UV CD spectra of the Abl-SH3 domain in the presence of saturating concentrations of 3BP-1 and 3BP-2. (top) Fluorescence spectra of 10 μM Abl-SH3 domain alone (-O-) or in the presence of 500 μM 3BP-1 (- - -) or 500 μM 3BP-2 (—). (Middle) Bound vs free 3BP-2 peptide measured by fluorescence changes as indicated in Experimental Procedures. (bottom) Near-UV CD spectrum of 100 μM Abl-SH3 domain alone (-O-) or in the presence of 100 μM 3BP-1 (- - -) or 100 μM 3BP-2 (—). All of the spectra were recorded in 5 mM phosphate buffer, pH 7.0, at 25 °C.

(B) Determination of K_d Values

It has been proposed that the aromatic-rich cleft of the SH3 domains could be involved in the interaction with target proteins (Musacchio *et al.*, 1992b). This surface has a conserved tryptophan residue, and we would expect large changes in tryptophan fluorescence upon binding of the target proteins or peptides on it. In Figure 3A, we show the changes in the fluorescence spectrum of the Abl domain after addition of saturating concentrations of 3BP-1 or 3BP-2. The maximum of the fluorescence emission shifts to a shorter wavelength, indicating that tryptophan residues become more deeply buried. Fitting of the fluorescence data, determined

Table 3: Summary of the Dissociation Constants (K_d) Obtained for the Complex between Proline-Rich Peptides and SH3 Domains[a]

peptide	spectrin (μM)	Fyn (μM)	Abl (μM)	c-src (μM)	n-src
3BP-1	2000	34	34	200	
3BP-2		34	5	300	
p85α-1		83		1000	
p85α-2		10		700	
Sos1-3		48		27	
Sos1-4		20		26	

[a] The values were determined by measuring the changes in fluorescence emission of the respective SH3 domain upon adding increasing concentrations of the peptide (see Experimental Procedures). No value is given for cases in which the K_d was higher than 2 mM.

Table 4: Corrected Fluorescence Spectra Maxima of the Free and Bound Domains (nm)[a]

peptide	spectrin	fyn	abl	c-src	n-src
no peptide	336	337	339	337	336
3BP-1		330	325	332	
3BP-2		330	321	330	
P85α-1		332		334	
P85α-2		331		329	
Sos1-3		330		332	
Sos1-4		332		332	

[a] The error in the determination of the maximum is ±1 nm. No value is given for those cases in which complete saturation of the corresponding SH3 domain with the peptide was not reached.

for all possible combinations of six peptides and five SH3 domains, to eq 1 (see Experimental Procedures) results in the K_d values shown in Table 3. As an example we show the fitting to the interaction between 3BP-2 and Abl-SH3 (Figure 3, middle).

These values are obtained by assuming that peptides bind in a one-to-one molar ratio to monomeric SH3 domains. Errors in the determination of the peptide concentration do not allow accurate measurements of the molar ratios. However, an NMR structure of the complex between a proline-rich peptide and the PI3K SH3-domain indicates that the assumption of a one-to-one molar ratio is correct (Yu *et al.*, 1994). Conversely, some domains (Abl, Fyn, and c-src) behaved as dimers in size-exclusion chromatography during purification in low ionic strength at high protein concentration (>10 mg/mL). However in the concentration range used in the experiments (10–20 μM), all SH3 domains behave as monomers as determined by changes in the fluorescence emission and cross-linking experiments at different concentrations (10–200 μM), data not shown.

(C) Conformational Changes in the SH3/Peptide Complexes

(1) Fluorescence Spectra. Table 4 shows the fluorescence emission maxima of the free and peptide-bound domains. The maxima for all free domains are characteristic of partly exposed tryptophan residues (Burstein *et al.*, 1973). This is in agreement with the calculated solvent-accessible areas of Trp41 and Trp42 in the spectrin-SH3 domain (80 and 38 Å^2). In the Abl domain, there is another Trp residue which replaces Phe52 of spectrin-SH3, and a Cys residue is in place of spectrin-SH3 Trp42 (see the alignment above).

Upon binding of proline-rich peptides to SH3 domains, there is an increase of the fluorescence emission as well as a blue shift of the maxima to 329–334 nm. The exception is Abl-SH3 in which the maximum is shifted to 325 nm by 3BP-1 and to 321 nm by 3BP-2 (in this latter case there is a decrease

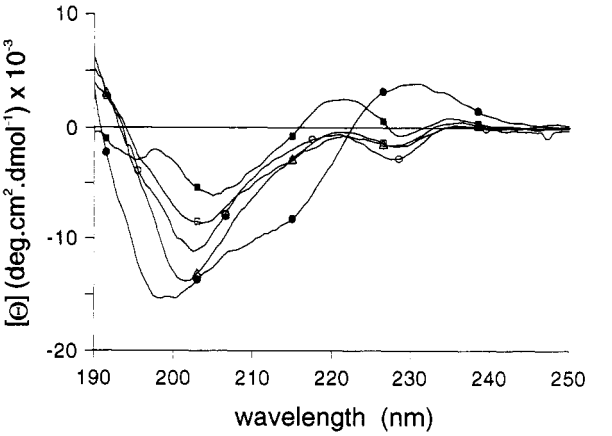

FIGURE 4: Far-UV CD spectra of the five different SH3 domains used in this study. All spectra were recorded at 25 °C, with a protein concentration of 200 μM, in 5 mM phosphate buffer, pH 7.0: (●) Abl-SH3, (■) Fyn-SH3, (□) c-src-SH3, (△) n-src-SH3, and (○) spectrin-SH3.

of the flurorescence emission) (Figure 3, top). These changes indicate that the interaction between proline-rich peptides and the SH3 domains involves the burial of the solvent accessible area of one or two tryptophan residues present in the domains. In the case of Abl-SH3, the blue shift to 321 nm suggests that both tryptophans are almost completely buried in the complex with 3BP-2.

(2) Circular Dichroic Spectra. The far-UV CD spectra of five SH3 domains in the absence of peptides are shown in Figure 4. Although the three structurally characterized domains (spectrin, Fyn, and Src; Musacchio *et al.*, 1992b; Noble *et al.*, 1993; Yu *et al.*, 1992) are very similar in overall fold, their CD spectra are quite different. In particular, the CD spectrum of the Abl-SH3 domain is rather distinct from the others. This could be due to the unequal Trp residue sequence positions as discussed above.

The addition of saturating concentrations of 3BP-1 or 3BP-2 peptide to the Abl-SH3 domain results in a change of the near-UV (Figure 3, bottom) and the far-UV CD spectra (Figure 5A,B). 3BP-2 produces stronger effects than 3BP-1. The increase of the negative ellipticity at 285–300 nm indicates that the peptide changes the enviroment of at least one tryptophan, and the increase of the positive ellipticity at 260–280 nm suggests changes also in the enviroment of Tyr and/or Phe residues. In the far-UV spectrum, the difference band at 230 nm shows that there is a change in the environment of aromatic residues in Abl-SH3 (Woody, 1978; Vuilleumier *et al.*, 1993). On the other hand, the difference band at 205 nm is reminiscent of the PPII CD spectrum. Yu *et al.* (1994) have observed that the perturbation of chemical shifts in the NMR spectrum of the PI3K-SH3 domain by ligand binding is highly localized within the binding site, suggesting that the conformations of the free and ligand-bound domains are essentially identical. If this is also true for the Abl-SH3 complex, the difference band at 205 nm could mean that binding increases the PPII conformation of 3BP-1 and 3BP-2.

The far-UV CD spectra of the complexes of the Sos1-3 and Sos1-4 peptides with c-src-SH3 indicate that the overall conformational changes induced upon binding are quite different from those detected in the complexes of Abl-SH3 with 3BP-1 and 3BP-2 (Figure 5C,D). In this case, the difference spectrum has a minimum close to 196 nm, while the 230-nm region is not significantly affected. Most ordered secondary structure elements have a positive spectral signal

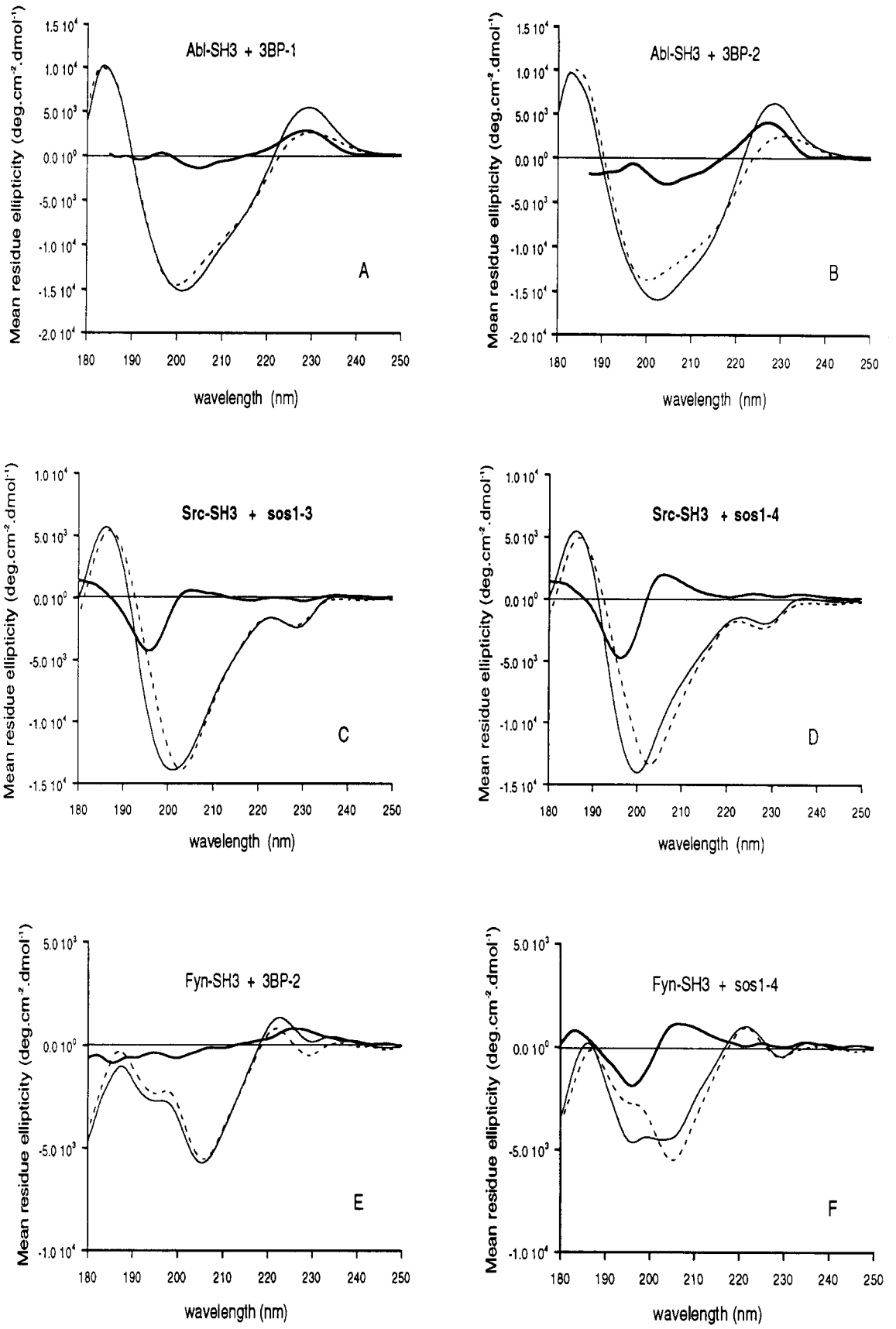

FIGURE 5: Far-UV CD analysis of the complexes between the proline-rich peptides and the SH3 domains. (A) Abl-SH3 domain with 3BP-1. (B) Abl-SH3 domain with 3BP-2. (C) c-src-SH3 domain with Sos1-3. (D) c-src-SH3 domain with Sos1-4. (E) Fyn-SH3 domain with 3BP-2. (F) Fyn-SH3 domain with Sos1-4. The units on the *y*-axis are for the protein without ligand. The thick line in each panel represents the difference between the spectrum of the complex (thin continuous line) and the sum of the spectra of the free peptide and the SH3 domain (broken line). All the experiments were performed at 25 °C, in 5 mM phosphate buffer, pH 7.0.

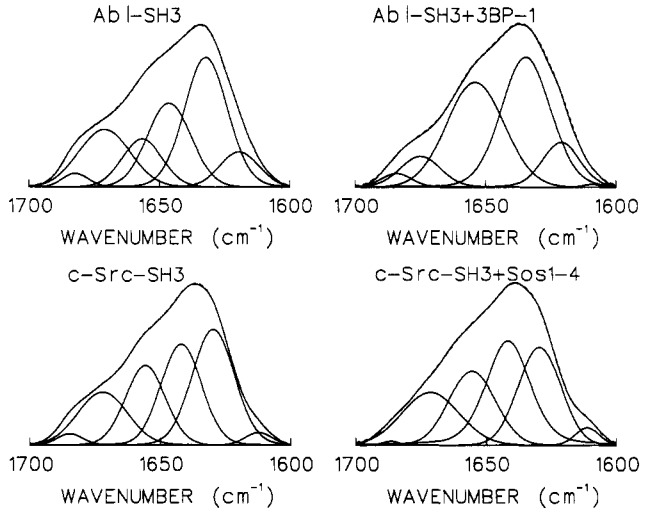

FIGURE 6: Band decomposition of the amide I region corresponding to Abl-SH3 alone (top left) *or* with the 3BP-1 peptide (top right) and of c-src-SH3 alone (bottom left) or with the Sos1-4 peptide (bottom right). The original envelope and the fit are plotted. The number and position of the components are obtained through deconvolution and derivation of the spectra. The experimental conditions are indicated in Experimental Procedures.

in the 196 nm region, whereas the random coil has a clearly negative signal at this wavelength (Manning & Woody, 1991; Jonhson, 1988; Perczel *et al.*, 1991, 1992). Therefore thedifference spectrum of these two complexes is compatible with an increase in unordered structure.

The Fyn-SH3 domain can help us to understand the CD effects observed in Abl- or src-SH3, since the K_d values for binding of 3BP-1, 3BP-2, Sos1-3, and Sos1-4 to it are all smaller than 50 μM. 3BP-1 and 3BP-2 provoke small changes in the aromatic region, increasing the observed positive maximum, but there is no difference signal around 205 nm (Figure 5E) such as that found in the spectrum of the Abl-SH3 complex. However, the formation of complexes with Sos1-3 and Sos1-4 results in the same difference spectra observed with the c-src-SH3 domain (Figure 5F). This means that the far-UV difference spectra of the 3BP-1 and 3BP-2 complexes or the Sos1–3 and Sos1–4 complexes could be due to the two sets of peptides adopting different conformations, or to a conformational change in the Fyn-SH3 domain that is differently induced by 3BP-1 and 3BP-2 and by Sos1-3 and Sos1-4.

(3) Infrared Spectra. On the basis of the CD data discussed above we performed an infrared analysis of two of the SH3–peptide complexes which exhibited the most distinctive behaviors, Abl-SH3 with 3BP-1 and c-src-SH3 with Sos1-4. In Figure 6 (top) we show the infrared spectra of the Abl-SH3 domain and of the complex between 3BP-1 and Abl-SH3, as well as the band decomposition of the spectra. The infrared spectra of c-src-SH3 and of its complex with Sos1-4 peptide are also shown in Figure 6 (bottom). The fact that the infrared technique is only relatively quantitative makes difficult the simple arithmetic manipulation of the spectra in order to analyze the difference between the complex and the isolated components, as in the case of CD. To study the spectra of the SH3 domains in the presence of peptide, it must be considered that the bands corresponding to the protein or the peptide can overlap. Therefore to compare the spectra of the SH3 domains with or without the peptide it is necessary to have a reference. In our case we have used the band around 1635 cm^{-1} as a reference. This band is related to the amount of intramolecular β-structure (Krimm & Bandekar, 1986; Arrondo *et al.*, 1993), which is the main secondary structure component of the SH3 domains, and consequently it is the one that should be less affected by binding of the peptide. The results of this analysis are shown in Table 5. In the case of the 3BP-1 complex with Abl-SH3, there is no band around 1640 cm^{-1} (the derivative spectrum does not show any band at this wavenumber), while the band at 1620 cm^{-1} slightly increases upon formation of the complex. The analysis of the complex with 3BP-2 shows similar results (data not shown). Band decomposition of the c-src-SH3 complex with Sos1-4 shows a different behavior in the case of the band around 1643 cm^{-1} (Figure 6, bottom;), which shows a large relative increase (Table 5).

Table 5: Relative Areas of the Bands around 1620 and 1645 cm^{-1} with Respect to the One around 1635 cm^{-1} for the Abl and c-src SH3 Domains and Their Complexes with 3BP-1 and Sos1-4, Respectively

system	1645/1635	1620/1635
Abl	0.66	0.24
Abl-3BP-1	0.00	0.36
c-src	1.18	0.05
c-src-Sos1-4	1.71	0.12

These results also indicate that the conformational changes that occur in the peptide and/or the domain upon binding are not the same in all the examples studied.

DISCUSSION

Free polyproline sequences extracted from proteins are likely to mimic their conformation in the entire protein. They can be expected to fold independently within a protein molecule, since they generally form extended structures as parts of loops and linker regions (Adzhubei & Sternberg, 1993). The study of a peptide–protein model as a simplified version of the interaction between two proteins seems more pertinent in this case, although the relevance of these peptide studies to the binding of the actual protein ligands is still an open question and needs further studies.

(A) Conformational Analysis. Structural analysis indicates that the structures of the free and bound SH3 domains are very similar and that peptide binding does not induce large conformational rearrangements. In the complex between the PI3K-SH3 domain and a proline-rich peptide, it was found that the bound peptide adopted a PPII conformation. On the basis of these results, it was postulated that this could be the general way in which other proline-rich peptides interact with the SH3 domains (Yu *et al.*, 1994). The CD and infrared analysis of the six peptides studied here indicates that all of them could adopt to different extents this conformation, with 3BP-1 and 3BP-2 having the larger content of it. The above hypothesis is compatible with our results on the binding of 3BP-1 and 3BP-2 to Abl-SH3, since the difference CD and FTIR spectrum of the complexes suggests an increase in the PPII conformation of the bound peptide.

The changes in fluorescence emission of Abl-SH3 in the presence of 3BP-2 indicate that both tryptophan residues in this protein are buried. Moreover, the near-UV CD spectrum suggests that also at least one Tyr or Phe residue is involved in the interaction. This clearly defines the region of Abl-SH3 involved in the binding of 3BP-2 as the aromatic surface. This comprises Y10, F12, W39, W50, and Y55 in the spectrin SH3. This region is also responsible for the interaction of src- and PI3K-SH3 domains with proline-rich peptides (Yu *et al.*, 1992, 1994).

CD difference spectra indicate that binding of Sos1-3 and Sos1-4 to Fyn-SH3 or c-src-SH3 induces similar large

conformational changes. These changes can be interpreted as an increase of aperiodic structure. Different changes are found when 3BP-1 and 3BP-2 interact with Fyn-SH3. It is unlikely that the binding of the Sos1-3 and Sos1-4 peptides to Fyn-SH3 would change the conformation of the domain in a way different from the binding of 3BP-1 and 3BP-2. Then these results suggest that these two families of peptides, 3BP-1 and -2 and Sos1-3 and -4, might have different conformations when bound to the Fyn- or Src-SH3 domains.

The infrared studies show that the changes induced by formation of the complexes with respect to the individual components are also different in the two representative examples analyzed here (Abl-SH3 with 3BP-1 and c-src-SH3 with Sos1-4). In the first case the results are compatible with a relative decrease in disordered structure (the band at 1643 cm^{-1} dissapears) and an increase in ordered structure which might be due to the formation of PPII structure (a slight relative increase in the band around 1620 cm^{-1}). A different behavior is found in the complex between c-src-SH3 and the Sos1-4 peptide. Both results are in principle compatible with the CD analysis of the same complexes. A straightforward interpretation of these results in structural terms is difficult since we can never rule out confomational changes in the domain. However, the fact that the induced changes are very different in both cases indicates that the conformation of the bound peptides could not be the same.

The CD and FTIR analysis of the complexes raises the possibility that the proline-rich peptides do not necessarily adopt a PPII secondary structure over their whole length when bound to the different SH3 domains.

(B) Affinity of Interaction. Affinities of the proline-rich peptides for different SH3 domains are relatively low, in the micromolar range (Table 3; Yu *et al.*, 1994). They do not compare with those found for the binding of phosphotyrosine peptides to SH2-domains (e.g., 0.3–3 nM; Felder *et al.*, 1993). Whether these values are physiologically significant is unclear. It is possible that the interactions between the SH3 domains and proline-rich targets take place in a zone of the cell where their local concentrations are high, for example, close to the membrane or in the cytoskeleton. It is also possible that the free peptides do not exactly represent the binding regions of target proteins because other residues of the protein or even other cellular components could be involved in the complex.

(C) Low Specificity. The n-src- and spectrin-SH3 domains do not bind well to any of the peptides we have used (Table 3). Spectrin is a cytoskeletal protein, and it is conceivable that its physiological partners may be different from those of the tyrosine kinase SH3 domains. The hydrophilic six amino acid insertion in the n-src domain dramatically alters its binding specificity (Ren *et al.*, 1993). The location of this insertion is adjacent to the proposed ligand-binding site, also revealing the importance of the interaction of nonaromatic side chains with the peptide.

Abl-SH3 has good affinity only for the 3BP-1 and 3BP-2 peptides. It is able to discriminate between 3BP-1 and 3BP-2, being the most selective among the domains tested here. Sos1-3 also contains the critical prolines at positions 2, 7, and 10, but its binding constant is in the millimolar range. Preliminary results (Pisabarro et al., 1994) indicate that a modified 3BP-1 with a mutation to tyrosine in the fourth position (Tyr is the residue in 3BP-2) is able to bind with the same affinity as 3BP-2. Placing a His residue in this position reduces the affinity to 90 μM. Moreover, Sos1-3 has a glutamate at this position. This suggests that residues in the fourth position may be critical for peptide binding to the Abl-SH3.

The c-src-SH3 domain binds all six peptides with affinities ranging from 25 μM to 1 mM. Two peptides with a higher affinity for this domain are Sos1-3 and Sos1-4 which have been described to inhibit the interaction between Sos and GRB2 (Rozakis-Adcock *et al.*, 1993). p85α-1 and p85α-2 inhibit the interaction between v-src and p85 PI3K (Liu *et al.*, 1993) but have a low affinity for the c-src-SH3 domain (about 1 mM). Peptide consensus sequences for c-src- and PI3K-SH3 are RXLPPLP (class I) and PPLPR (class II) (Yu *et al.*, 1994). Sos1-3 and Sos1-4 are closer to the class II peptides. These two peptides have a different consensus sequence (P$\Psi\Psi$PPR); however, their affinities (Table 3) are 3 times lower than those of the class I peptides and three times higher than those of the class II peptides. Therefore, we could define these peptides as a new class (class III). The only common feature of the three classes is the presence of an arginine residue and some prolines but not their relative positions. These results indicate that the requirement of proline residues at fixed positions may not be absolute for this SH3-domain, and other side chains may play the same role as proline residues.

Fyn-SH3 is the least selective domain. It binds to all peptides with K_d values lower than 100 μM. The lowest K_d is observed with p85α-2, which has the highest positive net charge (3+). Sos1-4 also shows good affinity and has a net charge 2+. This agrees with the observation that the vicinity of the binding region has a high negative charge density (5–) (Borchert *et al.*, 1994). p85α-1 and p85α-2 have been used in competition experiments to inhibit the interaction between v-src and PI3K. The measured IC_{50} values were 250 and 100 μM, respectively (Liu *et al.*, 1993), whereas we find the K_d values 1 and 0.7 mM, respectively. However, it is difficult to compare these values since IC_{50} depends on both K_d and the relative concentration of both ligands. Longer versions of the same peptides compete with the interaction between PI3K p85 subunit and the Fyn- and Lyn-SH3 domains (Pleiman *et al.*, 1994). A peptide related to p85α-2 is able to inhibit this interaction better than a version of p85α-1, which is in accordance with the K_d values of 10 and 83 μM, respectively (Table 3).

SUMMARY

It is not easy to obtain general conclusions about the interaction between proline-rich peptides and SH3 domains. One SH3 domain can interact with several different proline-rich regions, and one ligand can interact with several SH3 domains. For example, the guanine nucleotide realeasing factor Sos may be able to interact with either the Fyn or the Src domain via its proline-rich regions with almost equal affinity. The same can be true for 3BP-1 interacting with the Fyn or Abl domain. Moreover, the low affinity displayed by the proline-rich peptides toward SH3 domains raises the possibility that some of the interactions described here and in other studies could result from nonspecific interactions which are due to the presence of a high number of proline residues.

ACKNOWLEDGMENT

We are grateful to Dr. Sara Courtneidge for the generous gift of c-DNA encoding c-src.

REFERENCES

Adzhubei, A. A., & Sternberg, M. J. E. (1993) *J. Mol. Biol. 229*, 472–493.

Arrondo, J. L. R., Muga, A., Castresana, J., & Goñi, F. M. (1993) *Prog. Biophys. Mol. Biol. 53*, 23–56.

Brochert, T. V., Mathieu, M., Zeelen, J. Ph., Courtneidge, S. A., & Wierenga, R. K. (1994) *FEBS Lett. 341*, 79–85.
Burstein, E. A., Vedenkina, N. S., & Ivkowa, M. N. (1973) *Photochem. Photobiol. 18*, 263–279.
Byler, D. M., & Susi, H. (1986) *Biopolymers 25*, 469–487.
Chen, J. K., Lane, W. S., Brauer, A. W., Tanaka, A., & Schreiber, S. L. (1993) *J. Am. Chem. Soc. 115*, 12591–12592.
Cicchetti, P., Mayer, B. J., Thiel, G., & Baltimore, D. (1992) *Science 257*, 803–806.
Felder, S., Zhou, M., Hu, P., Ureña, J., Ullrich, A., Chaudhuri, M., White, M., Shoelson, E., & Schlessinger, J. (1993) *Mol. Cell. Biol. 13*, 1449–1455.
Fernandez-Ballester, G., Castresana, J., Fernandez, A. M., Arrondo, J. L. R., Ferragut, J. A., & Gonzalez-Ros, J. M. (1994) *Biochemistry 33*, 4065–4071.
Filimonov, V. V., Prieto, J., Martinez, J. C., Bruix, M. & Serrano, L. (1993) *Biochemistry 32*, 12906–12921.
Fry, M. J., Panayotou, G., Booker, G. W., & Waterfield, M. D. (1993) *Protein Sci. 2*, 1785–1797.
Gill, S. C., & von Hippel, P. H.(1989) *Anal. Biochem. 182*, 319.
Gout, I., Dhand, R., Hiles, I. D., Fry, M. J., Panayotou, I., Das, P., Troung, O., Totty, N. F., Hsuan, J., Booker, G. W., Campbell, I. D., & Waterfield, M. D. (1993) *Cell 75*, 25–36.
Johnson, W. C. J. (1988) *Annu. Rev. Biophys. Biophys. Chem. 17*, 145–166.
Krimm, S., & Bandekar, J. (1986) *Adv. Protein Chem. 38*, 181–364.
Liu, X., Marengere, L. E. M., Koch, C. A., & Pawson, T. (1993) *Mol. Cell. Biol. 13*, 5225–5232.
Manning, C., & Woody, R. W. (1991) *Biopolymers 31*, 569–586.
Martinez, R., Methey-Prevut, B., Bernards, A., & Baltimore, D. (1987) *Science 237*, 411–415.
Mayer, B. J., & Baltimore, D. (1993) *Trends Cell Biol. 3*, 8–13.
Mayer, B. J., Hamaguchi, M., & Hanafusa, H. (1988) *Nature 332*, 272–275.
Miller, S., Janin, J., Lesk, A. M., & Chothia, C. J. (1987) *J. Mol. Biol. 196*, 641–656.
Musacchio, A., Gibson, T., Lehto, V.-P., & Saraste, M. (1992a) *FEBS Lett. 307*, 55.
Musacchio, A., Noble, M. E. M., Pautit, R., Wierenga, R., & Saraste, M. (1992b) *Nature 359*, 851.
Nelson, J. W., & Kallenbach, N. R. (1986) *Proteins 1*, 211–217.
Noble, M. E. M., Musacchio, A., Saraste, M., Courtneidge, S. A., & Wierenga, R. K. (1993) *EMBO J. 12*, 2617.
Pawson, T. (1992) *Curr. Opin. Struct. Biol. 2*, 432–437.
Pawson, T., & Gish, G. D. (1992) *Cell 71*, 359–362.
Pawson, T., & Schlessinger, J. (1993) *Curr. Biol. 3*, 434–445.
Perczel, A., Hollósi, M., Tusnády, G., & Fasman, G. D. (1991) *Protein Eng. 4*, 669–679.
Perczel, A., Park, K., & Fasman, G. D. (1992) *Proteins 13*, 57–69.
Pisabarro, M., Ortiz, A., Viguera, A. R., Gago, F., & Serrano, L. (1994) *Protein Eng.* (in press).
Pleiman, C. M., Hertz, W. M., & Cambier, J. C. (1994) *Science 263*, 1609–1612.
Rabanal, F., Ludevid, M. D., Pons, M., & Giralt, E. (1993) *Biopolymers 33*, 1019–1028.
Ren, R., Mayer, B. J., Cicchetti, P., & Baltimore, D. (1993) *Science 259*, 1157–1161.
Rozakis-Adcock, Fernley, R., Wade, J., Pawson, T., & Bowtell, D. (1993) *Nature 363*, 83–85.
Serrano, L., & Avila, J. (1985) *Biochem. J. 230*, 551.
Shrake, A., & Rupley, J. A. (1973) *J. Mol. Biol. 79*, 351–371.
Sönnichsen, F. D., Van Eyk, J. E., Hodges, R. S., & Sykes, B. D. (1992) *Biochemistry 31*, 8790–8798.
Vuilleumier, S., Sancho, J., Loewenthal, R., & Fersht, A. R. (1993) *Biochemistry 32*, 10303–10313.
Williamson, M. P. (1994) *Biochem. J. 297*, 249–260.
Woody, R. W. (1974) in *Peptides, Polypeptides and Proteins* (Blout, E. R., Borey, F. A., Goodman, M., & Lotan, N., Eds.) pp 339–349, John Wiley & Sons, Inc., New York.
Woody, R. W. (1978) *Biopolimers 17*, 1471–1476.
Yu, H., Rosen, M. K., Shin, T. B., Siedel-Dugan, C., Brugge, J. S., & Schreiber, S. L. (1992) *Science 258*, 1665–1668.
Yu, H., Chen, J. K., Feng, S., Dalgarno, D. C., Brauer, A. W., & Scheiber, S. L. (1994) *Cell 76*, 933–945.