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Affinity Capillary Electrophoresis: Insights into the Binding of SH3 Domains by Peptides Derived from an SH3-Binding Protein

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Src Homology III (SH3) domains are small (55–70 amino acids) receptor domains that are found in many signaling proteins and are involved in intracellular signaling events.[1,2] For example, the SH3 domain in the adaptor protein GRB-2 facilitates the coupling of growth factor receptors to an essential switch in growth-related signaling pathways—the guanine nucleotide binding protein Ras.[3] Another example is the SH3 domain in the signaling protein PLC-γ; this domain is essential for its subcellular localization at actin microfilaments, where signaling results in PLC-γ-mediated cytoskeletal rearrangements.[4] Other SH3 domains such as those in the Abl and Src tyrosine kinases have been implicated in oncogenic pathways.[5] The molecular details of SH3-mediated signaling, however, remain elusive.

Recent evidence suggests that SH3 domains bind to proline-rich peptide motifs found in SH3-binding proteins (3BPs). By screening a λgt11 cDNA expression library with the SH3 domain of Abl fused to glutathione *S*-transferase (GST), Baltimore and co-workers were able to isolate an SH3-binding protein, 3BP1.[6] Subsequent filter binding assays using GST–3BP1 and several biotinylated GST–SH3 fusion proteins revealed that 3BP1 also binds specifically to the SH3 domain of Src. Colorimetric detection of the 3BP1–SH3 complexes using streptavidin alkaline phosphatase yielded similar intensities for the SH3 domains of Abl and Src, indicating that they have comparable affinities for 3BP1. Other GST–fusion protein probes containing the SH3 domains of neural Src and Crk produced much weaker signals. Similar experiments localized the binding site of 3BP1 for the Abl SH3 domain to a ten amino acid region, APTMPPPLPP.[7] The

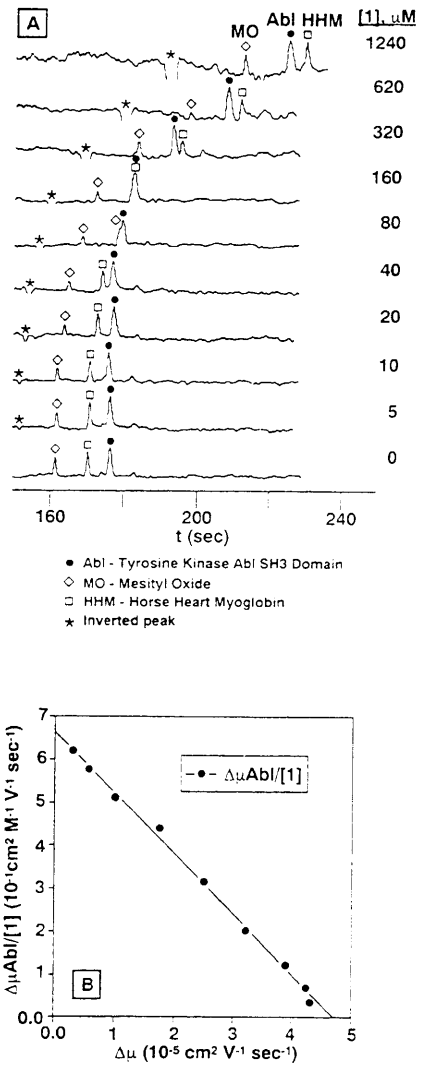

Figure 1. Affinity capillary electrophoresis (ACE) of the Abl SH3 domain in 192 mM glycine, 25 mM Tris (pH 8.3) containing various concentrations of 1. The total analysis time in each experiment was approximately 4.0 min at 30 kV using a 50 cm (inlet to detector), 50 μm diameter quartz capillary. Mesityl oxide (MO) and horse heart myoglobin (HHM) were used as internal standards to estimate the velocity of electroosmotic (EO) flow. (A) A representative set of electropherograms with increasing ligand concentration in the buffer. (B) Scatchard plot of the data, obtained after correcting for variable EO flow.[11] The inverted peaks in the electropherograms resulted from the dilution of the electrophoresis buffer at the point of injection.

qualitative nature of these binding assays, however, precludes quantitative assessments of SH3–ligand affinities. Little is known about the specificity or strength of these interactions.

We have used the SH3 domains of Abl and Src and a fragment of 3BP1 as a model system for investigating SH3–peptide interactions. To evaluate the specificity and avidity of these interactions, we measured the affinities of Abl and Src SH3 domains for peptide 1 using affinity capillary electrophoresis (ACE).[8] This peptide contains the proline-rich region of 3BP1 responsible for Abl SH3 binding and four additional residues from the 3BP1 sequence. A1G, P2A, P7A, and P10A mutations in 1 were

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Table 1. Dissociation Constants K_d (μM), Values for $\Delta\mu_{P,D}^{max}$ [a] (10^{-5} cm^2 V^{-1} s^{-1}), and $\delta\mu_{P,L}$ of Peptide Ligands for the SH3 Domains of the Protein Tyrosine Kinases Abl and Src[b]

peptide ligand[c]			Abl					Src			
			ACE			F[d]		ACE			
sequence	z	m	K_d	$\Delta\mu_{P,L}^{max}$	$\delta\mu_{P,L}$[e]	$\delta\mu_{P,L}$[f,g]	K_d	K_d	$\Delta\mu_{P,L}^{max}$	$\delta\mu_{P,D}$[e]	$\delta\mu_{P,L}$[f]
R-APTMPPPLPPVPPQ-NH_2 (**1**)	+1	1665	67	4.8	−0.60	−0.58	40	110[h]	5.6	−0.38	−0.35
R-*G*PTMPPPLPPVPPQ-NH_2 (**1-A1G**)	+1	1651	52	4.5	−0.58	−0.58	26	120	5.2	−0.37	−0.35
R-A*A*TMPPPLPPVPPQ-NH_2 (**1-P2A**)	+1	1639	>1000					140	5.5	−0.39	−0.35
R-APTMPP*A*LPPVPPQ-NH_2 (**1-P7A**)	+1	1639	410	4.4	−0.56	−0.58		160	5.3	−0.40	−0.35
R-APTMPPPLP*A*VPPQ-NH_2 (**1-P10A**)	+1	1639	190	4.7	−0.59	−0.58	110	240	5.3	−0.39	−0.35
R-APTMPPPLPP-NH_2 (**2**)	+1	1243	72	4.6	−0.59	−0.56	37	940	4.2	−0.31	−0.33

[a] $\Delta\mu_{P,L}^{max}$ maximum change in the electrophoretic mobility of the protein on binding the ligand. [b] The recombinant Src SH3 domain was actually two isoforms due to stop codon read-through. The results from one are shown. The values of K_d are similar for both isoforms. [c] R = $CF_3CO_2^-$ $^+H_3N(CH_2)_5C(O)$–; m is the mass; z is the net charge of the carboxyamide peptide calculated from the sequence. The amino acid that differs from **1** is italicized. [d] F = fluorescence assay. [e] This value of $\delta\mu_{P,L} = \Delta\mu_{P,L}^{max}/\mu_P$ was obtained from the Scatchard analysis: μ_P is the electrophoretic mobility of the protein in the absence of ligand. [f] This value was estimated from the sequence of the peptide using the expression $\delta\mu_{P,L} \cong (1 + (z/Z)(M/M + m))^{2/3} - 1$, where Z and M (−1.9 and 6632 for Abl; −3.9 and 7099 for Src) are the charge and mass of the protein, respectively. [g] A value of −0.53 was estimated using the expression $\delta\mu_{P,L} \simeq (C/L)(m/M)^{2/3}(\mu_L/\mu_P)$ where C and L are constants that relate the charge and mass of the protein and ligand to their respective mobilities and μ_P is the electrophoretic mobility of the protein. [h] A fluorescence assay gave a K_d of 89 μM.

also analyzed since these single amino acid changes were reported by Ren *et al.* to abolish binding to the Abl SH3 domain.[7] Figure 1A shows representative electropherograms for the interactions between **1** and the SH3 domain of Abl. Figure 1B is a Scatchard plot of the data.[9] Experimentally observed changes in the electrophoretic mobility ($\Delta\mu_{P,L}$) of the SH3 domains agree with semiquantitative estimations based on mass and change (Table 1). The dissociation constants (K_d) obtained by ACE are comparable to those measured by fluorescence spectroscopy.[10]

Consistent with the results of Ren *et al.*, our experiments with the Abl SH3 domain demonstrate that the P2A, P7A, and P10A mutations significantly diminish binding affinity (Figure 2). In contrast with these results, our findings indicate that the A1G mutation slightly *increases* the affinity of the peptide for the Abl SH3 domain. The reason for this discrepancy is unclear. Surprisingly, the Src SH3 domain exhibits a different binding profile for **1** and its mutants. The A1G, P2A, and P7A mutations in **1** do not significantly affect binding affinity. Only the P10A mutation reduces the peptide's affinity for Src SH3. The SH3 domains of Src and Abl also interact differently with a truncated ligand **2**. The Abl SH3 domain binds to **1** and **2** with similar affinities. The ability of the Src SH3 domain to bind **2** is compromised, demonstrating the importance of the VPPQ sequence for binding.

The divergent affinities of the Abl and Src SH3 domains for **1**, its mutated derivatives, and **2** indicate that the two SH3 domains bind the 3BP1 fragment differently. Several mutations in **1** that abolish Abl SH3 binding have little effect on Src SH3 bindng. Likewise, deleting the four C-terminal residues of **1** only reduces its affinity for the SH3 domain of Src. These results demonstrate that the

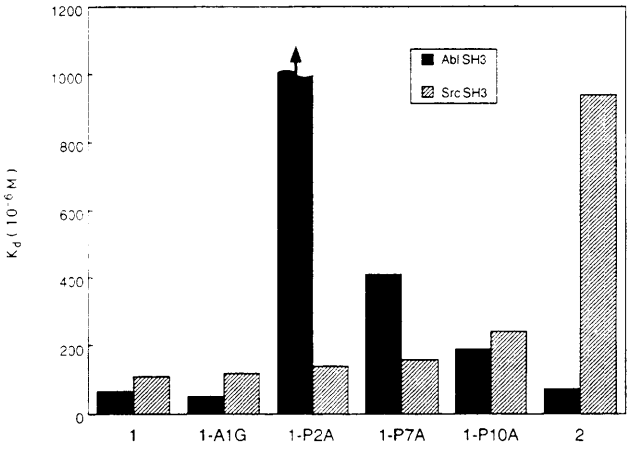

Figure 2. Comparison of the affinities of Src and Abl SH3 domains for peptides derived from 3BP1.

SH3 domains of Abl and Src bind to two distinct sites in the 3BP1 fragment.

In summary, this paper demonstrates that ACE is a useful method for the quantitative evaluation of peptide ligands for SH3 domains and suggests the value of this new analytical methodology for other problems involving the interactions of proteins with peptides. Based on the measured values of binding constants, we propose that the SH3 domains of Abl and Src bind to distinct sites of the 3BP1-derived peptide and therefore probably to distinct sites on the 3BP1 protein.

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Supplementary Material Available: General procedure and characterization data for **1** and **2** and capillary electrophoresis experimental procedures (3 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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