Journal of Biochemistry and Molecular Biology, Vol. 31, No. 4, July 1998, pp. 370–376

JOURNAL OF
Biochemistry
and
Molecular Biology

Establishment of a Binding Assay System for Screening of the Inhibitors of p56^{lck} SH2 Domain

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Received 28 March 1998
Revised 18 April 1998

Src-Homology 2 (SH2) domains have a capacity to bind phosphotyrosine-containing sequence context and play essential roles in various cellular signaling pathways. Due to the specific nature of the binding between SH2 domains and their counterpart proteins, inhibitors of SH2 domain binding have drawn extensive attention as a potential candidate for therapeutic agents. Here, we describe the binding assay system to screen for the ligands or blockers of the SH2 domains with an emphasis on the p56^{lck} SH2 domain. In our assay system, SH2 domains expressed and purified as fusion proteins to Glutathione-S-transferase (GST) were covalently attached to 96-well microtitre plates through amide bond formation, which were subsequently allowed to bind the biotinylated phosphotyrosine (pY)-containing synthetic peptides. The binding of biotinylated pY peptides was detected by the horseradish peroxidase (HRP)-conjugated streptavidin. Using the various combinations of SH2 domain-pY peptides, we observed that: (1) The binding of pY-peptides to its counterpart SH2 domain is concentration-dependent and saturable; (2) The binding is highly specific for a particular combination of SH2 domain-pY peptide pair; and (3) The binding of Lck SH2-cognate pY-peptides is specifically competed by the nonbiotinylated peptides with expected relative affinity. These results indicate that the established assay system detects the SH2-pY peptide interaction with reproducible sensitivity and specificity and is suitable for screening the specific inhibitors of p56^{lck} SH2 function.

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Keywords: Binding assay, Lck, Protein-to-protein interaction, Screening, SH2.

Introduction

Src homology 2 (SH2) domain is a protein module of approximately 100 amino acids and mediates protein-to-protein interaction through binding phosphotyrosine-containing proteins (Koch *et al.*, 1991). Many important intracellular signaling molecules such as phospholipase Cγ1 (PLC γ1), ras GTPase activating protein (GAP), Phosphatidyl Inositol 3-Kinase (PI3K), and Src-type tyrosine kinases contain one or two SH2 domains (Koch *et al.*, 1991; Cantley *et al.*, 1991; Pawson and Gish, 1992). These SH2-containing molecules play important roles in signal transduction pathways leading to the regulation of cell growth, differentiation, and development (Pawson, 1995).

Previous studies have shown that SH2 domains bind to phosphotyrosine (pY)-containing protein targets with a significant degree of specificity (Fantle *et al.*, 1992; Kashihian *et al.*, 1992; Rotin *et al.*, 1992). The specificity of these SH2- phosphotyrosine (pY) interactions is defined by the sequence and structural context of the pY (Kazlauskas *et al.*, 1990; Fantle *et al.*, 1992; Kazlauskas *et al.*, 1992;). More importantly, the SH2-pY interactions can be reconstituted by the use of short peptides containing phosphotyrosine (Escobedo *et al.*, 1991; Fantle *et al.*, 1992). Selections of preferable binding motifs from randomized phosphotyrosine-containing peptide libraries have shown that specific motifs are recognized by different SH2 domains (Songyang *et al.*, 1993). In particlar, the three residues located at the carboxyl-terminal side of phosphotyrosine are important in determining the specificity of binding (Payne *et al.*, 1994).

p56^{lck} (Lck) is a lymphocyte-specific member of a Src-type tyrosine kinase playing an essential role in T-cell

activation, proliferation, and development (Turner *et al.*, 1990; Glaichenhaus *et al.*, 1991; Molina *et al.*, 1992; Straus and Weiss, 1992). Like the other Src-type tyrosine kinases, Lck contains SH3, SH2, catalytic domains as well as a regulatory tyrosine phosphorylation site (pY505) at the carboxy-terminus. From the randomized pY-containing peptide mixtures, Lck SH2 selected pYEEI motif as the high affinity ligand, which was also found in the hamster polyomavirus middle-sized tumor antigen (hmT). On the other hand, phosphorylation of the regulatory site, pY^{505}QPQ, allows intramolecular interaction with its own SH2 domain, resulting in the negative regulation of kinase activity (Peri *et al.*, 1993). Studies using pY-containing synthetic peptides have shown that the pY^{505}QPQ motif binds to the Lck SH2 domain with relatively weak affinity (Payne *et al.*, 1993; Joung *et al.*, 1995).

The specificity of binding implies that the specific inhibitors of the SH2 domain function may be developed as drugs to treat the diseases caused by the abnormal regulation of signal transduction pathways. In particular, an inhibitor of Lck SH2 function may have therapeutic potential as an immunosuppressant insomuch as Lck SH2 function is important in T-cell activation (Veillette *et al.*, 1992). Previously, several assay methods were developed to study or screen inhibitors of SH2-phosphoprotein interaction (Wood *et al.*, 1992; Bibbins *et al.*, 1993; Piccione *et al.*, 1993; Zhu *et al.*, 1993; Gilmer *et al.*, 1994).

Here, we present a rapid and simple method for mass screening of the inhibitors of the Lck SH2 domain function. We employed a modified ELISA assay with recombinant SH2 domains covalently attached onto microtiter plates through an amide bond. Upon testing the assay system with various combinations of SH2 domains and pY-containing peptides, we observed that the established assay detects the binding of cognate pY-peptides to each SH2 domain with reproducible sensitivity and specificity.

Materials and Methods

Expression and purification of recombinant SH2 domains The SH2 domains were expressed as fusion proteins to glutathione-S-transferase (GST) employing the pGEX-2T bacterial expression vector (Pharmacia, Uppsala, Sweden). Fragments encoding amino acids 119–224 of human Lck (Perlmutter *et al.*, 1988), 322–441 of human PI3K (Coughlin *et al.*, 1989), and 176–275 of human GAP (Molloy *et al.*, 1989) were amplified by PCR. PCR primers contain *Eco*RI or *Bam*HI extensions introduced for the facilitation of subcloning. PCR products were digested with appropriate enzymes and subcloned into the corresponding sites of pGEX-2T. For the expression of PLC-γ SH2, a fragment encoding amino acids 517–676 was obtained by digestion of rat PLC-γ cDNA (Kumjian *et al.*, 1989) with *BglII*/*Sac*I and subcloned into the corresponding sites of pGEX-2T.

Fusion proteins were induced by the addition of 1 mM IPTG (isopropyl-β-D-Thiogalactoside) followed by incubation at 37°C for an additional 4 h. The lysates were clarified by centrifugation, and the supernatant was incubated with glutathione-sepharose beads at 4°C for 1 h. The beads were washed extensively and the fusion protein was eluted with 10 mM glutathione. The eluted fusion protein was dialyzed and concentrated with Centriprep-10 (Amicon, Beverly, USA). Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, USA). Purified GST-SH2 was stored at 4°C for immediate use or at −70°C in 10 mM DTT.

Peptide synthesis Synthesis of tyrosine-phosphorylated peptides was performed using a ABI 431A synthesizer with peptide amide resin (PAL resin, Milligen/Biosearch Division, Burlington, USA) following the established method (Furka *et al.*, 1991). The N-α-Fmoc protecting group was used throughout the synthesis in conjunction with standard side-chain protecting groups. N-α-Fmoc-O-(O,O-dimethoxyphosphoryl)-L-tyrosine(Fmoc-Tyr (OP(OCH3)2)) (Sigma, St. Louis, USA) was used for the incorporation of phosphotyrosine. After synthesis and cleavage from the resin, side-chain protecting groups were removed by treatment with a mixture of trifluoroacetic acid, thioanisole, ethanedithiol, and ainsole (90:5:3:2) for 2 h at room temperature. Methyl protecting groups on phosphotyrosine were removed during a second stage of deprotection with trimethylsilyl bromide. Peptides were precipitated with diethyl ether and purified further using a Waters' Prep HPLC equipped with a C18 column. The identity of peptides was confirmed by standard biochemical analysis, including fast atom bombardment mass spectrometry and amino acids composition analysis. The phosphate content of peptides was also verified by the inorganic phosphate assay (Vaskovsky *et al.*, 1975). When necessary, peptides were coupled with biotin at their N-terminus. For biotinylation, biotin (Sigma, St Louis, USA) was activated overnight at room temperature with DCC (dicyclohexyl carbodiimide) and HOBt (1-hydroxybenzo triazole). The biotinylated peptides contain linker amino aid residues, SGSG, to prevent the possible inhibition of binding by steric hindrance from biotin–streptavidin interaction.

Establishment of the binding assay Maleic-anhydride activated plate (Pierce, Rockford, USA) was employed for the binding assay. SH2 domains were diluted to 1 μM in PBS, and a 100-μl aliquot was placed in each well of 96-well maleic anhydride-activated plates in duplicate. After 2 h incubation, the plates were washed three times with PBST (0.05% Tween-20 in PBS). The wells were then filled with 200 μl of blocking buffer (3% BSA in PBS) and incubated for 2 h at 37°C. After removal of blocking buffer, various concentrations of biotinylated phosphotyrosyl peptide in PBST/0.5% BSA was added, incubated for 1 h at room temperature, and then rinsed three times with PBST. The binding of biotinylated phosphopeptides was detected by horseradish peroxidase-conjugated streptavidin (Pierce, Rockford, USA) diluted 1:20,000 (0.2 μg/ml) in PBST containing 0.5% BSA. Subsequently, the plates were washed four times with PBS/0.05% Tween-20 and incubated for 30 min at room temperature in the presence of 100 μl of 2 mg/ml TMB (tetramethyl-benzidine) in HRP buffer. The reaction was stopped by the addition of 0.8 N H_2SO_4 and the plates were read on a plate reader at 450 nm with reference at 620 nm.

Competitive binding assay Equal volumes of 1 μM B-pY324 and serial dilutions of nonbiotinylated competing peptides with final concentrations ranging from 10^{-3} μM to 103 μM were mixed and 100 μl of the mixture was added in duplicate to each well of a 96-well plate bound with the Lck SH2 domain. The binding was detected as described above.

Results and Discussion

Establishment of the binding assay The principle of the binding assay is shown in Fig. 1. The method is similar to a modified ELISA system widely applied in biochemical assays (Jang *et al.*, 1996; Kim, 1997). The assay measures the binding of biotin-labeled, phosphotyrosine-containing peptides to the GST-SH2 proteins covalently bound to the maleic anhydride-activated plates. The maleic anhydride group on the plates reacts with amine groups on the SH2 fusion proteins resulting in the formation of an amide bond. Compared to the simple adsorption method, the covalent binding is expected to be resistant to repeated washing and to prevent the conformational change of SH2 domain, as well as the steric hindrance, allowing improved specificity and binding.

Previously, a high-affinity ligand for the Lck SH2 domain was identified as -pYEEI- by affinity selection from a degenerate phosphopeptide mixture (Songyang *et al.*, 1993). Based on this information, in this study, we employed an hmT pY324 peptide, EEPQpYEEIPI, as a high affinity ligand for the Lck SH2 domain. The sequences and sources of the other peptides used in this study are listed in Fig. 2A. Recombinant GST-SH2 fusion proteins were expressed in *E. coli* and purified to near homogeneity using the glutathione agarose beads (Fig. 2B).

To test the feasibility of the binding assay, recombinant GST or GST Lck SH2 was immobilized onto microtiter plates and, subsequently, increasing concentrations of biotinylated pY324 peptide (biotin-SGSGEEPQpYEEIPI) were added. The binding was detected by the measurement of the remaining peroxidase activity conjugated to streptavidin. As shown in Fig. 3, the binding of biotinylated pY324 was concentration-dependent with half-maximal binding occurring at 150–200 nM and was saturated within the peptide concentrations used in this study. The experimental data define the lower limit of sensitivity of the assay as approximately 10^{-8} M. No binding was detected with GST. These results indicate that the binding assay system is suitable for the detection of SH2-pY peptide binding.

Binding specificity As a next step, we tested the binding specificity of the Lck SH2 domain with its cognate phosphotyrosine-containing peptide sequence (Fig. 4). GST-Lck SH2 domain was bound to plates and three different biotinylated phosphopeptides were tested. Biotin-pY324 is derived from hmT and known as the high affinity

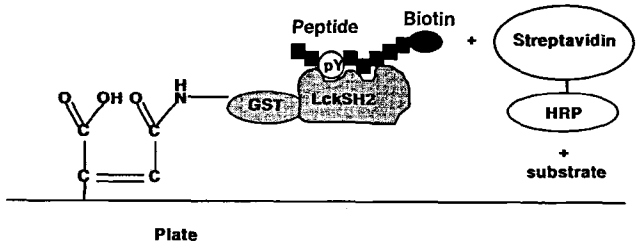


Fig. 1. Schematic representation of the assay principle. Maleic anhydride group attached onto microtitre plates interacts with amine group on the GST–SH2 fusion proteins resulting in the formation of an amide bond. The biotin-labeled, phosphotyrosine (pY)-containing peptide is allowed to bind the SH2 domain attached covalently to plates and the binding is detected by horseradish peroxidase (HRP)-conjugated streptavidin. pY; phosphotyrosine.

A

Name	Sequence	Source
pY324	EEPQpYEEIPI	hmT (Y324)
pY505	TEGQpYQPQPA	Lck (Y505)
pY740	SDGGpYMDMSK	PDGFR-β (Y740)
B-pY324	biotin-SGSGEEPQpYEEIPI	
B-pY505	biotin-SGSGTEGQpYQPQPA	
B-pY740	biotin-SGSGSDGGpYMDMSK	

B

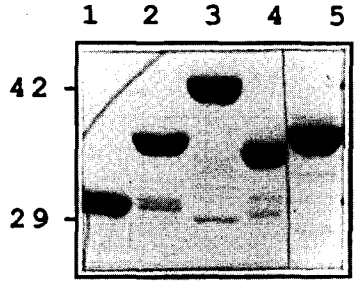


Fig. 2. Phosphotyrosine-containing peptides and purification of various SH2 domains. A. The sequences and source of peptides used in this study are listed. HmT, hamster polyoma middle sized antigen; Lck, p56^{lck}; PDGFR-β; platelet derived growth factor receptor-β. B. Purification of SH2 fusion proteins. Each SH2 domain was expressed in *E. coli* as a fusion protein to glutathione-S-transferase (GST). Fusion proteins were purified by affinity chromatography on glutathione-agarose as described under Materials and Methods. Purified proteins were electrophoresed on a 10% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. Lane 1, GST; Lane 2, GST-Lck SH2; Lane 3, GST-PLC-γ1 SH2 N; Lane 4, GST-PI3-Kinase SH2 N; Lane 5, GST-GAP SH2 N. The size of molecular standards in kDa is shown on the left side of the panel.

ligand of LckSH2. Biotin-pY505 is from the kinase tail of Lck and was previously shown to bind Lck SH2 with an intermediate affinity (Payne *et al.*, 1993). Biotin-pY740 is from the kinase insert region of PDGFR-β and was shown as the high affinity ligand of PI3-Kinase SH2 (Kashishian *et al.*, 1992). Upon testing the three peptides, we observed that only biotin-pY324 showed specific, high affinity binding to Lck SH2 (Fig. 4A), which is in good agreement with the previously reported results described above. Next, we also compared the binding specificity of biotin-pY324 with various SH2 domains. As shown in Fig. 4B, the biotin-pY324 displayed specific and concentration-dependent binding only with GST-Lck SH2. At high concentrations, weak but negligible binding was observed with GST-GAP SH2N (N-terminal SH2 domain). No detectable binding was observed with GST, GST-PI3-Kinase SH2N, or GST-PLC-γ1 SH2N. These results indicate that the established binding assay is highly specific for a particular combination of phosphotyrosyl peptide-SH2 domain.

Competitive binding assay To confirm the binding specificity as well as to compare the relative affinities of phosphopeptides, the competitive binding assay was developed (Fig. 5). Unlabeled pY324, pY505, and pY740 were tested for their ability to compete with B-pY324 for binding to immobilized GST-SH2. The results show that the ID_{50} values for pY324, pY505, and pY740 were approximately 10 μM, 100 μM, and 600 μM, respectively. Because the sensitivity of this assay requires that the concentration of phosphopeptide is much greater than K_d, the ID_{50} values provide only the relative affinity of phosphopeptides for the SH2 domain. The relative affinity

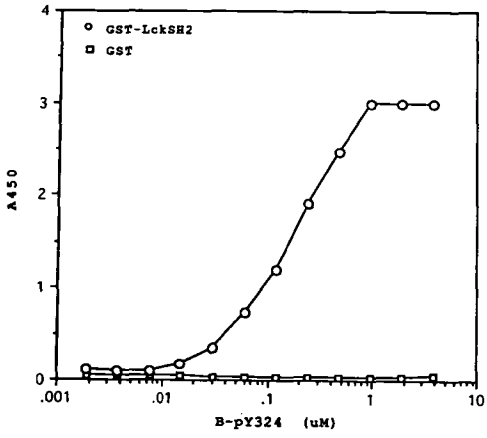
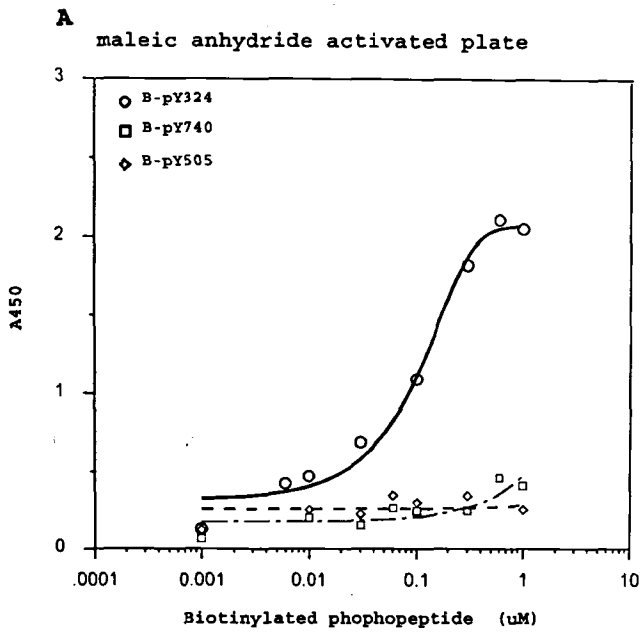


Fig. 3. Binding of biotinylated pY324 peptide to Lck SH2 domain is concentration-dependent and saturated. GST or GST-Lck SH2 was immobilized onto 96-well microtitre plates in duplicate and increasing concentrations of biotinylated pY324 peptide were added. The binding was quantitated by the measurement of remaining peroxidase activity conjugated to streptavidin with TMB as a substrate.



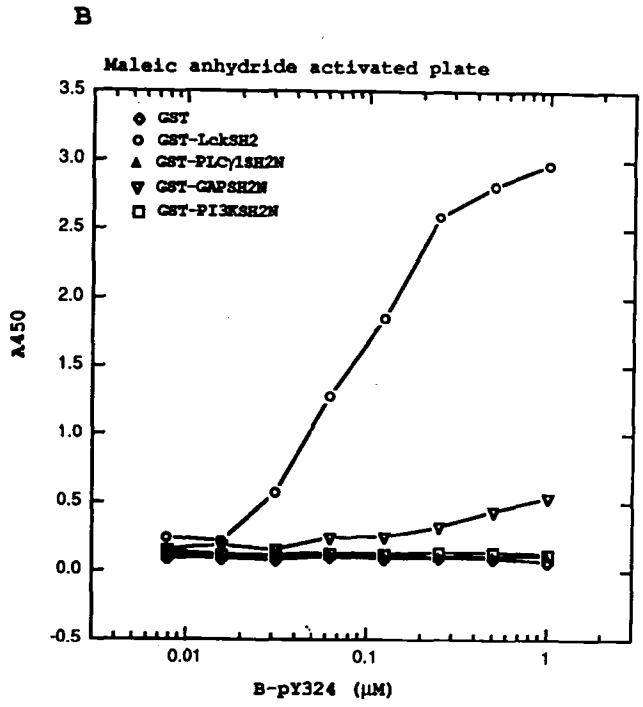


Fig. 4. Binding specificity. A. Binding specificity of GST-Lck SH2 domain was tested with various phosphotyrosine-containing peptides. Binding of three different biotinylated peptides, B-pY324, B-pY505, and B-pY740, to GST-Lck SH2 were determined. Among the peptides tested, only the biotin-pY324 peptide displayed a specific binding to GST-Lck SH2 in a concentration-dependent manner. B. Binding specificity of biotin-pY324 peptide to various SH2 domains. After coating of plates with four different GST-SH2 fusion proteins, increasing concentrations of biotinylated pY324 peptides were applied and assayed for binding.

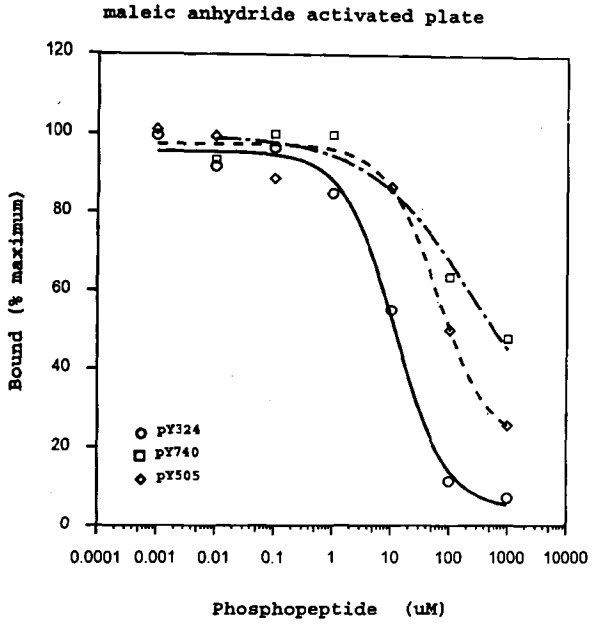


Fig. 5. Competitive inhibition assay. Three unlabeled peptides, pY324, pY740, and pY505, were tested for their ability to inhibit the binding of B-pY324 to GST-Lck SH2. Increasing concentrations of competitor peptides were added to a solution containing 1.0 μM of biotinylated pY324 and the mixtures were incubated for 10 min at room temperature. The mixtures were applied to GST-Lck SH2-coated wells in duplicate and the binding was measured by the method described in Materials and Methods. The competitor peptides were tested in 10-fold increments ranging from 10^{-3} μM to 10^{3} μM.

observed in this study is in good agreement with those of previous studies. Payne *et al.* (1993) observed that the relative affinities of pY505 and pY740 were 5-fold and 80-fold lower than that of pY324, which is comparable to the 10-fold an 60-fold difference observed using the established assay. In addition, using the techniques of surface plasmon resonance and isothermal titration calorimetry, Ladbury *et al.* (1995) observed the 8-fold lower affinity of pY505 compared to pY324, which is also in good agreement with the 10-fold difference in our assay system. Taken together, the competitive binding assay results indicate that the established binding assay system reflects accurately the binding specificity and the relative affinity of SH2-pY peptide interactions.

Here, we established a binding assay system suitable for mass screening of the inhibitors of SH2 domain function. Previously there were several reports (Wood *et al.*, 1992; Bibbins *et al.*, 1993; Piccoione *et al.*, 1993; Zhu *et al.*, 1993; Gilmer *et al.*, 1994; Koh *et al.*, 1997) describing the assay systems to identify SH2 or SH3 inhibitors. Even though each system has its own merit, they are not ideal for the mass screening of the inhibitors. Many of the systems employed either the radiolabeled SH2 domains (Bibbins *et al.*, 1993; Piccoione *et al.*, 1993)/ pY-peptides/ proteins (Wood *et al.*, 1992; Zhu *et al.*, 1993), which is not cost-effective and hazardous to prepare, or the purified proteins for a pY-containing counterpart which requires elaborate receptor purification (Wood *et al.*, 1992; Zhu *et al.*, 1993; Gilmer *et al.*, 1994). In contrast, the system described in this report uses simple components, excluding the requirement of radiochemicals or cumbersome protein purification step. Furthermore, the system is suitable for automated, high throughput screening insomuch as the assay is performed on a 96-well microtiter plate. In addition, we observed the improved specificity of SH2-pY binding with maleic anhydride-activated plates compared to the simple absorption method. For example, when the pY324 peptide was tested using the polysorp plate, a relatively high level of nonspecific binding was detected with GAPSH2N and PI3K SH2N (data not shown). Besides, in the competition assay on polysorp plates, the relative affinity of binding was in disagreement with the previously reported value (data not shown). The improvement of binding specificity with maleic anhydride-activated plates is probably due to the prevention of the conformational change of the SH2 domains during the attachment step. Currently, the established system is being employed to screen the inhibitors of the Lck SH2 binding which may work as a novel type of immunosuppressant.

Acknowledgements We are grateful to K-H. Lee for help in the synthesis of peptides and to J. Shin for various materials. We thank other members of the laboratory, especially Y. Choi and C. Park, for their help. This work was supported in part by funds from the Korea Green Cross Co. and the Korea Ministry of Science and Technology.

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