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Solid-phase binding assays of peptides using EGFP-Src SH2 domain fusion protein and biotinylated Src SH2 domain

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Abstract—Two solid-phase binding assays were designed and evaluated for their potential use in comparing the affinity of peptides to the Src SH2 domain. Resin beads attached to peptides were incubated with the enhanced green fluorescence protein (EGFP)-Src SH2 domain fusion protein or the biotinylated Src SH2 domain and extensively washed. The beads-attached tetrapeptides with high affinities to the EGFP-Src SH2 domain showed more fluorescence intensity than those beads containing tetrapeptides with weak binding affinities, as shown by fluorescence microscopy and fluorescence imaging system. Only the beads attached to pYEEI produced a dark purple color on incubation of the beads, respectively, with the biotinylated Src kinases SH2 domain, alkaline phosphatase-coupled streptavidin, and BCIP/NBT. These solid-phase binding assays may have potential applications for the screening of peptides for the Src kinases SH2 domains.

Src, one of the first studied protein tyrosine kinases, exists as an intriguing therapeutic target for drug discovery with respect to cancer, osteoporosis, and inflammation-mediated bone loss.[1,2] Src tyrosine kinase contains five subdomains, in the order from N- to C-terminal, a short N-terminal membrane anchor domains, followed by two Src homology domains, SH3 and SH2 domains, a kinase domain, and a C-terminal regulatory region. SH2 domains are relatively small protein modules of approximately 100 amino acids and have an extraordinary ability to specifically recognize sequences containing phosphotyrosine (pTyr), thereby facilitating phosphorylation-dependent protein–protein interactions that result in signal propagation.[3] Small-molecule inhibitors, capable of disrupting these interactions, would be useful as probes for studying the mechanism of SH2 domain signaling and as potential inhibitors for further development. The Src SH2 domain preferentially binds peptides with the sequence pTyr-Glu-Glu-Ile (pYEEI) with high affinity (K_D = 100 nM).[4]

A number of methods have been introduced for the identification of protein kinase inhibitors over the past few years.[5–11] Several of these assays have been specifically designed for high-throughput screening of the protein kinase inhibitor libraries. Scintillation proximity assay (SPA) technology has been proved to be very robotic and accurate.[5,6] This assay has the advantages of the absence of washing steps and a solution-based phosphorylation reaction. However, SPA is based on radioactive techniques. Several other non-radioactive techniques have been used for identification of protein kinase inhibitors, such as the ELISA, by phosphotyrosine antibodies.[7,8] Fluorescence polarization (FP) assay is another commonly used assay based on the generation of high molecular weight species of fluorescent probes with protein kinases that generate high polarization intensity.[9] FP assay is an easy and well-established method for identification of the peptides binding to the Src SH2 domain, but it requires the purified peptides in solution. The cleavage of peptides from solid-phase and their purification are usually time-consuming processes.

In search for more efficient non-radioactive binding assays, we developed and evaluated two solid-phase-based

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binding assays for the potential use in comparing the affinity of peptides to the Src SH2 domain.

The Src SH2 domain was expressed and purified as enhanced green fluorescence protein (EGFP)-Src SH2 domain fusion protein. Alternatively, the Src SH2 domain was biotinylated using the Pierce biotinylation kit. These two proteins were incubated with peptides attached to resin beads and subjected to extensive washing. The resin beads were evaluated using fluorescent- and color-based techniques according to protein tagging and biotinylation protocols, respectively.

The tetrapeptides, pYEEI, IEEpY, YEEI, IEEY, FEEI, and IEEF, were synthesized by the solid-phase peptide synthesis strategy on a PS3 automated peptide synthesizer (Rainin Instrument Co., Inc.) employing *N*-(9-fluorenyl)-methoxycarbonyl (Fmoc)-based chemistry on 0.1 mmol of Novasyn® TG amino resin (loading capacity 0.56 mmol/g). 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and NMM (0.4 M) in *N*,*N*-dimethylformamide (DMF) were used as coupling and activating reagents, respectively. Novasyn® TG amino resin (0.1 mmol), coupling reagents (0.4 mmol), and Fmoc-amino acid building blocks (0.4 mmol) were purchased from Novabiochem. Fmoc deprotection at each step was carried out using 20% piperidine/DMF. The peptides remained attached to the Novasyn® TG amino resin beads and washed subsequently with DMF, MeOH, and DCM. The side-chain protecting groups were removed using trifluoroacetic acid/anisole/water (90:5:5). The Tentagel resin was selected due to its swelling property in aqueous solutions required for incubation with the Src SH2 domain. Alternatively, the corresponding control peptides were synthesized using Fmoc-Wang resin and cleaved from resin beads, purified by HPLC and their structures were confirmed using electrospray mass spectrometry according to a previously reported procedure.[12]

1. Solid-phase binding assay using EGFP-Src SH2 domain fusion protein

To generate glutathione *S*-transferase (GST)-Src SH2 domain-EGFP fusion protein, the EGFP was isolated by PCR from the pEGFP vector (BD Biosciences) using EGFP oligonucleotides (5′GCATGAATTCATGGTG AGCA-AGGGCGAGGAGCTG-3′ and 5′-GCATAA GCTTT-TACTTGTACAGCTCGTCCATGC-3′), and the product was cleaved with *Eco*RI and *Hin*dIII. It was ligated into the pGEX-Src SH2 vector at *Eco*RI and *Hin*dIII sites. The nucleotide sequence of pGEX-Src SH2-EGFP was confirmed by sequencing and expressed in *Escherichia coli* DH5α.

Bacteria harboring pGEX-Src SH2-EGFP plasmid were cultured in LB medium at 37 °C with shaking at 250 rpm overnight. The overnight culture was then mixed with an equal volume of fresh LB medium and cooled to 20 °C. IPTG (0.2 mM) was added to the culture to induce recombinant protein expression at 20 °C for 6 h. The GST fusion protein was purified by glutathione affinity chromatography. Protein concentration was determined by the Bradford assay and the purity was assessed by SDS–PAGE with Coomassie blue staining.

The resin beads attached to different peptide sequences (4 mg) were incubated with the EGFP-Src SH2 domain fusion protein (40 μl, 83 μg/mL) for 3 h at 4 °C. The mixtures were subjected to washing with Tris buffer (pH 8.0) three times to remove any unbound fusion protein (Fig. 1). Then, the resins were mounted on a glass slide and observed under a fluorescent microscope. All the microscopic observations of the peptide-attached resins were carried out using the Nikon Fluorescent Microscope under a FITC channel (480/520). By using the function of 'Z Projection' of the software ImageJ (NIH), 30 pictures at different focuses (scanning the surface) were stacked together to reflect the fluorescence distribution on the beads more precisely.

From all the synthesized peptides attached to the resin beads, pYEEI is known to have high affinity to the Src SH2 domain (IC_{50} = 6.5 μM) in solution-based FP assays.[12] Other control peptides, IEEpY, YEEI, IEEY, FEEI, and IEEF, showed very weak binding affinity or no affinity to the Src SH2 domain (IC_{50} > 100 μM). The presence of the pTyr group in the N-terminal of the tetrapeptide is required for binding to the pTyr-binding pocket of the Src SH2 domain. Under the fluorescence microscope using FITC channel, the EGFP protein can be activated to give fluorescence.

Figure 2 shows all the stacked images of all the resin beads-attached peptides. All beads exhibited autofluorescence that was unavoidable, but there was a significant difference in fluorescence intensity between peptides with strong binding affinity and those with weak or no binding affinity to the Src SH2 domain. The picture of the bead attached to pYEEI exhibited a much higher fluorescence intensity than the other beads. Unequal distribution and localization of fluorescent spots were probably caused by an unequal distribution of the bounded EGFP-Src SH2 domain fusion proteins

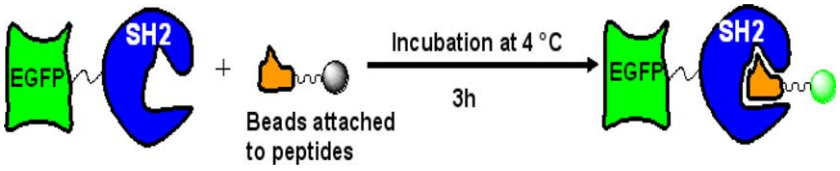

Figure 1. Solid-phase binding assay of the resin beads-attached peptides with the EGFP-Src SH2 domain fusion protein. The beads containing peptides with high affinity to the Src SH2 domain showed high fluorescent intensity upon binding to the EGFP-Src SH2 domain fusion protein.

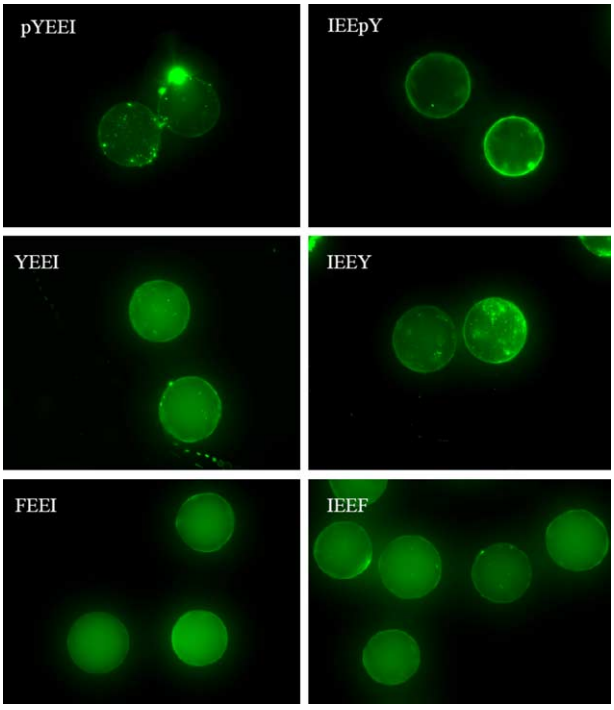

Figure 2. The fluorescent images of the resin beads after incubating with the EGFP-SrcSH2 fusion protein and extensive washing.

on the bead surface. During the solid-phase synthesis, the peptides are not synthesized equally on the bead surface. If relatively more peptides are formed at the same area on the surface, this may trigger a strong signal of fluorescence intensity, as seen with beads attached to pYEEI. All other resin beads-attached peptides showed a much weaker fluorescence intensity. Fluorescence detected in these beads was on account of the autofluorescence characteristics of the Novasyn® TG amino resin. There was a significant difference between the fluorescence which resulted from the binding of EGFP and autofluorescence. The resin-beads-attached IEEY also showed some fluorescence spots on their surface probably due to the non-specific binding of the fusion protein to the bead containing this peptide sequence.

Alternatively, the fluorescence intensity was measured using the Kodak Imaging System 2000. The EGFP-Src SH2 domain fusion protein and beads-attached peptides were loaded together into a 96-well bottom filtered microplate. After 3 h of incubation, the protein solution was filtered under vacuum. The mixtures were subjected to extensive washing with Tris-buffer (pH 8.0). The washing and filtration were repeated six times. The fluorescence intensity of the beads was measured using the Kodak Image System 2000. Figure 3 shows the picture taken by the Kodak Image System.

The measured fluorescence intensity of resin beads attached to pYEEI was approximately twofold higher than that of beads attached to IEEpY, YEEI, IEEY, FEEI, and IEEF. The fluorescence intensity in beads attached to peptides with weak binding affinity to the Src SH2 domain is mainly due to the autofluorescence property of the Novasyn® TG amino resin. Although these

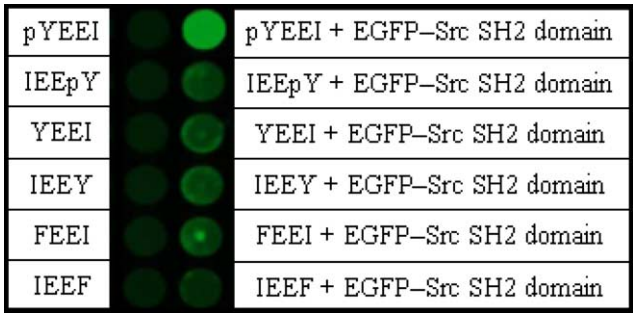

Figure 3. The fluorescence-based assay using the Kodak Imaging System: the column on the right shows the resin beads-attached peptides incubated with the EGFP-Src SH2 domain fusion protein. The left column shows the same resin-beads attached to the peptides alone.

fluorescence-based assays were able to identify peptide analogs with a higher binding affinity to the Src SH2 domain, we investigated whether a solid-phase color-based assay can be used to avoid the problem of high autofluorescence and non-specific binding associated with these fluorescence-based assays.

2. Solid-phase binding assay using biotinylated Src SH2 domain

The Src SH2 domain was biotinylated using the biotinylation kit from Pierce Co. (Catalog # 21420). Biotin can be used to detect the receptor–ligand interaction. *N*-Hydroxysuccinimide (NHS) esters of biotin are commonly used as biotinylation reagents. Primary amino groups ($-NH_2$) of proteins or peptides form stable amide bonds upon reaction with NHS-activated biotins in pH 7–9. In proteins, such as the Src SH2 domain, both the lysine residues and the *N*-terminal of the protein can provide the primary amino groups for reaction with NHS esters of biotin. The Src SH2 domain (10 mg) was dissolved in PBS buffer (2.0 mL, pH 7.2). Sulfo-NHS-LC-Biotin solution (200 μl, 10 mM) was added to the protein solution. The reaction mixture was incubated for 2 h at 0 °C to generate the biotinylated SH2 domain.

The resin beads attached to peptide sequences, pYEEI, IEEpY, YEEI, IEEY, FEEI, and IEEF (10 mg), were washed three times with PBS buffer (1 mL, pH 7.4). Then, the resin beads were incubated with the biotinylated Src SH2 domain (1 mL) at room temperature for 3 h on a rotary shaker and subsequently washed three times with 1 mL PBS buffer (pH 7.4) to remove the unbound protein. The beads were incubated with alkaline phosphatase-coupled streptavidin (1 mL) for 20 min on a rotary shaker at room temperature. A solution (200 μl) containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) was added and the mixtures were compared for the production of purple color (Fig. 4).

It is expected that for the peptide sequences with high affinity for binding to the Src SH2 domain, the biotinylated Src SH2 domain remains on the resin

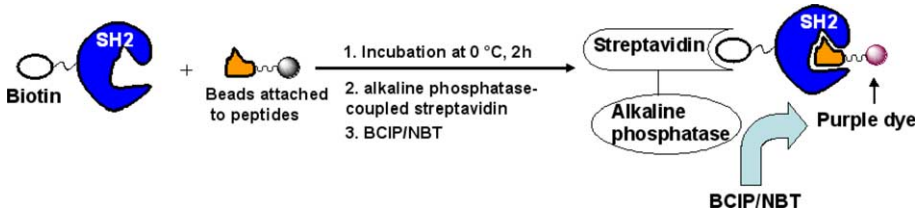

Figure 4. Solid-phase binding assay of the resin-beads-attached peptides with the biotinylated Src SH2 domain. The beads containing peptides with high binding affinity for the SH2 domain exhibited a purple color upon incubation with the biotinylated Src SH2 domain, followed by addition of alkaline-phophatase-coupled streptavidin and BCIP/NBT.

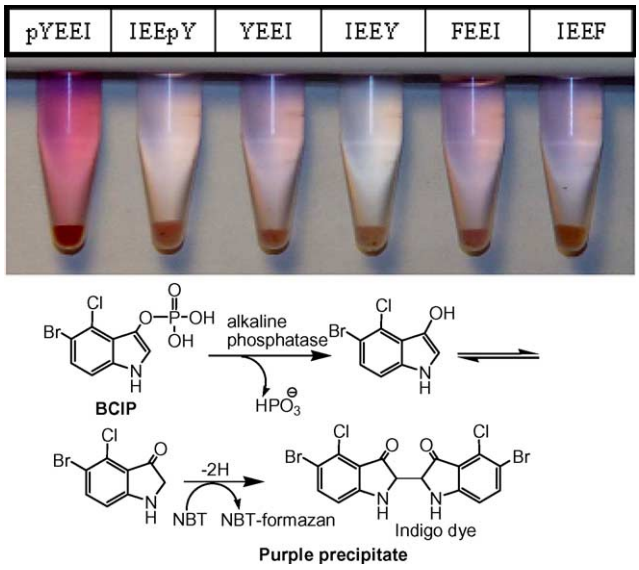

Figure 5. Solid-phase binding affinity assay using the biotinylated Src SH2 domain with resin-beads attached to peptides.

beads, even after extensive washing. Subsequent reactions with alkaline phosphatase-coupled streptavidin, BCIP, and NBT create a dye color. Figure 5 shows the results after final reactions that lead to the production of colors. The resin-beads attached to pYEEI in the first tube showed purple color with a significantly higher intensity than other tubes containing resin-beads attached to other peptides, suggesting that this solid-phase binding assay can identify beads containing peptides with high affinity to the Src SH2 domain. The presence of weak purple color in tubes 2–6 indicates the presence of some insignificant non-specific binding to the Src SH2 domain.

In summary, fluorescence and color-based solid-phase assays were designed and evaluated for the possible use in the identification of peptides with high binding affinity to the Src SH2 domain. The color-based solid-phase assay appears to be more reliable due to the absence of autofluorescence and reduced non-specific binding. These solid-phase assays when optimized may have potential application for high-throughput screening of resin-beads attached peptide libraries for the Src SH2 domain and other proteins of interest.

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