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Development of ELISA System for Screening of Specific Binding Inhibitors for Src Homology (SH)2 Domain and Phosphotyrosine Interactions

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In the present study, an *in vitro* ELISA system to assess the interaction between Src homology (SH)2 domains and phosphotyrosine that contain peptides was established using purified GST-conjugated SH2 proteins and synthetic biotinylated phosphotyrosine that contain oligopeptides. The SH2 domains bound the relevant phosphopeptides that were immobilized in the streptavidin-coated microtiter plate in a highly specific and dose-dependent manner. The epidermal growth factor receptor (EGFR)-, T antigen (T Ag)-, and platelet-derived growth factor receptor (PDGFR)-derived phosphopeptides interacted with the growth factor receptor binding protein (Grb)2/SH2, Lck/SH2, and phosphatidyl inositol 3-kinase (PI3K) p85/SH2, respectively. No cross-reactions were observed. Competitive inhibition experiments showed that a short phosphopeptide of only four amino acids was long enough to determine the binding specificity. Optimal concentrations of the GST-SH2 fusion protein and phosphopeptide in this new ELISA system for screening the binding blockers were chosen at 2nM and 500nM, respectively. When two candidate compounds were tested in our ELISA system, they specifically inhibited the Lck/SH2 and/or p85/SH2 binding to the relevant phosphopeptides. Our results indicate that this ELISA system could be used as an easy screening method for the discovery of specific binding blockers of protein-protein interactions via SH2 domains.

Keywords: Binding inhibitor, ELISA, Screening, SH2, Protein-protein interaction

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Introduction

Binding of growth factors, such as the epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) to their respective receptors induces receptor dimerization. This is associated with autophosphorylation on tyrosine residues in the receptor dimer (Ullich and Schlessinger, 1990). Following autophosphorylation, individual phosphotyrosine residues that are located in the cytoplasmic domain of the receptors serve as highly selective binding sites that interact with the specific cytoplasmic signaling molecules. These signaling molecules include phosphoinositide-3-kinase (PI3K), phospholipase Cγ (PLCγ), GTPase activating protein (GAP), growth factor receptor-binding protein 2 (Grb2), etc (Coughlin *et al*, 1989; Margolis *et al.*, 1989; Bjorge *et al.*, 1990; Kaplan *et al.*, 1990; Morrison *et al.*, 1990). The high affinity of these molecules for the receptor tyrosine kinase is due to the presence of a highly conserved region, so-called SH2 domain, in these molecules. SH2 domains are composed of about 100 amino acids that are homologous to a noncatalytic region that is present in the c-*src* protooncoprotein (Pawson and Gish, 1992). After the first report on the SH2 domain that contains protein-crk SH2 (Matsuda *et al.*, 1992), a large number of proteins containing SH2 domains have been identified. Each SH2 domain in various molecules binds to phosphotyrosine residues in a highly specific manner (Fantl *et al.*, 1992; Songyang *et al*, 1993).

To better understand the molecular basis for signal discrimination, several *in vitro* assays for SH2 binding were developed (Wood *et al.*, 1992; Piccione *et al.*, 1993; Sonatore *et al.*, 1996; Koh *et al.*, 1997b). The results from these studies demonstrated that a specific interaction between SH2-containing proteins and phosphoproteins can be reconstructed with the isolated the SH2 domain and the corresponding phosphopeptide. In our previous report on the SH2 binding

assay, specific binding was calculated from the radioactivity of [^{3}H]-labeled phosphopeptide that is bound to the immobilized GST fusion protein (Koh *et al.*, 1997b). However, all of these previous assays have limitations, such as the use of radioactive labels, lack of sensitivity, a long time requirement, or the high cost. In order to avoid use of radioactive materials, we recently reported an SH3 binding assay that employs biotinylated phosphopeptide instead of [^{3}H]-labeled phosphopeptide (Koh *et al.*, 1997a). In the report, biotinylated phosphopeptide, which bound to the immobilized GST fusion protein, was detected by peroxidase-conjugated streptavidin. Here, we report the establishment of an ELISA method to assess the interactions between several SH2 domains and phosphotyrosine-containing peptides, and the use of this method to screen inhibitors of the protein-protein interactions. Our ELISA system takes advantage of the strong interaction between the antibody and antigen, which enables the signal to have a high amplification. It also avoids the use of radioactive probes without the loss of sensitivity. SH2 binding inhibitors may be good candidates for anticancer drugs since, for example, the activation of the ras protein that is located downstream of Grb2 is known to be related to some tumors (Bos, 1989). More direct evidence for the important role of Grb2 in tumorigenesis comes from the studies of BCR-ABL. BCR-ABL is a chimeric oncoprotein that exhibits deregulated tyrosine kinase activity, and is implicated in the pathogenesis of Philadelphia chromosome-positive human leukemia. Mutation of tyrosine (Y = 177) to phenylalanine abolishes Grb2 binding. This BCR-ABL mutant is unable to transform primary bone marrow cultures (Gishizky *et al.*, 1995), indicating that Grb2 is required for cellular transformation by BCR-ABL. In the case of other SH2 that contain proteins, it is the general idea that they usually have important roles in cell growth and differentiation. For these reasons, the assay system to screen blockers of the SH2 domain-mediated signal transduction will be a useful tool for drug discovery. The data presented here indicate that our assay system is sensitive, reproducible, easy, and flexible. It can be used as a suitable method for screening specific signal blockers.

Materials and Methods

GST-Grb2/SH2, GST-Lck/SH2, and GST-p85/SH2 fusion protein The GST-SH2 fusion proteins were generated by PCR using Grb2, Lck, and p85 (a regulatory subunit of PI3K) cDNAs as templates and pairs of gene-specific primers (Koh *et al.*, 1997b). The PCR products were digested with *BamH*I and *EcoR*I. They were subsequently subcloned into the pGEX-2T bacterial expression vector (Pharmacia Biotechnology, Uppsala, Sweden). GST fusion proteins were expressed in *E. coli* and purified by affinity chromatography on glutathione-agarose (Pharmacia Biotechnology).

Biotinylated peptides The biotinylated phosphotyrosine-containing peptides (Alpha Diagnostic International) were designed as described elsewhere (Garcia-Echeveria *et al.*, 1997). The sequences of the peptides were as follows: Biotin-DDTFLPVPEpY INQSVPK-NH_2 (derived from the EGF receptor, pY1068) (each capital letter, except NH_2, is expressed by a single letter code for amino acid and the small letter 'p' denotes the modification by phosphorylation) for Grb2/SH2 binding, Biotin-EEEPQpYEEIPIY L-NH_2 (derived from polyoma middle T antigen, pY324) for Lck/SH2 binding, Biotin-DMSKDESVDpYVPMLDMK-NH_2 (derived from PDGF receptor, pY751) for p85/SH2. Shc-derived phosphopeptides that were used for the competitive binding assay were DPSpYVNVQNLDK-NH2 (Shc p52, pY317), Acetyl-SpYVNVK-NH2, and Acetyl-pYVNV-NH2. The PDGFR-derived phosphopeptide for competition was SVDpYVPMLDMK-NH_2 (pY751). Lysine (K) on the carboxy-terminus of each peptide was not present in natural sequences, but was added for efficient labeling of its terminus for separate experiments. All of the oligopeptides were highly pure (90-95%; HPLC grade).

ELISA assay The biotinylated phosphopeptide was first diluted in 50 mM Tris-HCl, pH7.5. The phosphopeptide solution (100 µl) was then added into the wells of streptavidin-coated plates (Boehringer Mannheim, Indianapolis, USA). The plate was incubated overnight at 4°C, then rinsed 3 times with 50 mM Tris-HCl, pH7.5, 150 mM NaCl, 0.1% Tween (TBS-T). The incubation time for coating can be reduced as much as 1 h (at room temperature) without a significant loss of coating efficiency. The plate was further blocked with 300 ml of the blocking reagent for ELISA (Boehringer Mannheim) for 15 min at room temperature. GST-SH2 was then added into the wells in a 100 µl/well total volume of 0.1% BSA-TBS-T and incubated for 2 h on a rocker. The assay proceeded with a primary rabbit anti-GST antibody (1 : 1000 in 3% BSA-TBS-T for 1 h at room temperature) and a secondary peroxidase-conjugated goat-anti-rabbit IgG (1 : 1000 in 0.1% BSA-TBS-T for 1 h at room temperature). After a series of final washes, the peroxidase activity was monitored at 492 nm (with sulfuric acid), or 450 nm (without sulfuric acid) on a plate reader after the addition of 100 µl/well of the reaction solution (0.04% H_2O_2, 0.04%(w/v) *o*-phenylene-diaminedihydrochloride (OPD), citrate-phosphate buffer, pH5.0).

Test compounds A partially purified compound (PPC) (under investigation for a patent pending for the binding blocker of protein-protein interactions) was kindly provided from Mokam Institute of Bioengineering. AII was isolated from a strain *Penicillium multicolor* through preliminary studies for searching bioactive molecules in our lab. Its structure was determined by NMR spectroscopy and chemical evidence (Nam *et al.*, 2000). The two compounds were preliminarily found to inhibit more or less SH2-phosphotyrosine interactions. Test compounds were diluted in dimethylsulfoxide (DMSO) and added to the phosphopeptide solution prior to the binding reaction with SH2 fusion proteins. The final concentration of DMSO was less than 1%.

Results and Discussion

Construction and optimization of ELISA system With the help of their SH2 domains, Grb2, Lck, and p85 have been known to bind respectively on specific tyrosine residues phosphorylated by upstream regulators (Garcia-Echeveria *et*

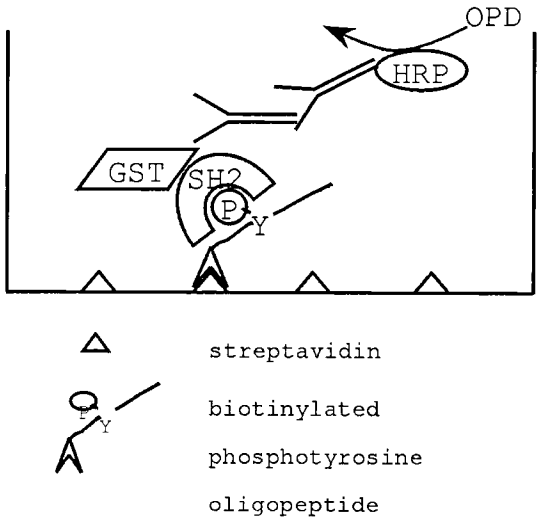

Fig. 1. Schematic diagram of new ELISA system. GST-conjugated SH2 proteins bind to phosphotyrosine-containing oligopeptide immobilized on the microtiter plate by a strong interaction between biotin of the oligopeptide and streptavidin of the plate. Bound SH2 proteins were quantified by an anti-GST antibody and a peroxidase conjugated secondary antibody.

al., 1997). Previous studies located the specific tyrosines of EGFR at pY1068 for the interaction with Grb2/SH2, T Ag at pY324 for Lck/SH2, and PDGFR at pY751 for p85/SH2. We selected three examples for establishment of the ELISA method, since Grb2 and p85 were known to have important roles in transducing signals from growth factor receptors to downstream effectors in order to regulate the cellular proliferation and differentiation (Carpenter and Cantley, 1996; Feng *et al.*, 1996; Gu *et al.*, 2000). Recently, the importance of PI3K in the transformation was also suggested (Akagi *et al.*, 2000; Wellbrock *et al.*, 2000). Lck has a critical role in the differentiation of T lymphocytes (Weiss, 1993; Choi *et al.*, 1999; Sundvold *et al.*, 2000). Therefore, strong blockers of these interactions may be good candidates for an anti-cancer drug or immunomodulator. To mimic the interactions between the whole SH2 domain-containing proteins and whole phosphotyrosine-containing proteins, the purified SH2 domains of each protein that was conjugated to GST and phosphotyrosine-containing oligopeptides were used for construction of an *in vitro* ELISA system. To improve the coating efficiency, phosphopeptides were synthesized with the modification of biotinylation at their N-termini. The biotinylated phosphopeptides were subsequently added onto wells of streptavidin-coated plates. The overall scheme of the ELISA method for a screening system was depicted in Fig. 1. As the first step toward the establishment of an assay system, optimal concentrations of both the biotinylated phosphopeptide and GST-SH2 fusion protein were determined by testing various combinations of their concentrations (Fig. 2). In all of the cases, no binding of SH2 peptides was observed in the absence of immobilized phosphopeptides. Also, in the absence of SH2 containing peptides, no cross-reactions of both the primary and secondary antibodies that were used in the present study with the microtiter plate were observed. As anticipated, the interaction between phosphopeptides and SH2 increased in proportion to the increasing concentrations of added phosphopeptides and SH2 containing peptides. From the results in Fig. 2, we chose efficient and economical concentrations of SH2 fusion proteins and phosphopeptides as 80 ng/ml and 1 μg/ml, respectively, since about 80% of the saturated binding was observed at these concentrations. The use of higher concentrations than these may decrease the sensitivity of the assay. If the binding is saturated, then larger amounts of the inhibitors are required to effectively interfere with the interaction between SH2s and phosphopeptides in order to achieve the same extent of inhibition that is found at the lower concentrations of our choice. Also, the use of lower concentrations may make it difficult to detect the inhibitory effects of test chemicals, due to the low binding level of the control group. Therefore, it seemed proper that the concentrations of the SH2 fusion protein and phosphopeptide should be chosen at the level of 80 ng/ml and 1 μg/ml, respectively. The concentration of 80 ng/ml GST fusion protein and 1 μg/ml phosphopeptide is equivalent to 2 ± 0.5 pM and 500 ± 50 nM in the molar concentration, respectively. At these concentrations, three different pairs of SH2/phosphopeptide showed considerable bindings of similar

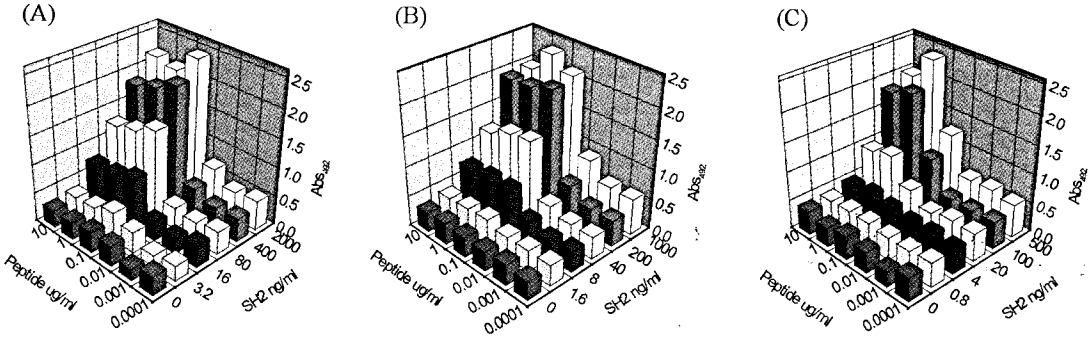

Fig. 2. Effect of phosphopeptide and SH2 domain-containing protein concentrations on binding interactions. A, Grb2/SH2 binding to EGFR pY; B, Lck/SH2 binding to T Ag pY; C, p85/SH2 binding to PDGFR pY.

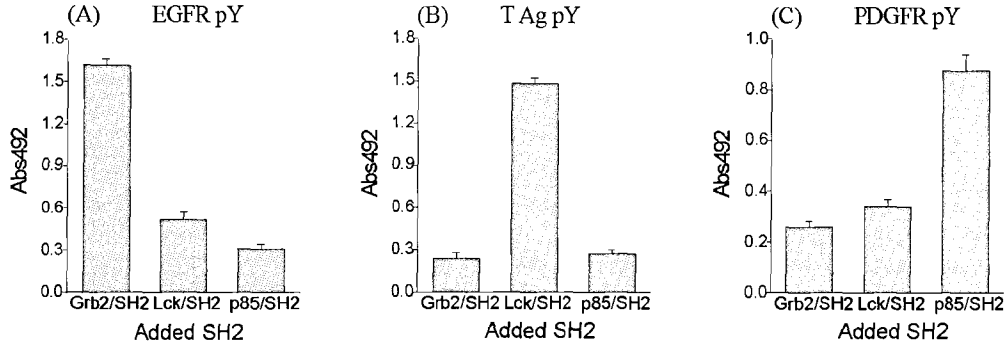

Fig. 3. Effect of phosphopeptide sequence on the SH2 domain binding activity. After three kinds of phosphopeptide (each 1 μg/ml) were immobilized onto the ELISA plate, Grb2/SH2, Lck/SH2, and p85/SH2 (each 80 ng/ml) were added into each well. The coated phosphopeptides were as following: A, EGFR pY; B, T Ag pY; C, PDGFR pY.

extent. This implied that the binding kinetics between purified SH2 proteins and its corresponding phosphopeptides was quite similar, regardless of their origins.

In our previous paper on the SH2 binding assay, we used the GST fusion protein and biotinylated phosphopeptide at concentrations of 3 μg/ml and 4 ng/ml, respectively. The reason for the requirement of a high concentration of the GST fusion protein is due to the scheme of the binding assay. According to the scheme, the GST fusion protein, not phosphopeptide, had to be immobilized onto the Immunomodule. In our new ELISA system, we found that the short phosphopeptide that was immobilized on the plate was highly effective in the binding reaction. The use of biotinylated phosphopeptide instead of the GST-fusion protein for the immobilized substratum makes the assay faster and more reliable, because more than 8 hrs is required for efficient coating in the case of the GST-fusion protein. In contrast, the time for coating in the new ELISA system could be reduced as much as 1 h without a significant loss of coating efficiency (data not shown). This improvement might be due to the strong interaction between biotin and streptavidin. Also, the use of phosphopeptide for the immobilization substrate eliminates the requirement for a large amount of the purified SH2 fusion protein.

Specificity of the interactions between each SH2 domain and phosphopeptides To investigate the specificity of the protein-phosphopeptide interactions, each SH2 protein was tested for the binding of all three of the phosphopeptides. After the three kinds of phosphopeptides (1 μg/ml) were immobilized onto an ELISA plate, 80 ng/ml of each SH2 protein was added to be assayed for its binding affinity to the phosphopeptides. As shown in Fig. 3, pairs of Grb2/SH2 and EGFR pY1068, Lck/SH2 and middle T Ag pY324, and p85/SH2 and PDGFR pY751 showed apparent high degrees of binding. No nonspecific cross interactions were observed, as expected. The interactions between each SH2 protein and the two irrelevant phosphopeptides were very low to ensure the specificity of our ELISA system. These results indicated that

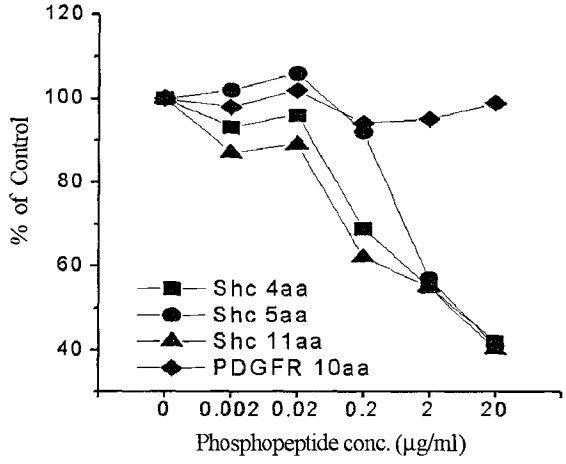

Fig. 4. Inhibition of Grb2/SH2 binding to EGFR pY by Shc-derived oligopeptides. Shc-derived oligopeptides with different lengths were added to the binding reaction solution with increasing concentrations, as indicated. As a control, the PDGFR-derived oligopeptide was also tested in the same range of concentrations. The Grb2/SH2 protein and EGFR pY were used at 80 ng/ml and 1 μg/ml, respectively.

the interactions between protein-phosphopeptide were highly specific, in spite of the short length of the synthetic phosphopeptide, as well as the fact that the three-dimensional structure of the short oligopeptides resembled their natural structure within the whole protein. In addition, this implied that other SH2 protein-phosphotyrosine peptide interactions could be reconstructed *in vitro* without loss of their specificity.

Screening of specific SH2 binding inhibitors The highly specific binding between the SH2 domain and phosphotyrosine is provided especially by the phosphotyrosine residue itself, as well as its neighboring residues carboxyterminal within the phosphotyrosine-containing proteins (Fantl *et al.*, 1992; Songyang *et al.*, 1993; Hart *et al.*, 2000; Beebe *et al.*, 2000). Based on this property, a series of phosphopeptides with different lengths (different

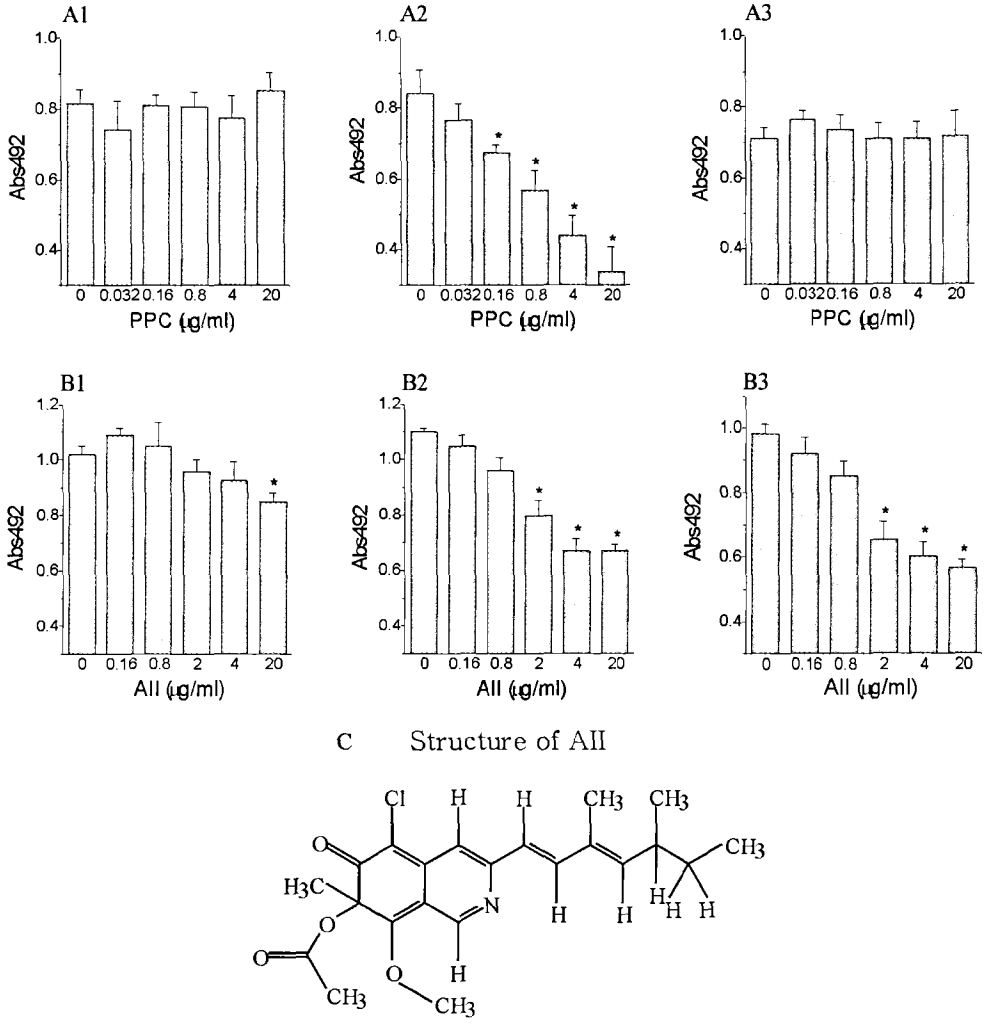

Fig. 5. Effects of PPC and AII on SH2-phosphopeptide interactions. Two kinds of compounds were tested for their binding inhibitory effects, as indicated in Materials and Methods. The GST-fusion proteins were used as 80 ng/ml, and phosphopeptides were coated at 1 μg/ml. Increasing concentrations of PPC (A) or AII (B) were added into the binding solution-A1 and B1, Grb2/SH2+EGFR pY; A2 and B2, Lck/SH2+T Ag pY; A3 and B3, p85/SH2+PDGFR pY. One experiment is shown. It is representative of the three independent tests. The asterisks indicate the significance ($P<0.05$) relative to no addition of test compounds. C, The structure of AII determined by NMR analysis and chemical evidence.

molecular weights) was designed to determine the effects of molecular size on SH2 binding without the loss of specificity. The Shc p52 protein is known to interact with Grb2 in the signal pathways that are activated by several receptors (Park *et al.*, 1996). Interestingly, Shc pY317 has the same SH2 specificity as EGFR pY1068. Three Shc-derived phosphopeptides of different lengths, which commonly contained the phosphotyrosine pY317 and its neighboring three amino acids carboxyterminal, were tested to competitively inhibit the Grb2/SH2-EGFR pY interaction. Fig. 4 shows that a very short peptide of only four amino acids had a high affinity to Grb2/SH2, as proved by its efficient blocking of the Grb2/SH2 binding to EGFR pY1068. Although the eleven-residue peptide was the strongest competitor, the efficiency of the acetylated four-residue peptide as a competitor was also substantially high. At a 2 μg/ml concentration, Shc-derived oligopeptides inhibited Grb2/SH2 binding by 45%, regardless of their length. When an irrelevant PDGFR-derived ten-residue phosphopeptide (pY751) was added, the Grb2/SH2-EGFR pY interaction was unaffected, even at a high concentration (20 μg/ml) of PDGFR pY. These results indicate that the four amino acid residues of phosphoprotein are sufficient for discriminating between irrelevant SH2 and specific SH2. Also, small molecules with low molecular weight can inhibit SH2 binding without the loss of specificity.

The results from the competitive inhibition experiment implied that our ELISA system might serve as a good screening method to find blockers of the protein-protein interactions. For example, if certain compounds are

structurally analogous to the Shc oligopeptide, the compounds may specifically inhibit the Grb2/SH2-EGFR peptide interaction. Many investigators are attempting to discover this kind of inhibitor from the peptide library (Beebe *et al.*, 2000; Hart *et al.*, 2000). One laboratory at the Mokam Institute of Biotechnology, Korea, has sought such compounds. Several candidates that might inhibit the Lck/SH2-phosphopeptide were partially purified. In order to prove the applicability of our ELISA system, one of those compounds (named PPC here) was tested for its inhibitory effect on the SH2-phosphopeptide interactions. PPC significantly blocked the interaction between Lck/SH2 and middle T Ag pY, as shown in Fig. 5A2. At a very low concentration of 0.16 μg/ml, the inhibition of the interaction was significant ($P<0.05$). This blockage was very specific because PPC had no inhibitory effects, even at a high concentration of 20 μg/ml on both the Grb2/SH2-EGFR pY interaction and the p85/SH2-PDGFR pY interaction (Fig. 5A1, 5A3). Since PPC was partially purified, further purification processes will identify the active component(s). Another chemical was AII (Fig. 5C), which was isolated from *Penicillium multicolor*. The structure of AII was determined using NMR spectroscopy and chemical evidence (Nam *et al.*, 2000). As shown in Fig. 5B1-B3, it was clear that AII did inhibit the interactions between Lck/SH2 and middle T Ag pY, and between p85/SH2 and PDGFR pY in a dose-dependent manner. The inhibition became significant at concentrations higher than 2 μg/ml. Since the concentration of AII (2 μg/ml) is about 5 μM in a molar concentration, the inhibition was very specific and strong. The Grb2/SH2 binding was moderately, but significantly ($P<0.05$) inhibited (about 10-15%) by high concentrations (20 μg/ml) of AII through repetitive experiments. Because AII was previously selected for its inhibitory action on Lck binding, it was an unexpected result that it also showed a strong inhibitory effect on p85 binding, as well as a moderate effect on the Grb2 binding. Whether this chemical could inhibit other SH2 protein-phosphopeptide interactions though has still not been tested. The broad binding specificity of AII contrasted well with the highly specific binding inhibition of Lck by PPC (Fig. 5A2). The reason for different specificities of the SH2 binding inhibition between PPC and AII still needs to be solved. In our opinion, AII is thought to structurally mimic only the phosphotyrosine residue and partially neighboring residues, due to its very small size (MW = 391). Active component(s) in the PPC mixture though may be large enough to discriminate each SH2 domain. Further studies will reveal the mechanism of the multiple specificities of the inhibition by AII.

In summary, as proved by the test results of the two compounds, our ELISA method was successful as a simple method to screen specific inhibitors of protein-protein interactions. The advantages of this method are that they are reproducibility and versatility. The use of biotinylated phosphopeptide secures a high coating efficiency. The use of antibodies amplifies the signal of the interaction high enough to be detectable without the use of radioactive probes. Compared to other methods that sometimes require protein purification and/or use of radioactive materials, our new ELISA system is very safe and specific though using short synthetic phosphopeptides.

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