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Development of a solid-phase binding assay and identification of nonpeptide ligands for the FynB Src homology 2 domain

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Abstract

The nonreceptor tyrosine kinase FynB is known to be required in the induction of long-term potentiation (LTP), a cellular mechanism for learning and memory. Ligands of the FynB SH2 domain as a possible FynB activator are, thus, of great interest. In this study, a solid-phase ligand binding assay was established to meet the screening requirement of high-throughput and ease of use, and in an attempt to find the specific ligands for the FynB SH2 domain. This assay measures the competitive inhibition of the binding of the biotinylated phosphopeptide (GGSET-DDY*AEIID), derived from a binding sequence in human focal adhesion kinase, to the SH2 domain of FynB precoated as a glutathione S-transferase fusion protein on a solid-phase. Using this high-throughput screening method for SH2 ligands, a modest size of chemical library was screened, and two non-peptide compounds, 4-acetamidobenzene sulfinic acid and 1-allylpyridinium 3-sulfonate, were identified by their strong binding affinity to the FynB SH2 domain. This result demonstrates the feasibility of the developed assay in high-throughput screening. Further studies on the molecular structures of the identified SH2-binding ligands will allow presentation of specific models for ligand-domain complexes for improving the ligands and will help to develop a potential lead compound for improving LTP.

Keywords: FynB; High-throughput screening; Biotinylated phosphopeptide; Src Homology 2; Solid-phase

Abbreviations: ABTS, 2,2′-Azino-di-[3-ethylbenzthiazoline sulfonate (6)]; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; GS4B, glutathione sepharose 4B; GST, glutathione S-transferase; LTP, long-term potentiation; SH, src homology; SA-POD, streptavidin-peroxidase; SDS-PAGE, sodium dodesyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

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1. Introduction

The Src family of protein tyrosine kinases plays an important role in regulating intracellular signaling events [1]. Mice studies of FynB, a 59 kDa Src-related nonreceptor tyrosine kinase present mostly in the brain [2–5], have proposed a particularly important role for Fyn kinase in learning and memory [6–9] and made FynB an attractive target for the development of therapeutics to improve learning and memory. FynB contains a highly conserved region termed SH2 (src-homology 2) shared in all protein tyrosine kinases of the src family [2–4,10,11]. The SH2 domain plays an essential role in the selective and specific inhibition of the kinase activity [10–12]. The modulation of enzyme activity by regulatory SH2 domain is thought to control the subsequent intracellular signaling events and represent a promising step for therapeutic intervention.

Several peptides have been identified as potential ligands for the SH2 domains of Src kinase family [13–17]. However, it is not shown whether these peptides targeting Src SH2 or Grb SH2 could be also effective to the FynB SH2 domain. Furthermore, they inhibit tyrosine kinases although a phosphopeptide ligand is known to stimulate kinase activity [18] because the therapeutic goal was the development of anticancer agents by inhibiting Src activity. In addition, these natural peptides have inherent limits to being developed as pharmaceutical drugs since they can be easily degraded after oral digestion. Therefore, there are increasing demands for the nonpeptide SH2 ligands specific for FynB, resulting in the stimulation of FynB tyrosine kinase activity.

The commercial availability of huge, defined collections of chemical libraries now provide an unlimited source for drug discovery and development [19]. Identification of therapeutic compounds, however, is currently hampered by the lack of proper assay systems to permit rapid high-throughput screening of large number of chemicals. In this study, we describe the development of a solid-phase ligand binding assay that enables rapid, high-throughput screening of chemical libraries to identify ligands for the FynB SH2 domain. In an initial screening of a 1132-compound chemical library, we identified synthetic compounds, which bind to the FynB SH2 domain as shown in the competitive inhibition of the binding of the natural ligand, a phosphopeptide GGSETDDY*AEIID, where Y* is phosphotyrosine. The experimental results prove that our solid-phase ligand binding system makes possible a new high-throughput screening approach for finding ligands.

2. Materials and methods

2.1. Expression and purification of the GST-SH2 recombinant proteins

The pGEM-SH2 recombinant plasmids were kind gifts from Dr A. Veillette at McGill University, Canada. The pGEX-2T bacterial expression system (Pharmacia Biotech Inc., Peapack, NJ) was used to express and purify human FynB SH2 domain as a glutathione S-transferase (GST) fusion protein. Plasmid pGST-SH2 was constructed by inserting the FynB-SH2 fragment of pGEM-SH2 into the *Bam*HI and *Eco*RI restriction site of pGEX-2T and introduced into *Escherichia coli* JM109. Restriction enzyme digestion, ligation, and bacterial transformation were carried out as described [20]. The expressed GST-SH2 fusion proteins were purified on a glutathione sepharose 4B (GS4B) column (Pharmacia Biotech Inc.) according to the procedures provided by the supplier.

2.1.1. A solid-phase ligand binding assay

The GST-SH2 fusion protein was diluted to 40 μg/ml in Tris-buffered saline, TBS (150 mM NaCl, 20 mM Tris-HCl; pH7.5) containing 1 mM Na_3VO_4, and a 50 μl aliquot was placed in each well of 96-well Nunc Maxisorp microtiterplates. After 1 h at room temperature, the plates were washed three times with TBS containing 0.2% Tween-20. After addition of 300 μl of blocking buffer (3% wt./vol. of bovine serum albumin and 1 mM Na_3VO_4 in TBS), the plates were incubated at 4 °C overnight and washed three times.

The phosphopeptide sequence (GGSETDDY*AEIID, Y* is phosphorylated tyrosine) in

focal adhesion kinase is known to bind to FynB SH2. The synthetic phosphopeptides, either non-biotinylated or functionalized with biotinylation (B-GGSETDDY*AEIID, B is biotin) were synthesized by Korea Basic Science Institute (Seoul, South Korea). Peptides were dissolved in dimethyl sulfoxide (DMSO) to 1 mg/ml and further diluted to 100 nM in the blocking buffer containing 5 mM dithiothreitol (DTT). Fifty microliters of the peptide solution, containing varying amount of the biotinylated phosphopeptides as indicated, were added to each well of GST-SH2-coated plates. After 1 h incubation with agitation, the plates were washed three times with TBS and subjected to streptavidin-peroxidase (SA-POD) conjugate (Boehringer Mannheim, Indianapolis, IN). To measure the peroxidase-driven indicator reaction, ABTS (2,2′-Azino-di-[3-ethylbenzthiazoline sulfonate (6)]), a peroxidase substrate was used and the color development at 405 nm with the reference of 490 nm was recorded.

2.1.2. Identification of candidate ligands to FynB SH2

A portion of the CNS-Set chemical library, containing 1100 small molecules (ChemBridge, San Diego, CA) was screened for their competitive binding to the SH2 domain using the solid-phase ligand binding assay. Additional 32-small molecules possessed in our laboratory were also screened for the binding. Compounds were dissolved directly in DMSO to 1 mg/ml and further diluted to 200 μM with a blocking buffer containing 5 mM DTT. Twenty-five μl of 200 nM biotinylated phosphopeptide solution and same volume of the compound solutions (200 μM) were added to each well of microtiterplates. After 1 h incubation, SA-POD and ABTS reaction system was applied and color development was measured after 20 min.

3. Results

3.1. Expression and purification of pGST-SH2 clones as GST-SH2 fusion proteins

pGST-SH2 recombinant clones were screened for the expression level by 1-chloro-2,4-dinitrobenzene (CDNB) analysis (Pharmacia) and the clones with the highest expression level were induced to express GST-SH2 fusion protein. The final yield of GST-SH2 was 10-15 mg/5 l cultures. SDS-PAGE was carried out to verify the correct size and quality of the purified fusion protein (data not shown here).

3.2. Development of a solid-phase ligand binding assay for SH2 domain

In order to bypass the drawbacks of the cell-based or immunoprecipitation assays and to generate quantitative data for SH2 binding by various molecules, this study established a quantitative, high-throughput, solid-phase ligand-binding assay for SH2 ligands. The assay employs a biotinylated SH2-binding phosphopeptide, which can be subsequently detected with SA-POD conjugate (Fig. 1). When the biotinylated phosphopeptide is added to the microtiterplate precoated with GST-SH2, phosphopeptides bind to the immobilized SH2. Then the biotin of the SH2-bound phosphopeptide binds to SA-POD, allowing the formation of the color product from ABTS and this can be detected at 405 nm (Fig. 1(A)). Whenever any of these components of the binding system is missing, A_{405} will be the base level; as in the case of non-biotinylated phosphopeptides. In the screening for FynB SH2 ligands, the addition of compounds to this reaction system can lower the reading when the compounds compete for the same binding site with the biotinylated phosphopeptide and thus reduce the binding of the biotinylated phosphopeptide to SH2 (Fig. 1(B)). A compound, which have strong affinity to the SH2 domain and thus compete for FynB SH2 with the biotinylated phosphopeptide, will be identified as a FynB SH2 ligand.

To determine the assay condition, peptide binding was measured with the biotinylated phosphopeptide and non-biotinylated phosphopeptide (Fig. 2). When there are only non-biotinylated phosphopeptides in the reaction, the absorbance reading was maintained at the minimal level. Addition of the biotinylated phosphopeptide increased A_{405} although the increment was saturating when the amount of the biotinylated phosphopeptide was > 12.5 pmole. From this data, we

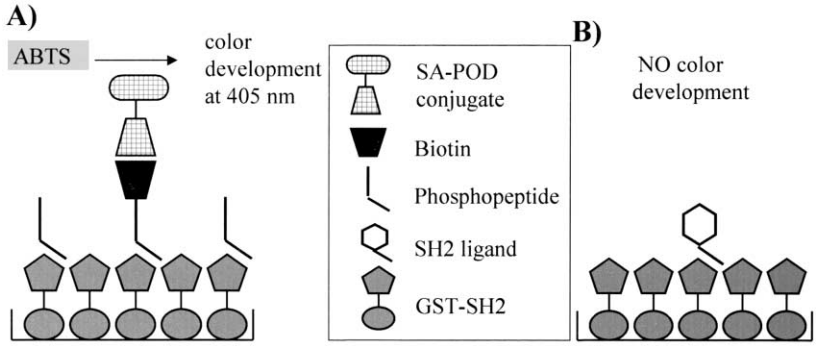

Fig. 1. Schematic diagram of the principle of a solid-phase ligand binding assay. The solid-phase ligand binding assay was composed of the GST-SH2 domain immobilized on a microtiterplate, a biotinylated phosphopeptide, and SA-POD conjugate that allows detection by ABTS reaction. (A) In the absence of competing ligands, the biotinylated phosphopeptide binds to immobilized SH2 domain. The binding of SA-POD to the biotin of the phosphopeptide immobilize SA-POD on the microtiterplate. This binding can be detected by the enzymatic reaction of ABTS that develops a blue-green product, ABTS radical ($ABTS^{\cdot}+$), when reacted with POD. (B) In the presence of the SH2-binding ligand, this compound will occupy the SH2 domain, inhibiting the binding of the biotinylated phosphopeptide and therefore lower the reading.

chose to use 5 pmole/50 μl (100 nM) as the concentration of the biotinylated phosphopeptide for screening of a chemical library because it appeared to be enough to make a specific and inhibitable peptide binding. When the biotinylated phosphopeptide and non-biotinylated phosphopeptide were added together, the A_{405} is dependent on the relative ratio between the biotinylated and non-biotinylated peptides because both peptides have same binding affinity to the SH2 domain. The competitive binding inhibition of the non-biotinylated phosphopeptides imply that the binding assay is specific to peptide ligand and able to screen possible ligands for the FynB SH2 domain. The relative intensity of the developed color is reversibly proportional to the binding of the small molecules to the SH2 domain competing with the phosphopeptide added.

3.3. Screening of a chemical library for FynB SH2 ligand by solid-phase binding assay

A chemical library, consisting of 1132 compounds, was screened by the developed solid-phase ligand binding assay. The absorbance from the reaction containing the non-biotinylated peptide was the basal level and A_{405} from the reaction containing only the biotinylated phosphopeptide served as the possible maximum control (Fig. 3(A)). When both biotinylated and non-biotiny-

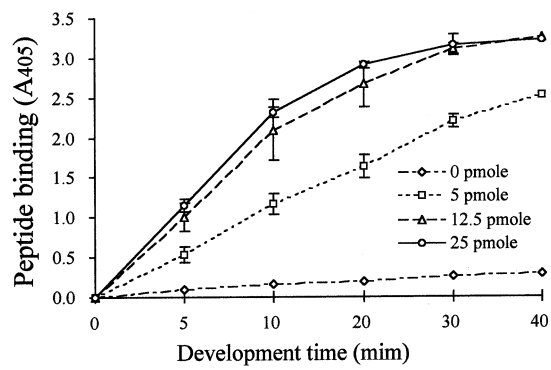

Fig. 2. The competitive binding of the biotinylated phosphopeptide to the SH2 domain of the immobilized GST-SH2 fusion protein on a microtiterplate. The purified GST-SH2 fusion protein was immobilized on 96-well plates. Competitive binding of the biotinylated peptides to the immobilized SH2 domain was assessed by measuring the color development of the POD and ABTS enzymatic reaction. The X-axis represents the color development time (min) and the Y-axis represents the peptide binding indicated as absorbance at 405 nm. Biotinylated peptide solution was added with increasing amount gradually from 0, 5, 12.5–50 pmole (inset) while non-biotinylated peptide was reduced concomitantly from 50 to 0 pmole. Each data point represents the results of triplicate samples ± the standard deviation.

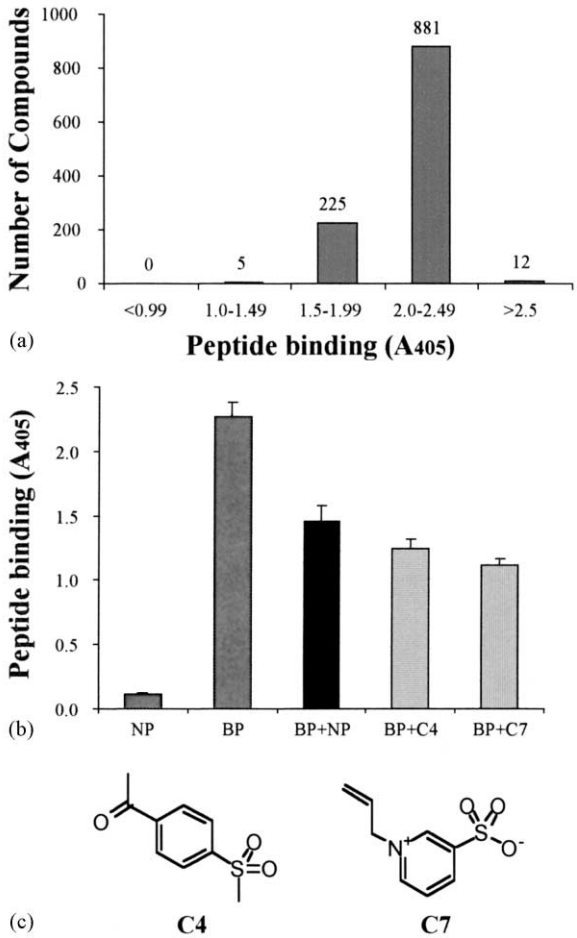

Fig. 3. The solid-phase ligand-binding assay for SH2 ligands. (A) The distribution of compounds in accordance to the absorbance at 405 nm. The library, consisting of 1123 compounds, was screened by their inhibition of the binding of the peptides to FynB SH2. (B) Ligand-binding assay of compounds to FynB-SH2. The X-axis represents controls and experimental groups; NP, 100 nM non-biotinylated peptide; BP, 200 nM biotinylated peptide; BP + NP, 100 nM biotinylated and non-biotinylated peptide; BP + C4, 100 nM biotinylated peptide and 100 μM 4-acetamidobenzene sulfinic acid; BP + C7, 100 nM biotinylated peptide and 100 μM allylpyridinium-3-sulfonate. The Y-axis represents the peptide binding indicated as absorbance at 405 nm. Each data represents the results of triplicate samples ± the standard deviation. (C) The molecular structures of 4-acetamidobenzene sulfinic acid (C4) and 1-allylpyridinium-3-sulfonate pyridinium-3-sulfonate (C7).

lated peptides present in the reaction, A_{405} was reduced due to the competitive inhibition of the non-biotinylated peptide. The reading from the reaction containing chemical was compared to this value to find potential ligands for SH2. Most chemicals showed the A_{405} between 1.5 and 2.49, while five compounds were associated with the absorbance value lower than 1.5, indicating strong binding to SH2 (see Fig. 3(B)). Particularly, two hit compounds, 4-acetamidobenzene sulfinic acid and 1-allylpyridinium 3-sulfonate, were able to inhibit the peptide binding to the level of the reaction containing the same amount of the non-biotinylated phosphopeptide (Fig. 3(A)). Interestingly, both hit compounds have the same molecular formula, $C_8H_9NO_3S$, and the structural similarity is clear as illustrated (Fig. 3(C)).

4. Discussion

High-throughput screening is an important tool for discovering new drugs that target various biomolecular sites [21]. Application of a quantitative, high-throughput, solid-phase ligand binding assay allows fast identification of lead compounds compared to the traditional time-consuming and tedious cell-based or immunoprecipitation assays. In this work, a method for the identification of high-affinity non-peptide ligands for FynB SH2 by a solid-phase binding assay was established. The assay involves immobilization of GST-SH2 protein onto the wells of microtiterplates, addition of the biotinylated phosphopeptide (a natural ligand for SH2) and non-peptide small molecules, and detection by SA-POD conjugates. The competitive binding between the biotinylated phosphopeptide and compounds to FynB SH2 was monitored colorimetrically and the measured absorbance is proportional to the binding of biotinylated phosphopeptides to SH2 domain. Binding was examined in the presence of various compounds in an attempt to identify non-peptide ligands for FynB SH2. An initial screening of a modest-sized chemical library of 1132 molecules resulted in several hits, demonstrating the feasibility of using this assay to perform a high-throughput screening. 4-acetamidobenzene sulfinic acid and 1-allylpyridinium 3-sulfonate were identified by their strong binding affinity to the FynB SH2

domain. Further studies on the molecular structures of the identified SH2-binding ligands will allow presentation of specific models for ligand-domain complexes for improving the ligands by chemical modification.

A physiological role for FynB in brain function was first suggested by the analysis of *fyn*-deficient mice, exhibiting an impaired long-term potentiation (LTP) and learning, suggesting Fyn signaling contributes to neuronal synaptic plasticity [6]. The understanding of the requirement of FynB in LTP proposed the possibility to consider FynB as a potential drug target. A compound that specifically binds to FynB SH2 and promotes the activity of FynB kinase should have a role in the induction and maintenance of LTP. Two compounds, identified in this work, could be tested for their presumed ability to free the kinase domain and thus activate FynB tyrosine kinase. The identified SH2 ligands could be tested to answer whether they have a potential role in the treatment of defects in learning and memory by improving LTP.

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