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A Screening Method for Src Homology 3 Domain Binding Blockers Based on Ras Signaling Pathway

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Abstract : Grb2, which is composed of a Src homology 2 (SH2) domain and two Src homology 3 (SH3) domains, is known to serve as an adaptor protein in signaling for Ras activation. Thus, a blocker of the Grb2 interactions with other proteins can be a potential candidate for an anticancer drug. In this study, we have developed a high throughput screening method for SH3 domain binding ligands and blockers. Firstly, we made and purified the glutathione S-transferase (GST)-fusion proteins with the Grb2 SH2 and SH3 domains, and the entire Grb2. This method measures the binding of a biotin-labeled oligopeptide, derived from a Grb2/SH3 binding motif in the hSos, to the GST-fusion proteins, which are precoated as glutathione S-transferase fusion protein on a solid phase. When 1 μg of each fusion protein was used to coat the wells, both N- and C-terminal SH3 the domains as well as the whole of Grb2 were able to interact with the biotin-conjugated ligand peptide, while the SH2 domain and GST alone showed no binding affinity. Although N- and C-terminal SH3 domains showed an increase of binding to the ligand peptide in proportion to the amount of peptide, the GST fusion protein with Grb2 demonstrated much higher binding affinity. GST-Grb2 coating on the solid phase showed a saturation curve; 66 and 84% of the maximal binding was observed at 100 and 300 ng/100 μl, respectively. This binding assay system was peptide sequence-specific, showing a dose-dependent inhibition with the unlabeled peptide of SH3 binding motif. Several other peptides, such as SH2 domain binding motifs and PTB domain binding motif, were ineffective to inhibit the binding to the biotin-conjugated ligand peptide. These results suggest that our method may be useful to screen for new anticancer drug candidates which can block the signaling pathways mediated by SH3 domain binding.
Key words : anticancer, Grb2, screening, Src homology 2

Grb2, a 25-kDa protein, is composed entirely of a Src homology 2 (SH2) domain flanked by two Src homology 3 (SH3) domains (Lowenstein, *et al.*, 1992). Since Grb2 does not have any catalytic domain, it is generally known to serve as an adapter protein in signaling from growth factor-activated tyrosine kinase receptors to Ras activation (Pawson and Gish, 1992; Lowy and Willumsen, 1993). While the SH2 domain of Grb2 binds to the carboxyl-terminal tail of activated receptor via interactions with a phosphotyrosine residue (Rozakis-Adcock, *et al.*, 1993; Songyang, *et al.*, 1993), the SH3 domains bind to proline-rich sequences in the carboxyl-terminal portion of hSos, the human homologue of Son of sevenless, which is a Ras guanine nucleotide-releasing factor (Li, *et al.*, 1993; Oliver, *et al.*, 1993). Thus Grb2 directly links receptor tyrosine kinases to the Ras signaling pathway. This recruitment of Grb2/Sos to the cell surface has been proposed to enhance the ability of Sos to activate Ras, which is associated with the plasma membrane, via its GTP/GDP exchange activity (Qulliam, *et al.*, 1994).

Ras activation induced by the close positioning of Sos, in turn, activates the kinase cascade including Raf, mitogen-activated protein kinase kinase, and mitogen activated protein kinase (Joneson, *et al.*, 1996; Khosravi-Far, *et al.*, 1996). Thus the Ras activation has been implicated in control of cell proliferation and oncogenesis. In a large number of human cancers, Ras is locked in its GTP-bound form due to mutations in amino acids 12, 13, or 61 (Gibbs, 1991). As a result, the Ras pathway

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no longer requires an upstream growth signal and the enzymes in this pathway are constitutively activated. These facts suggest the possibility that a blocker of Grb2 SH2 or SH3 domain binding can be a feasible candidate for an anticancer drug. Although there are many groups that have developed drug screening methods using the mechanism of protein and protein interaction including SH2 domain binding (Wood, *et al.*, 1992; Piccione, *et al.*, 1993; Sonatore, *et al.*, 1996), SH3 domain binding has been ignored. However, recent studies suggest that the SH3 domain as well as SH2 domain have profound roles in cell growth, differentiation, and oncogenic transformation (Baik, *et al.*, 1992; Bar-Sagi, *et al.*, 1993; Egan, *et al.*, 1993; Herskovits, *et al.*, 1993). Here, we report the development of a quantitative solid phase binding assay using purified glutathione S-transferase (GST)-Grb2 fusion protein and biotin-labeled ligand peptide whose sequence originated from the SH3 binding motif of Sos protein.

Materials and Methods

Bacterial expression and purification of GST fusion proteins

Sequences corresponding to the full length of Grb2 and its SH2 and SH3 domains (Lowenstein, *et al.*, 1992) were separately amplified by PCR and cloned into the expression vectors (Pharmacia, Uppsala, Sweden): *Eco*RI site of pGEX2TK (Grb2) and pGEX5T (SH2 domain) and BamH1 and EcoRI site of pGEX4T1 (SH3 domains). The GST fusion proteins of Grb2 (GST-Grb2), Grb2 SH2 domain (GST-Grb2/SH2), Grb2 N-terminal SH3 domain (GST-Grb2/SH3(N)), and Grb2 C-terminal SH3 domain (GST-Grb2/SH3(C)) expressed in *E. coli* DH5α were purified on glutathione-agarose beads (Pharmacia) as described previously (Smith and Johnson, 1988).

Peptide design and synthesis

The oligopeptide ligand was designed and synthesized to contain a Grb2 SH3 domain recognition motif (GTDEVPVPPPVPPRRRPESA) by Korea Basic Science Institute (Seoul, Korea). The sequences chosen were based on the sequence found in the human Sos (Rozaski-Adcock, *et al.*, 1993). For competitive inhibition studies, the oligopeptides corresponding to the Grb2/SH2 binding sequence in hShc (SpYVNVK) (Songyang, *et al.*, 1993), another SH2 binding sequence in the human platelet-derived growth factor receptor (PDGFR) (DpYIIPLPDPK) (Songyang, *et al.*, 1993), and a phosphotyrosine binding domain (PTB) binding motif in the human Neu (PTAENPEpYLGLK) (Kavanaugh, *et al.*, 1995) were used.

Biotinylation of oligopeptide

Peptide biotinylation was performed as described previously (Bayer and Wilchek, 1980). The oligopeptide containing an SH3 binding motif was dissolved in 1 mg/ml in sodium borate buffer (100 mM, pH 8.8). N-hydroxysuccinimide biotin (10 mg/ml in dimethylsulfoxide) (Sigma, St. Louis, USA) was added to the peptide solution at a ratio of 100 μg/mg of peptide and incubated at room temperature for 4 h. In order to remove the unused N-hydroxysuccinimide biotin in the reaction buffer, 10 μl of 1 M NH_4Cl per 100 μg N-hydroxysuccinimide biotin was added and incubated for 10 min at room temperature. The peptide solution was then dialyzed against phosphate-buffered saline (PBS) for 2 days.

SH3 domain binding assay

The principle of the binding assay for GST-Grb2 fusion protein and its ligand oligopeptide is illustrated in Fig. 1. Briefly, the fusion protein in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) was coated in a well of a 96-well ELISA plate (Nunc, Denmark) at 4°C for 16 h. After discarding the supernatant, the well was incubated with the blocking buffer, PBS containing 3% (w/v) BSA, at room temperature for 2 h. The incubation with the indicated amount of biotin-labeled peptide was performed following the blocking at 4°C for 8 h. The plate was then washed with PBS twice and incubated with horse radish peroxidase (HRP)-conjugated streptavidin (Sigma) at room temperature for 2 h. After the incubation, a 100 μl aliquot of substrate solution containing ortho-phenylenediamine and H_2O_2 was added into the well, and the absorbance at 492 nm was measured by ELISA reader (Titertek, Finland). For competitive binding analysis, the indicated concentrations of unlabeled of peptides were added into the well with the biotin-labeled ligand peptide.

Results and Discussion

To develop a method for SH3 domain binding assay, we made recombinant protein of SH2, SH3, or full domain of Grb2 as GST fusion proteins as designed in Fig.

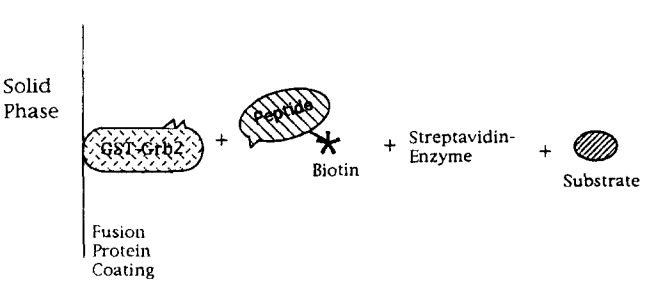

Fig. 1. Schematic diagram of the assay principle. The biotin-labeled phosphopeptide ligands bind to the SH3 domain of GST-Grb2 fusion proteins precoated on the solid phase.

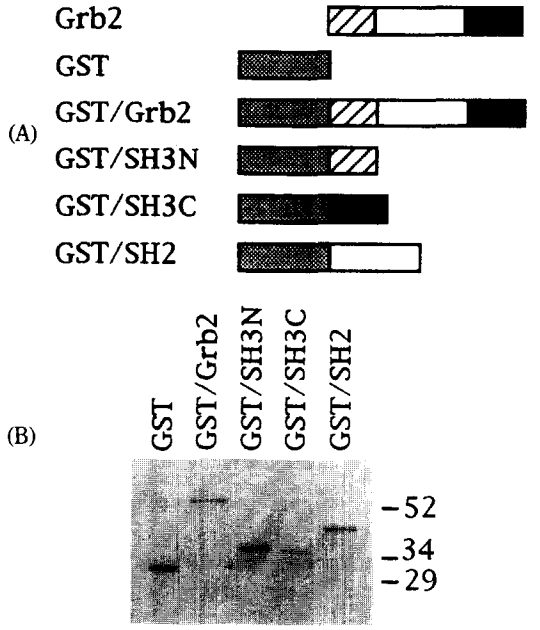

Fig. 2. Bacterial expression and purification of GST fusion proteins. (A) Schematic representation of the GST-fusion proteins. Sequences corresponding to the full length of Grb2 and its SH2 and SH3 domains were separately amplified by PCR and cloned into the expression vectors as described in Materials and Methods. (B) SDS-PAGE analysis of the fusion proteins. One μg of each protein GST, GST-Grb2, GST-Grb2/SH3(N), GST-Grb2/SH3(C), and GST-Grb2/SH2 after glutathione-agarose bead purification was applied to an SDS-PAGE and Coomassie blue staining.

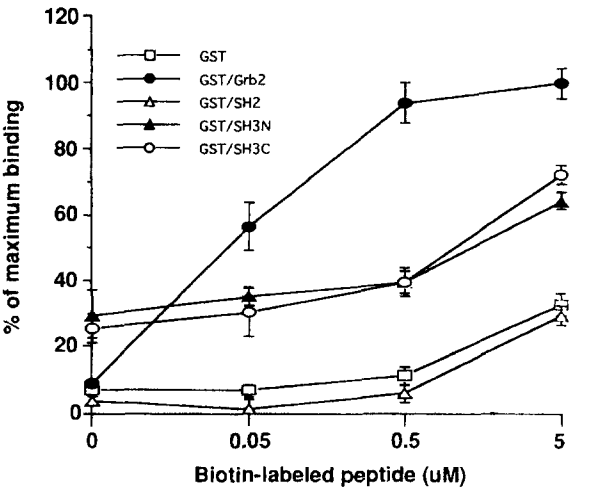

Fig. 3. Binding of biotin-labeled peptide to GST fusion proteins: Dependence on the concentration of ligand peptide. The wells of a 96-well plate were coated at 4 for 16 h with 1 μg of GST fusion proteins in coating buffer. After the well coating with the fusion proteins, and the incubations with 3% BSA, the indicated concentrations of biotin-labeled peptide, and HRP-conjugated streptavidin were followed as described in Materials and Methods. After the incubations, a 100 μl aliquot of substrate solution containing ortho-phenylenediamine and H_2O_2 was added into well, and the absorbance at 492 nm was measured by ELISA reader. Each point presented is the average± standard deviation of quadruplicate determinations.

2A. These GST fusion proteins were able to be purified with no significant protein contaminants (>95% purity) when applied on glutathione-agarose beads (Fig. 2B). The method measures the binding of a biotin-labeled oligopeptide derived from a Grb2/SH3 binding motif in the hSos to the GST-fusion proteins, which are precoated as GST fusion protein on the solid phase. When 1 μg of each fusion protein was used to coat the wells and various concentrations of biotin-labeled oligopeptide were incubated, three different binding patterns were observed (Fig. 3). First group includes GST and GST-Grb2/SH2; they showed no interaction with the biotin-labeled ligand peptide although low level of nonspecific binding was monitored with 5 μM of the ligand peptide. Second group is GST-Grb2/SH3; both GST-Grb2/SH3(N) and GST-Grb2/SH3(C) were able to interact with the biotin-conjugated ligand peptide in a dose-dependent manner. However, their binding affinities were relatively low when compared with the binding affinity of the GST-Grb2. Although N- and C-terminal SH3 domains showed an increase of binding to the ligand peptide in accordance to the amount of peptide, the GST-Grb2 demonstrated much higher binding affinity and also showed a saturation curve within the range of peptide concentration used in this study. These results suggest that Grb2 has not only two binding sites for SH3 motif but also has better three dimensional structure for the binding. Ligand peptide binding gradually increased up to 0.5 μM and appeared to reach to a plateau at 5 μM of ligand. This binding pattern of the fusion proteins clearly shows the GST-Grb2 is much bet- ter than GST-Grb2/SH3(N) and GST-Grb2/SH3(C) as the SH3 binding assay system.

Fig. 4 shows the binding curve of biotin-labeled peptide observed when a constant concentration (5 μM) of ligand peptide was incubated in the wells precoated with various concentrations (0~1000 ng/100 μl/well) of GST-Grb2. Since GST has a low level of nonspecific binding at high concentration of ligand peptide as shown in Fig. 3, a small portion of the GST-Grb2 binding capacity was from the GST part of the fusion protein. To get rid of this nonspecific binding, we performed the same binding assay with various concentrations of GST. Thus specific binding was determined from the binding values of GST-Grb2 subtracted by those obtained with GST alone. The specific binding curve in Fig. 4 basically represents the saturation of GST-Grb2 coating on the wall of the well. Sixty six and 84% binding of the maximum (the binding obtained with 1 μg/100 μl of GST-Grb2 for well coating) was monitored at 100 and

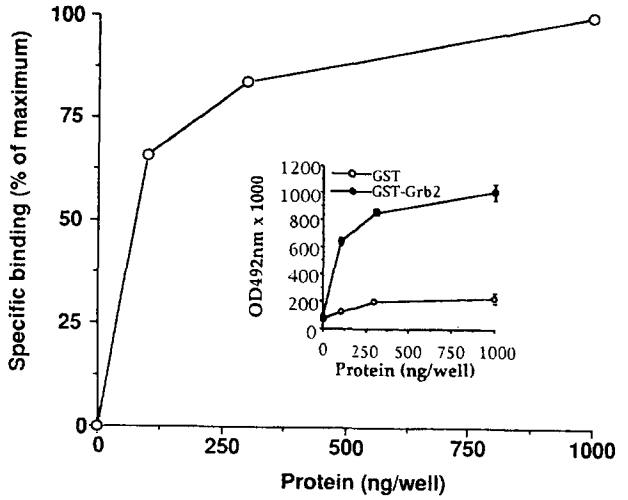

Fig. 4. Specific binding curve of biotin-labeled peptide to GST-Grb2 fusion protein. The wells of a 96-well plate were coated with various concentrations (0, 100, 300, and 1000 ng/100 ml/well) of GST or GST-Grb2 at 4°C for 16 h. The supernatant was aspirated, and the wells were incubated with 3% BSA, 5 μM of biotin-labeled peptide, and HRP-conjugated streptavidin. Color development was performed and measured as described in Materials and Methods. Specific binding represents the total binding (to GST-Grb2) subtracted with the nonspecific binding (to GST alone). Each point represents the average binding±SD of quadruplicate determinations.

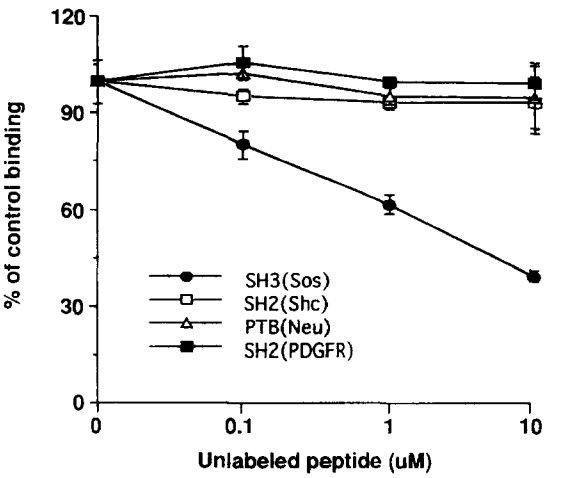

Fig. 5. Competitive inhibition of biotin-labeled ligand peptide binding. The wells of precoated with 300 ng/100 μl/well were incubated with biotin-labeled ligand peptide of 5 μM at 4°C for 8 h. One of the 4 different unlabeled peptides was added to the incubation buffer to determine whether it could displace the binding of biotin-labeled ligand peptide. Each point shows the average (the converted value as the percent of control binding)±SD of quadruplicate determinants.

300 ng/100 μl, respectively.

The time length for the well coating and ligand incubation was 18 and 8 hrs at 4°C, respectively, which has been determined in our previous work (Koh, *et al.*, 1997). In order to determine whether our binding assay system is specific and inhibitable by specific oligopeptides, competitive binding experiments were performed with four different kinds of unlabeled oligopeptides containing an SH3 binding motif found in Sos, SH2 binding motifs in Shc or PDGFR, or a phosphotyrosine binding motif in Neu, respectively. As shown in Fig. 5, the binding assay system was peptide sequence-specific since a dose-dependent inhibition of biotin-labeled peptide binding was observed only by the addition of unlabeled proline-rich oligopeptide (SH3 domain binding motif). The percent inhibition in 0.1, 1, and 10 μM of unlabeled peptide was 19, 36, and 58 of control, respectively. The other peptides, such as SH2 domain binding motifs and PTB domain binding motif, even at 10 μM were ineffective in inhibiting the binding to the biotin-conjugated ligand peptide. Since we used 5 μM of biotin-labeled ligand peptide, 58% inhibition at 10 μM was quite consistent with the result of simple calculation (66% inhibition). If we consider this peptide as an ideal ligand with the highest binding affinity, the 5 μM concentration is likely to be an extremely high concentration because even a blocker with the same affinity would have an IC_{50} of 5 μM. However, considering its role as a screening system, the adoption of such a high ligand concentration can exclude a possibility to screen out putative or weak binding blockers. In fact there have been few or no cases of development of anticancer drug screening method using the SH3 domain binding. It is probably because the precise role of protein-protein interaction mediated by SH3 binding is not clearly defined yet. However, the more studies on the role of the SH3 domain interaction proceed, the more attention is paid on its possibility as a target for anticancer drugs (Prendergast and Gibbs, 1994; Smithgall, 1995; Sastry, *et al.*, 1997). In other words, an antagonist screened for the SH3 domain of Grb2 may dissociate Sos from the Grb2 and activated receptor complex and block the signaling for Ras activation.

Our new method of SH3 binding assay does not require a filtration step and is simply conducted in the wells of the plate. Thus, even though there are lots of samples to be tested, we can keep the experimental space very small. In addition, this assay system was considerably resistant to the existence of organic solvents, such as methanol and dimethylsulfoxide in the binding buffer up to 5% (v/v) (data not shown). Thus, it appears to be useful for the screening of SH3 binding ligands and blockers from small hydrophobic compounds or natural compound libraries. This screening system is also very useful as a counter screening system of SH2 binding assay because a compound of SH2 binding blocker may need to be checked regarding its effect on the

SH3 binding. These results taken together suggest that our method may be useful to screen out new candidates of anticancer drug which can block or attenuate the signaling pathways mediated by SH3 domain binding.

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