APPLICATION NOTE

SPA Technology

The Application of SPA to SH2 and SH3 Domain Binding to Specific Peptide Sequences

Introduction

SH2 and SH3 domains are small, independent domains of about 100 or 70 amino acid residues respectively. They are found in a variety of proteins, and can occur together or separately. SH2 domains are thought to be involved in signal transduction mechanisms[1]. Some SH2 domains control enzyme activity by binding membrane-bound receptors to regulate downstream events such as kinase cascades. However, recently transcription factors (for example Signal Transducers and Activators of Transcription (STAT)) have been shown to contain SH2 domains[2]. SH3 domains are also thought to be a feature of signal transduction mechanisms, or to have a role in cytoskeletal organization[3].

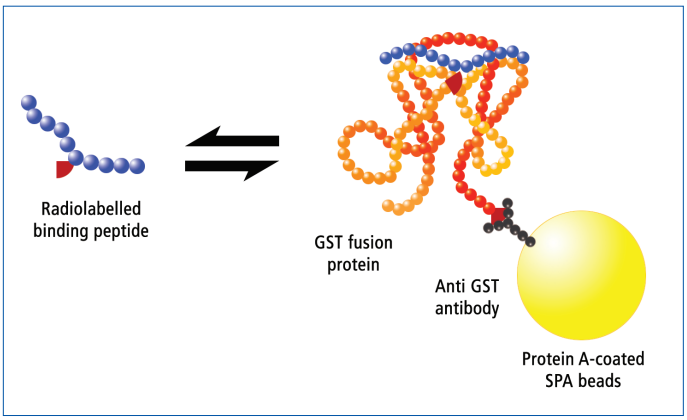

Figure 1. Diagrammatic representation of the antibody capture concept for detecting SH2 domain binding to peptides.

SH2 domains have been well characterized by *in vitro* mutagenesis studies, and are known to specifically bind phosphotyrosine residues with affinities in the low nanomolar range. Examples of proteins containing SH2 domains include enzymes (GTPase Activating Protein (GAP), Src), and non-enzymatic adapters (Growth Factor Receptor Binding-2 (GRB2), the p85 domain of phosphatidylinositol 3-kinase). SH3 domains bind proline rich sequences with a much lower affinity. Recently, the use of peptides as opposed to intact proteins for binding studies has received significant attention. Examples of proteins containing SH3 domains also include enzymes (Abl, GAP) and adapters (Crk and p85). Non-hydrolyzable peptide mimetics might represent an attractive therapeutic target.

SPA has successfully been used to quantitate SH2 and SH3 domain binding to radiolabelled peptides and, more recently, to detect binding between intact proteins.

Assay Concepts

All peptides used in these studies were synthesized using standard Fmoc chemistry, and then radiolabelled using [^{125}I] Bolton-Hunter reagent, or by catalytic reduction under tritium gas. Subsequently, peptides were purified by reverse phase HPLC.

Antibody Capture

The radiolabelled binding peptide was incubated with glutathione S-transferase (GST) SH2 domain, anti-GST antibody and protein A coated SPA beads (RPNQ0019, RPN143) in a suitable buffer (Figure 1).

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Streptavidin-biotin

The radiolabelled binding peptide was incubated with biotinylated GST SH2 domain, and streptavidin-coated SPA beads (RPNQ0007, RPNQ0012) in a suitable buffer (Figure 2).

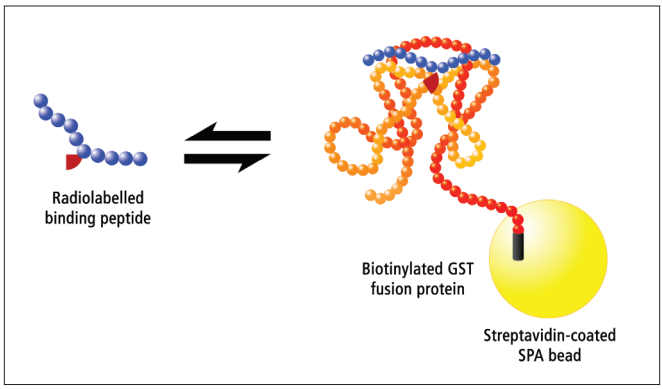

Figure 2. Diagrammatic representation of the streptavidin-biotin concept for detecting SH2 or SH3 domain binding to peptides.

Use of the antibody capture system with reference to Src (SH2-SH3)

The peptide 107P (sequence EPQpYEEIPIYL) derived from the hamster middle T antigen (hmT) and containing pY324 was used for these studies. The procedure for maximizing the signal involved titrating the amount of SH2 domain at a constant amount of tracer (Figure 3), and then adjusting the antibody concentration at a constant amount of tracer (Figure 4).

The signal to noise ratio for the antibody capture system can differ from the biotin-streptavidin system. This format has the advantage that the capture system should not directly interfere with binding to the SH2 domain. However, it is important to check that inhibitors identified by this method are inhibitors of the protein:peptide interaction, and not of the antibody:protein interaction.

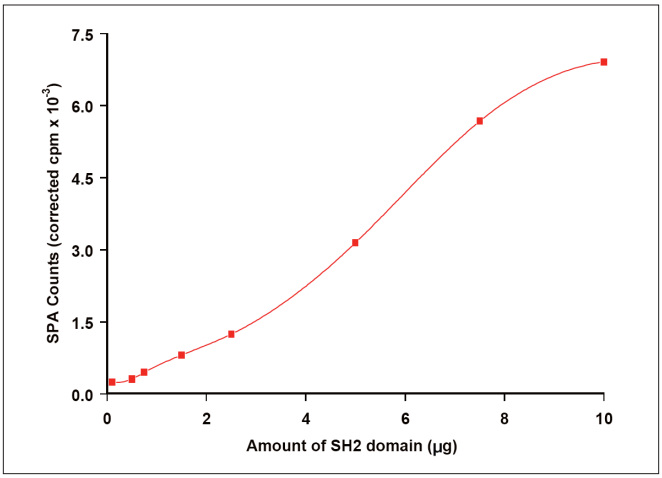

Figure 3. Titration of the Src SH2 domain at a constant amount of tracer and antibody.

Use of the biotin-streptavidin system: biotinylation of a fusion protein using GRB2 as a model system

Biotinylation of GST.GRB2 was tested at ratios of 6:1 to 75:1 molar excess of NHS-LC-biotin ester (Pierce) to GST.GRB2 in 200 mM borate buffer, pH 8.5, containing 1% Triton X-100. The reactions were roller-mixed at room temperature for 1 hour and free biotin was removed using pre-equilibrated NAP-5 columns. Biotinylation did not reduce the binding affinity of GST.GRB2 for the radiolabelled peptide. This was confirmed using an anti-GST antibody capture SPA format.

The biotin GST.GRB2 produced at the various ratios of protein to biotin ester were tested in the biotin-streptavidin SPA assay format. This involved incubating radiolabelled peptide with SPA beads and biotin GST.GRB2 fusion protein in a suitable assay buffer. On the basis of the highest counts achieved the optimum ratio of biotin ester to protein was found to be 10:1 in this case (Figure 5).

Figure 6 shows the effect of varying the amount of bead from 0-5 mg. It can be seen that the signal increased with the bead concentration, however the highest signal to noise ratio was at 1 mg of beads.

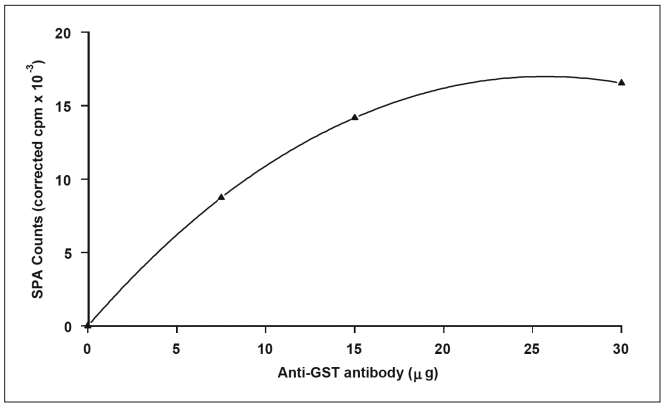

Figure 4. Titration of the anti-GST antibody at constant amounts of SH2 domain, and radiolabelled peptide.

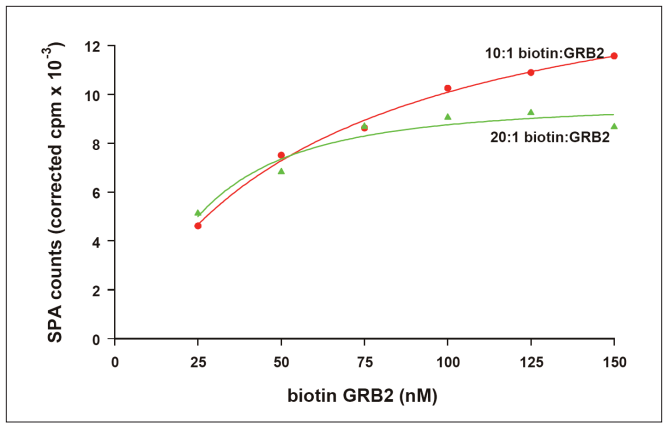

Figure 5: Titration of biotin GRB2 SH2 domain produced at 10:1 or 20:1 molar excess of biotin:protein.

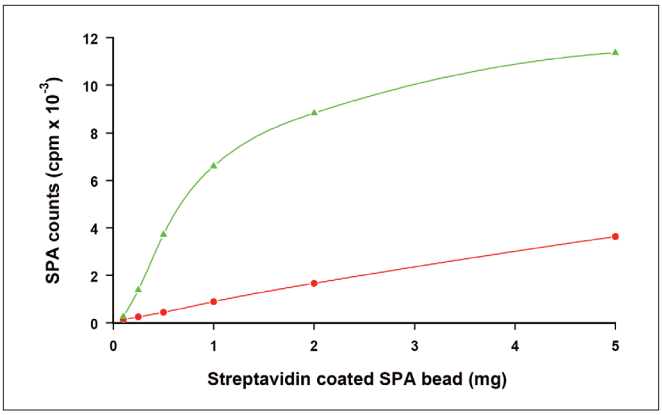

Figure 6. The effect of varied bead concentration on signal (B0) and nonspecific binding (NSB).

The effect of peptide length on binding with reference to p85 (SH2-SH2-SH2-SH3-Breakpoint Cluster Region)

The peptide consisting of residues 604-635 of Insulin Receptor Substrate-1 (IRS-1) binds the SH2 domain of the p85 protein. The sequence contains two phosphotyrosine residues, and various N or C terminal truncations were synthesized (see Table 1) to identify the critical binding regions. The short, C terminal peptide (121P) was extended at the N terminus in blocks of 4 residues and the effect on its binding affinity was tested by displacement of [^{125}I]121P. The results showed that as the peptide was extended to the N terminus, the affinity for the p85 SH2 domain increased significantly for the first extension, but subsequent extensions had little effect on the affinity.

An investigation was then carried out into the relative affinities of the N and C terminal phosphotyrosines for the SH2 domain. The ability of various peptides to compete with [^{125}I] ppY604-635 for binding to the p85 SH2 domain was tested. The results showed that the N terminal pY bound the SH2 domain with a slightly higher affinity than the C terminal pY. In addition, a preliminary experiment indicated that a mixture of the N and C terminal pY sequences did not compete with the radiolabel as efficiently as the intact sequence. This indicated that the sequence between the phosphotyrosines is important for binding, either directly, or by providing a conformational restraint.

The effect of temperature on binding with reference to GAP (SH2-SH3-SH2)

Three peptides sequences derived from the Platelet Derived Growth Factor receptor (PDGFr) (Table 2) were synthesized and tested for binding to the GAP SH2 domain.

An 11 residue sequence, a 21 residue extended sequence, and a cyclic version of the extended sequence were tested. Assays were set up and counted in a PerkinElmer TopCount® microtitre plate counter over a range of preset temperatures. The results (Figure 7) showed that the shorter sequence was more susceptible to temperature changes than the longer sequence, which in turn was more susceptible than the cyclic sequence. These results could be explained by progressively less conformational flexibility as the peptide was extended and then cyclized causing the peptide to take on the binding conformation. Temperature effects have been observed in other systems, however, the signal was always found to remain constant at any one temperature.

Table 1. Peptide sequences used for binding to the p85 SH2 domain.

	CODE
pYMPMSPKS	121P
GNGDpYMPMSPKS	122P
SNRKGNGDpYMPMSPKS	123P
ADVPSNRKGNGDpYMPMSPKS	124P
SPGVADVPSNRKGNGDpYMPMSPKS	125P
TDDGpYMPMSPGV	128P
TDDGpYMPMSPGVADVPSNRKGNGDpYMPMSPKS	127PP

Table 2. Peptide sequences used for testing binding to the GAP SH2 domain.

	CODE
SSNpYMAPYDNY	98P
GDIKYESSNpYMAPYDNYVPSA	117P
GDIKYESSNpYMAPYDNYVPSA	117P (cyclic)

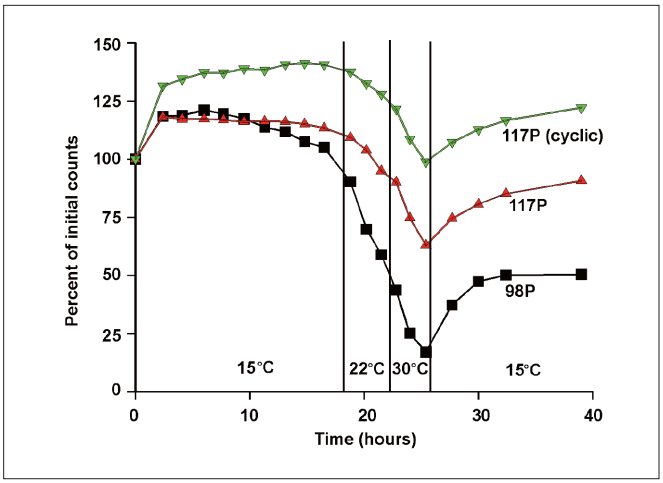

Figure 7. The effect of temperature on peptide binding to the GAP SH2 domain.

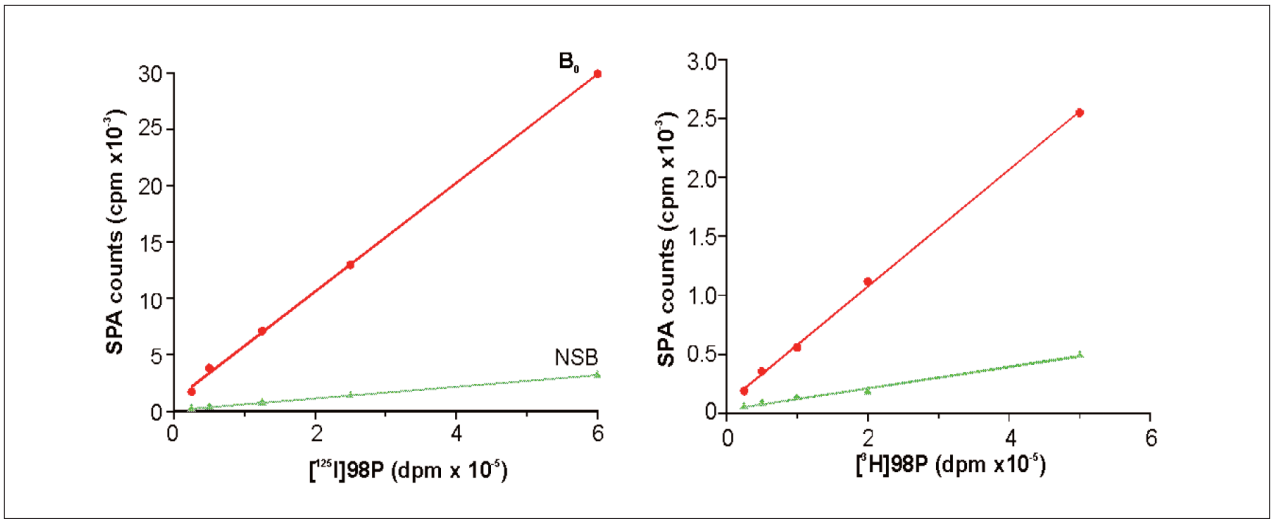

Figure 8. Choice of radiolabel for peptide binding to the GAP SH2 domain.

Choice of radiolabel with reference to GAP (SH2-SH3-SH2)

Figure 8 shows the effect of signal and background obtained for [^{3}H] or [^{125}I] labelled peptide. It can be seen that the signal and background are significantly lower for [^{3}H] than for [^{125}I], the choice of label will be determined by a combination of signal, and signal to noise ratio required, an acceptable half life, and whether there is any steric hindrance.

Low affinity SH3 domain binding with reference to the Crk/Ab1 interaction

The biotin-streptavidin system was selected for this assay as the reagents were supplied pre-biotinylated. This assay involved incubating biotinylated Crk SH3 domain with radiolabelled binding peptides derived from the Abl tyrosine kinase (Table 3). Because of the extremely low affinity of this interaction (micromolar), a relatively large amount of radiolabelled peptide had to be added to produce an acceptable signal. [^{3}H] and [^{125}I] labelled peptides were compared, and it was found that only the [^{3}H] labelled peptide produced a signal. Results suggested that the reason for this might be steric hindrance by the [^{125}I] atom.

Table 3. Peptide sequences used for testing binding to the Crk SH3 domain.

SOURCE	SEQUENCE	CODE	BINDS TO
Abl-1	QAPELPTKTRT	108	Crk SH3
Abl-2	SEPAVSPLLPRKER	109	Crk SH3
Btk-1	TKKPLPPTPEED	110	Hck SH3

Specificity was demonstrated by the Btk-1 peptide, known to bind the Hck SH3 domain, not binding the Crk SH3 domain. Figure 9 shows competition between unlabelled peptides and peptide [^{3}H]108.

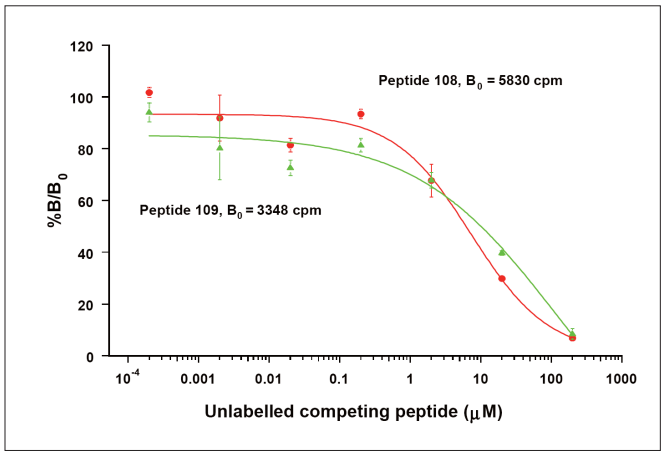

Figure 9. Unlabelled peptides competing with peptide [3H]108 for binding to the Crk SH3 domain.

The assay was found to exhibit a severe temperature dependency, which meant that the assay had to be incubated at 4 °C, and counted before the plate reached room temperature in order to produce a signal to noise ratio of 6:1. When the plate reached room temperature, the signal decreased by 50%. Despite this effect, SPA has enabled quantification of binding events with micromolar affinities. In addition, recent work has focused on protein:protein SH3 interactions, and initial results suggest that these have higher affinities than the equivalent protein:peptide interaction.

Typical assay conditions for SH2 and SH3 binding

- assay buffer
- biotinylated SH2/SH3 domain
- 0.1-1 µCi radiolabelled peptide
- 0.5-1 mg streptavidin-coated SPA beads

OR

- assay buffer
- SH2/SH3 domain
- 0.1-1 µCi radiolabelled peptide
- 0.5-1 mg protein A-coated SPA beads
- anti-GST antibody

Shake for 30 minutes at room temperature, count.

(Assay buffer: 20 mM MOPS, 10 mM $MgCl_2$, 10mM DTT, 0.1% (w/v) BSA, pH 7.4)

Conclusion

The assay concept of choice was found to be the biotin/streptavidin system, although the antibody capture system might be more suitable if biotinylation is known to affect binding of the protein to the peptide. A temperature dependency effect has been observed in some cases, so it is recommended that counting is carried out in a temperature controlled counter. The assay conditions have been found to be consistent with only minor alterations required between the systems tested to date. SPA has therefore been shown to be suitable for measuring SH2 and SH3 interactions with specific peptide sequences in the high, intermediate and low affinity range.

References

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940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

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008958_01 Printed in USA Oct. 2010