

A SCINTILLATION PROXIMITY ASSAY (SPA) TO QUANTITATE Src HOMOLOGY 2 (SH2) AND Src HOMOLOGY 3 (SH3) DOMAIN BINDING TO SPECIFIC PEPTIDE SEQUENCES

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Introduction

Scintillation proximity assays have been developed to study the binding between 1. the SH2 domain of c-Src and a specific phosphotyrosine-containing peptide, and 2. the SH3 domain of Crk and a specific proline-containing peptide.

1. c-Src

c-Src is a non-receptor tyrosine kinase involved in signal transduction pathways. The SH2 domain of c-Src binds to phosphotyrosine-containing peptides with high affinity^{13,2}, but dephosphorylation leads to dissociation. Peptides were synthesized using standard Fmoc chemistry (see Table 1) and radiolabelled with Bolton and Hunter reagent. Purification was by reverse phase HPLC. The assay concept was as shown in Figure 1.

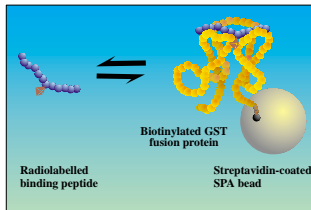


Figure 1. A diagrammatic representation of the assay concept used for Src Homology domain binding to radiolabelled peptides.

GST c-Src was biotinylated with NHS-LC biotin, and immobilised on streptavidin-coated SPA beads, in a suitable assay buffer.

Source	Code	Sequence	Binds to
hmT	pY324	EPQpYEEIPIYL	c-Src SH2
c-Src	pY527	EPQpYQPGENL	c-Src SH2
PDGFr	pY771	SSNpYMAPYDNY	GAP SH2
Abl-1	108	QAPELPTKTRT	Crk SH3
Abl-2	109	SEPAVSPLLPRKER	Crk SH3

Table 1. Peptide sequences used for binding the c-Src SH2 domain or the Crk SH3 domain.

Competition curves for c-Src

The specificity of peptide binding was demonstrated by poor displacement of peptide [¹²⁵I]pY324 by peptide pY771 which is

derived from the PDGF receptor and is reported to specifically bind to the GAP SH2 domain. Specificity of the interaction was further demonstrated by the significantly lower affinity of the Y324 peptide for biotinylated GST c-Src SH2 domain. Phosphotyrosine, when added in excess, also had a low affinity for the biotinylated GST c-Src SH2 domain under identical experimental conditions. The signal (B_s) has been shown to be stable with time, and highly specific, with a low background non-specific binding (NSB) of labelled peptide to bead.

The range of affinities which can be detected using this SPA assay format was investigated further. A second peptide based on the pY527 sequence at the carboxyl terminus of c-Src, is known to bind the c-Src SH2 domain with 104-fold lower affinity than the pY324 sequence. An N-terminal truncation of the pY527 peptide was therefore synthesized and compared directly with the pY324 (hmT sequence) peptide in the SPA system (see Figure 2). The results were as expected, the truncated pY527 peptide exhibited both lower counts and a higher IC₅₀ value.

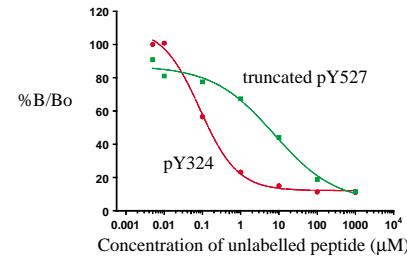


Figure 2. Competition between the [¹²⁵I]pY324 peptide and unlabelled peptide pY324 or truncated Y527.

Figure 3 shows the effect of altering the concentration of biotinylated GST c-Src SH2 domain from 0-84nM. The assay was incubated at room temperature and counted at intervals. The signal to noise ratio at 84nM was 12:1, but this increased to 21:1 after bead settling for 6 hours. The signal then remained constant over the 16 hour period investigated.

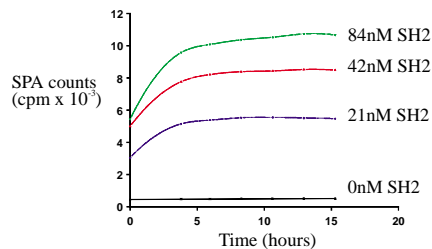


Figure 3. The effect on signal achieved of titrating biotinylated GST c-Src SH2 domain over a 16 hour period.

2. Crk

Crk is an ubiquitous protein consisting of an amino terminal SH2 domain followed by two SH3 domains. Current evidence suggests that the SH3 domains of Crk bind to proline-rich peptide sequences in Abl and are involved in protein-protein interactions^{13,4}. The validity of peptide sequences as models for SH3 domain binding has been questioned as some interactions could be non-specific due to the low micromolar affinity. The binding sequences chosen are shown in table 1. Peptides were synthesized and radiolabelled with Bolton and Hunter reagent (see above) or separately by catalytic reduction of dehydropoline with tritium gas. A comparison of ³H and ¹²⁵I labelled peptides was carried out using the assay format above (Figure 1).

Figure 4 shows the effect of increasing the amount of [³H]108 added to the assay. It can be seen that both the signal and the background increase as the amount of activity is increased.

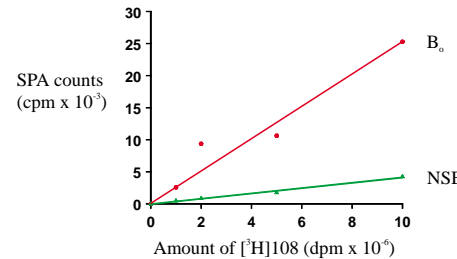


Figure 4. Titration of [³H]108 using 0.5µM biotinylated Crk SH3 domain.

Competition curves for Crk

Following mixing of assay components, the assay plate was shaken for 30 minutes at room temperature and then incubated at 4°C for 2-4hrs, or overnight, before counting.

Using the same amount of radioactivity, binding was observed with tritium-labelled, but not with ¹²⁵I-labelled peptides. This effect could be due to steric hindrance of the binding by the [¹²⁵I] Bolton and Hunter residue. Alternatively, the low molar quantity of peptide present in the assay, due to the high specific activity of the ¹²⁵I-labelled peptide, might be the cause of the low binding observed. On the basis of these results ³H-labelled peptides were selected for all further studies.

Results of a competition curve showing displacement of the [³H]108 peptide by peptides 108 or 109 are shown in Figure 5. Signal to noise ratios of 6:1 and 4:1 were achieved for peptides 108 and 109 respectively.

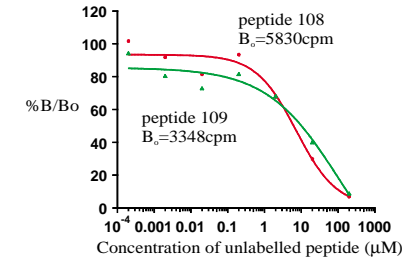


Figure 5. A graph showing competition between labelled and unlabelled peptides 108 and 109 for binding to biotinylated GST Crk SH3 domain.

In order to test the effect of temperature on the signal, the same assay plate was warmed to room temperature and counted again. The signal dropped significantly to about 50% of the initial value whereas the non-specific binding values remained constant. One theory to explain this effect is that as the temperature rises, the population of peptide in the thermodynamically favoured binding structure reduces as less favourable conformations are adopted. In addition, when adjacent prolines are found in a binding sequence, *cis*- and *trans*- proline conformers can exist. At any one time, only a proportion of the peptide is in a conformation capable of binding to the biotinylated SH3 domain.

Discussion

SPA has been used to measure the interactions of radiolabelled binding peptides with high affinity to the biotinylated GST c-Src SH2 domain and with low affinity to the biotinylated GST Crk SH3 domain. A separation step, which might disturb the weaker interactions in particular, is not required.

SPA may therefore provide a simple, rapid, homogeneous method for the high throughput screening of potential inhibitors of protein-peptide interactions. Inhibitors of these interactions might represent significant therapeutic targets, particularly in controlling the response of cells to growth factors.

References

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