Simultaneous Binding and Kinetic Analysis of Protein-Peptide Interactions on a Single Chip

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Abstract
Protein-protein interactions play a central role in almost all cellular responses. Specific recognition of signaling domains, such as SH2 and WW domains, with peptide motifs found on protein-binding partners is critical for a variety of cell-signaling pathways. Many techniques have been developed to study protein binding to peptide motifs. These include ELISA, far western, phage display, immunoprecipitation and pull-down assays. A new developed platform, the FLEX CHIP™ Kinetic Analysis System, developed in partnership with HTS Biosystems is ideal for analysis of protein-peptide interactions. This system uses Grating-Coupled Surface Plasmon Resonance (GC-SPR) for kinetic measurement of molecular interactions between unlabelled analytes and biomolecules immobilised on the surface of a chip having the dimensions of a standard microscope slide. The optical design of this platform enables simultaneous affinity characterisation of up to 400 targets immobilised on a single chip. We describe the application of GC-SPR technology for the characterisation of either full-length proteins or signal transduction domains, such as SH2 and 14-3-3, binding to a small peptide library immobilised on a single affinity chip in order to show both specificity of binding and affinity characterisation.

Introduction
Many protein-protein interactions can be probed by reducing the complexity of at least one of the protein partners to a peptide. Examples of such interactions include antibody interaction with linear epitopes on a given antigen and SH2, SH3, and WW domain interactions with small linear amino acid sequences found on interacting proteins. Motifs and/or post-translational modifications (PTMs) in such sequences allow for binding discrimination that ultimately leads to specificity. Historically, the comparison of protein interactions with peptide panels has been limited by both the high cost of peptide synthesis and the throughput capacity of the interaction assay. For kinetic analysis of protein-peptide interactions, researchers have been hindered by the low throughput of available SPR instrumentation. The Kretschmann configuration typically used in SPR instruments depends on a prism to measure SPR angles. Incident light strikes the gold layer on the opposite side of the immobilised biomolecules, causing strict requirements for chip design and for surface real-estate on which binding events can be monitored. In contrast, the FLEX CHIP™ Kinetic Analysis System utilizes a grating-coupled configuration in which a fine grating on the chip surface provides optical coupling (Figure 1a). Incident light illuminates the gold layer from the top and through the biomolecular layer avoiding both the need for a prism and strict limitation on the area of detection. SPR angle change is detected in real-time across a 1cm² surface area using a CCD camera. Thus, binding events can be simultaneously monitored for up to 400 targets immobilised on a single affinity chip. Each of the individual immobilised targets has its own set of nearest-neighbour references that enables one to distinguish binding events.

By making use of a newly available platform for high throughput peptide synthesis and GC-SPR, we show analysis of several different protein interactions with a small library of peptides. Both binding specificity and kinetics are demonstrated using parallel analyses.

Experimental Setup
Peptides were synthesised as 15- or 20-mers by Sigma-Genosys using the PEPScreen™ technology, a high-throughput peptide synthesis platform. Peptides contain an N-terminal biotin and a diethylene glycol linker between the biotin and first amino acid residue. The average peptide purity was approximately 73% for 15-mer and 61% for 20-mer peptides. Peptides were spotted in duplicate at 2mM, 10mM, 50mM, onto NeutrAvidin™ Affinity Chips using a Cartesian spotter. Chips were then inserted into the FLEX CHIP™ Kinetic Analysis System for experimental analysis. Unlabelled analytes, either protein domains or full-length proteins from various vendors, were flowed over the spotted peptide chips at indicated concentrations. Analyte binding to peptides was assessed using either off-point equilibrium binding (the average of the last minute of binding signal before dissociation begins) or kinetic analysis. Affinity constants were calculated using the FLEX CHIP™ Kinetic Analysis System software.

Results
Specificity of Protein-Peptide Binding
Over 50 different peptides representing a variety of protein binding motifs were spotted on a single affinity chip. Sequences that are known to bind to SH2 domains, 14-3-3 proteins, actin, WW domains and CAMP-dependent kinase were included (Figure 2). For sequences, such as SH2- and 14-3-3-binding motifs, in which binding to a given protein is regulated by phosphorylation on a specific residue, both the phosphorylated and non-phosphorylated peptides were represented.
Multiple protein and protein domain analytes were flowed over separate chips spotted in the same configuration in order to demonstrate binding specificity. Protein-binding to peptides immobilised on the affinity chip demonstrated striking specificity as shown in the examples in Figure 3. When the SH2 domain of Grb2 protein was flowed over the peptide chip, the protein bound specifically to peptides containing phosphotyrosine, and did not bind to the corresponding non-phosphorylated peptides. Binding of an anti-phosphotyrosine antibody also mirrored these results with only phosphotyrosine-containing peptides being recognised. Likewise, when a 14-3-3β-GST fusion protein was flowed over the chip, it specifically bound to phosphothreonine- and phosphoserine-containing peptides with cognate flanking sequences. Protein kinase A (PKA) catalytic subunit bound to a peptide derived from protein kinase A inhibitor (PKI), but not to other peptides immobilised on the chip.

Binding Specificity of SH2 Domains

The optimal binding motif for the Grb2 SH2 domain is pYXNX, which is present in all five SH2-binding peptides included in the peptide library (Table 1) (Oligino et al., 1997; Suenaga et al., 2003). As shown in Figure 3, Grb2 SH2 domain bound specifically to the phosphotyrosine version of each of these peptides. The relative amounts of binding, as indicated by endpoint RCU values, generally correlated with increasing peptide spotting concentration (Figure 4A). In contrast, when a GST fusion of the SH2 domain from protein-tyrosine phosphatase SH-PTP2 was flowed over the peptide chip, the protein bound only to peptides SH2Pep1 and SH2Pep2 (Figure 4B). This data is consistent with previous reports showing that the optimal binding motif for the SH-PTP2 SH2 domain is pY[I/V]X[V/I] which is present only in the SH2Pep1 and SH2Pep2 peptides (reviewed in Neel et al., 2003).

Table 1. Sequence of Grb2 SH2 domain-binding peptides. Phosphotyrosine containing peptides are in the SpotMatrix.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Full protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH2Pep1</td>
<td>[Biot]-[DEG]-GSGSMKPF[pTyr]VNVEF</td>
<td>ICB (Y177)</td>
</tr>
<tr>
<td>SH2Pep2</td>
<td>[Biot]-[DEG]-GSSGDPS[pTyr]VNYQ</td>
<td>SHC (Y117)</td>
</tr>
<tr>
<td>SH2Pep3</td>
<td>[Biot]-[DEG]-GSGLPN[pTyr]PQSV</td>
<td>EnB2 (Y1068)</td>
</tr>
<tr>
<td>SH2Pep4</td>
<td>[Biot]-[DEG]-GSGVPN[pTyr]TNYQ</td>
<td>EnB2 (Y1319)</td>
</tr>
<tr>
<td>SH2Pep5</td>
<td>[Biot]-[DEG]-OTLPVP[pTyr]NQSV</td>
<td>EnB2 (Y1068)</td>
</tr>
</tbody>
</table>

Figure 2. Peptide Chip Map. Peptides representing Grb2 SH2, 14-3-3 protein, WW domain, kinase, or antibody binding motifs were spotted at three different concentrations in replicate on a single affinity chip.

Figure 3. Specificity of different protein analytes for peptides spotted on the affinity chip. Analyte flowed over each chip is shown in red. SPR signal for each experiment is shown on the Y-axis.
Kinetic Analysis of SH2 Binding

The power of SPR analysis is the ability to determine kinetic rate constants and binding affinities between two unlabelled molecules. To determine the binding affinity of the Grb2 SH2 domain with phosphotyrosine-containing peptides, peptides were spotted on an affinity chip at multiple concentrations. Figure 5A shows the binding kinetics of Grb2 SH2 domain binding to the SH2Pep2 peptide. The derived equilibrium constants for Grb2 SH2 binding to all five phosphotyrosine-containing peptides are shown in Figure 5B. Similar kinetic values were obtained from the PEPscreen™-synthesised SH2Pep2 peptide and a control (HPLC-purified) SH2Pep2 peptide. These results lend confidence to the use of the PEPscreen™ platform for production of peptide libraries for SPR analysis. Kinetic results also agree with previous reports in the literature (Suenaga et al., 2003). Similar studies were not performed for the SH-PTP2 SH2 domain due to the avidity effects introduced by the dimeric GST fusion.

![Figure 5. Affinity analysis of Grb2-SH2 domain binding to pTyr peptides.](image)

A) Affinity trace of Grb2 SH2 domain protein binding to peptide SH2Pep2 spotted multiple concentrations.

B) Affinity calculations obtained by global analysis of affinity traces for Grb2-SH2 binding to phosphotyrosine-containing peptides.
Mapping the Actin-Binding Site of Thymosin β4.

Thymosin β4 is a ubiquitous 43 amino acid polypeptide that is an important mediator of cell proliferation, migration, and differentiation (reviewed by Goldstein, 2003). At the molecular level, it serves to sequester G-actin (Safer et al., 1991). In order to identify the sequence required for G-actin binding, a peptide scan was performed on the human thymosin β4 polypeptide (Figure 6A). End-point binding of each individual overlapping peptide comprising thymosin is shown in Figure 6B. Peptides 12-26 and 17-31 were shown to have the highest binding activity to G-actin, suggesting that the primary interaction site of thymosin is shown in Figure 6B. A) Six overlapping peptides were synthesized to span the 43 amino acid sequence of human Thymosin β4. B) Bovine G-actin was flowed over the peptide chip at 8.7 mM in the presence of calcium and ATP. End point binding of each peptide is shown as SPR units.

Conclusion

We have used a high throughput peptide synthesis platform together with GC-SPR analysis to characterize multiple protein-peptide interactions in parallel for both binding specificity and binding kinetics. Parallel analysis of protein binding to a peptide library enables one to probe for specificity of binding and contributions from PTMs, such as phosphorylation, methylation, or acetylation, as demonstrated here for SH2-domain and 14-3-3 domain binding to phosphotyrosine or phosphoserin/threonine modified peptides respectively. In addition, the approach can be used for mapping the site(s) of protein interaction between two proteins, demonstrated by the identification of the G-actin binding site of thymosin and for identifying linear epitopes for antibody binding to its antigen, demonstrated for an anti-phosphothreonine antibody. For any of these interactions, affinity comparisons including $k_{on}$, $k_{off}$, and KD, can be compared under identical experimental conditions including temperature, analyte concentration and analyte quality. Such conditions often vary slightly between separate experimental analyses leading to spurious results. The ability to simultaneously analyse protein interactions with a large panel of peptides also allows for inclusion of numerous controls that ultimately lead to an increased confidence in the experimental results. For instance, both appropriate positive and negative controls can be included in a single experiment, as well as multiple peptide spotting concentrations and replicates. Having made use of such controls in our own experimental set-up, we have demonstrated binding specificity for a variety of different protein-peptide interactions.

GC-SPR enables the ability to confidently interrogate protein-peptide interactions in a high throughput manner. Variations in specificity and affinity parameters detected by such analyses, may ultimately play key physiological roles in cell signaling events.

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