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Combinatorial Peptide Microarrays for the Rapid Determination of Kinase Specificity

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Abstract—We report a rapid method for profiling of kinases using a strategy that couples the merits of combinatorics (in rapid diversity generation) with the throughput attainable using microarrays (in parallel screening). Alanine-scanning, deletion and positional-scanning peptide libraries of a kinase substrate were synthesized and site-specifically arrayed onto glass slides. The phosphorylation pattern of target sequences detected using fluorescently-labeled antiphosphoamino acid antibodies revealed the substrate preference of the kinase through its activity profile.

Intricate control of cell signaling and signal transduction pathways is mediated by kinases which confer phosphate groups to cognate amino acid sequences, thereby regulating protein activity and function. As with other enzymes, the overriding principle that governs the activity is the affinity of the enzyme to the substrate. Deciphering preferred substrates for different kinases thus not only provides invaluable information in establishing their roles in complex biological pathways, but also enables discovery of inhibitors or drug candidates with which to regulate their enzymatic activity. Spatially resolved peptides immobilized in high-density microarrays provide miniaturized platforms upon which parallel screenings of bioactive peptides, novel ligand binders, antigenic epitopes as well as studies of enzyme function and specificity may be conducted at high throughput.[1–3] The manifold advantages peptide microarray presents over antecedent strategies, like SPOT™,[4] on-bead peptide libraries[5] and peptide phage display,[6] make it an attractive and promising tool in the burgeoning proteomics arena. Traditional microarrays employ a 'one spot–one compound' strategy that allows direct identification of positive hits. This, however, limits the diversity of compounds studied in arrays to what may be synthesized individually, thus severely limiting throughput. In order to study a greater diversity of compounds more rapidly and efficiently, it is thus imperative that alternative strategies like combinatorial peptide synthesis be suitably employed together with array-based methods to facilitate the development of a new generation of peptide arrays. We show here that, by spotting libraries with various combinations of peptide sequences, it is possible to draw conclusions about positive hits or substrate specificity without generating large numbers of peptide sequences individually. The augmentation of combinatorial strategies for rapid diversity production with the throughput attainable using microarrays provides for an even more rapid means of generating and applying peptide microarrays in high-throughput studies. The combinatorial chip we present herein will have wide ranging applications in array-based research, and we demonstrate its utility in screening for the preferred substrate of a tyrosine kinase, p60c-src. Three different classes of combinatorial libraries, namely alanine-scanning (where alanine is systematically substituted in substrate sequences), deletion libraries (where incremental truncation of flanking residues of the phosphorylation site establishes the length of peptide required for mediating kinase activity) and positional-scanning libraries[7] (to assess preferred amino acid residues at specific positions) were generated and reacted with the kinase on chip.

A putative substrate of p60c-src, YIYGSFK was used as the starting template from which all libraries were designed and constructed.[8] The set of libraries generated

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is shown in Table 1. Synthesis was performed using a Pioneer™ automatic peptide synthesizer (Applied Biosystems, USA) on Rink amide support using standard Fmoc chemistry. *O*-Benzothiazol-1-yl-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate (TBTU), 1-hydroxybenzotriazole (HOBt) and *N,N*-diisopropylethylamine (DIEA) coupling chemistry was employed.[9,10] A proportioned mixture of amino acids was reacted at positions denoted X to ensure equivalent representation of 18 standard amino acids (comprising the 20 proteinogenic amino acids with the exclusion of cysteine and tyrosine). All peptides were engineered with N-terminal cysteine residues and an intermediary sequence of two glycine residues functioning as a spacer.[9] The terminal cysteine residue acts as an anchor which chemoselectively reacts with thioester moieties functionalized on the slide surface. This site-specific immobilization strategy homogeneously orientates the peptide substrates on array for reaction with the kinase.[9] Cleavage of peptides was performed using reagent R (90% TFA, 5% thioanisole, 3% anisole and 2% ethanedithiol) and the filtrate was precipitated in cold ether to isolate the peptide. Individual peptides were purified by reverse-phase HPLC using a Phenomenex C_{18} semi-preparative column. Their identities were further confirmed by mass spectroscopy. Libraries with diverse members were mixtures that were not purified but shown to give a normally distributed mass spectrum about the mean theoretical peptide mass. All peptides were subsequently lyophilized and stored at −20°C under nitrogen until ready for use.

PEGylated thioester slides were used for all array-based experiments described herein, and were generated as previously described.[9] Briefly, slides were first cleaned in piranha solution and treated with a 1% solution of 3-glycidopropyltrimethoxysilane in 95% ethanol containing 16 mM acetic acid. After curing at 150 °C, the resulting epoxy functionalized slides were reacted with diamine PEG (M_r 3400) in 0.1 M $NaHCO_3$, pH 9, for 30 min. The slides were subsequently placed in a solution of 180 mM succinic anhydride in DMF, pH 9, for 30 min and thereafter in boiling water for 2 min. The resulting carboxylic acid derivatized surface was then activated using TBTU/HOBt/DIEA for 3 h and reacted overnight with a solution of 120 mM DIEA and 100 mM benzyl mercaptan in DMF. PEGylated slides were preferred over unPEGylated alternatives as the provision of a polymeric cushion is useful in increasing the hydrophilicity of the slide surface, and also prevents non-specific binding by antibodies used in the screening steps.[11] It also extends the peptides away from the slide surface, better presenting them for reaction and further eliminates the requirement of BSA for blocking.[11] Peptides were prepared fresh to 3–5 mM in PBS, pH 7.4, and printed onto thioester slides using an ESI SMA™ arrayer (Ontario, Canada) with a pitch of 180 μm between the spots.[12] The slides were incubated for 4–8 h before washing with PBS, water and drying. p60c-src kinase was then applied in a solution containing 25 mM Tris, pH 7.4 buffer with 15 mM $MgCl_2$, 7 mM $MnCl_2$, 0.5 mM EGTA, 100 μM ATP and 2 U p60c-src. A fluorescein-tagged antiphosphotyrosine antibody was then applied and, after a 1-h incubation, the slides were rinsed with PBST for 15 min. This was followed by a final wash with water before drying and analyzing using an ArrayWoRx™ microarray scanner (Applied Precision, USA).

Table 1. Peptide sequences and libraries constructed

No.	Peptide Sequences	No.	Peptide Sequences
1	IYGSFK	**14**	XXXXYXXXX
2	YGSFK	**15**	YIYGSFK
3	YIYGSF	**16**	XXYIXFK
4	YIYGS	**17**	XXYSXFK
5	YIYG	**18**	XXYKXFK
6	YIY	**19**	XXYEXFK
7	YIYGSFA	**20**	XXYFXFK
8	YIYGSAK	**21**	XIYXXFK
9	YIYGAFK	**22**	XSYXXFK
10	YIYASFK	**23**	XKYXXFK
11	YAYGSFK	**24**	XEYXXFK
12	AIYGSFK	**25**	XFYXXFK
13	YIAGSFK		

1-6 Deletion libraries (red), **7-13** Alanine-scanning libraries (blue), **16-25** Positional-scanning libraries (green), **14** Full combinatorial mixture peptide library (pink), **15** Original putative substrate (orange). The target phosphorylation site tyrosine residue is highlighted in yellow. Positions denoted X represent sites where an 18 amino acid mixture was coupled (20 standard amino acids - Cys, - Tyr). All peptides were engineered with CGG at the N-terminus.

Results obtained are shown in Figure 1. The putative substrate, (**15**) which was highlighted in orange, gave a fluorescence intensity value of 246.5 units that was used as the benchmark for comparison and evaluation of other peptides. Examination of the reduction library reveals that a truncation exceeding one residue at either terminus renders the substrate non-reactive with the kinase (**2**, **4**–**6**). A minimum recognition motif of six residues is thus necessary to mediate the action of p60c-src kinase (**1**, **3**). Elimination of the N-terminal tyrosine or the C-terminal lysine gave a strongly fluorescent readout with a 70.3 and 49.8% increase in fluorescence level respectively relative to the original substrate. This may be attributed to the following reasons: (1) these residues inhibit the phosphorylation activity of the kinase; (2) the kinase prefers a shorter recognition motif of six compared to seven residues—as in the original

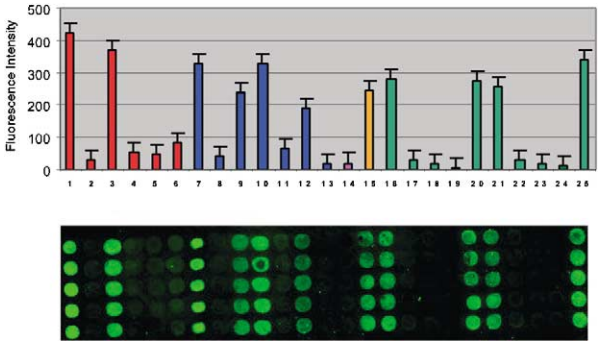

Figure 1. Peptide combinatorial libraries immobilized on PEGylated thioester slides in replicates of five and reacted with p60c-src kinase. **1**–**6** Deletion libraries (red), **7**–**13** Alanine-scanning libraries (blue), **16**–**25** Positional-scanning libraries (green), **14** Full combinatorial mixture peptide library (pink), **15** Original putative substrate (orange). The slides were probed with antiphosphotyrosine antibody to afford the kinase fingerprint presented.

substrate or (3) a combination of 1 and 2. The results obtained with the alanine-scanning library provided some clues as to which of these explanations is most plausible. The replacement of the terminal tyrosine with an alanine (**12**) slightly reduced the intensity obtained in relation to the original substrate. This indicates that a tyrosine residue is preferred at the N-terminus (relative to alanine) but is not critical for phosphorylation, as its removal did not eliminate activity. At the C-terminal end of the substrate, the replacement of lysine with alanine (**7**) increased the fluorescence readout obtained by 33% over the native substrate. Alanine is thus a preferred residue relative to lysine at this position. Given these results, it seems that the removal of lysine consistently gives above average signals in both reduction and alanine-scanning libraries. We further identified residues that were critical in preserving phosphorylation, namely phenylalanine (**8**) and isoleucine (**11**). Replacing these residues brought the fluorescence readout observed to levels under 25% of the native substrate. Compounds **9** and **10** were not significantly affected by alanine replacements, establishing that glycine and serine are not critical in mediating phosphorylation. The negative control (**13**) with its active tyrosine residue replaced with alanine was not phosphorylated, confirming that all kinase-mediated phosphorylation took place only at this target locus.

We also generated a full-spectrum diversity library (**14**) comprising over 1.1×10^{10} members (18^8) with a tyrosine residue at the central position. This library, however, did not give a positive signal when tested with the p60c-src kinase. It is likely that cognate peptides in the mixture (that possessed key residues at suitable positions) were severely under-represented. Consequently, these target substrates, even if phosphorylated, may not have been detected. We thus generated positional-scanning libraries by maintaining the C-terminal FK motif of the native substrate while systematically scanning other positions (phenylalanine was established as one of the key residues in mediating phosphorylation with p60c-src). Each positional-scanning library had a diversity of almost 6000 members (18^3), and was designed to identify preferred amino acids that immediately flank the tyrosine phosphorylation site. Five representative amino acids, namely Ile, Ser, Lys, Glu and Phe, were systematically substituted adjacent to the phosphorylation site, giving a set of 10 positional-scanning libraries which were subsequently screened with the p60c-src kinase (**16**–**25**). Results indicated that only Ile (**16**, **21**) or Phe (**20**, **25**) when adjacent to the target tyrosine residue render the substrate reactive with the kinase. It was observed that, in the presence of these residues, a fluorescence intensity of at least ten fold stronger than with the six remaining libraries was obtained. This result is consistent with the earlier finding that isoleucine is important at the N-terminal position adjacent to the target tyrosine residue for phosphorylation to occur.

To further validate the results, we synthesized fluorescently tagged positional-scanning libraries, with CK*GG used in place of CGG linker sequence. The lysine residue, K*, incorporated in this set of libraries was conjugated with a fluorophore, Cy3.[13] Using these tagged libraries, we confirmed that the peptides were evenly spotted on the array. Furthermore, these peptides gave the same pattern of fluorescence upon reaction with the kinase, thus supporting the results obtained earlier. Cy3 did not interfere with detection of peptide phosphorylation using the fluorescein-tagged antiphosphotyrosine antibody, thus presenting a useful strategy to both study enzyme activity and quantitate the immobilized peptide simultaneously. We also verified that, without reaction with the kinase, no labeling with the antibody was observed, further confirming that the antibody is specific in targeting only phosphorylated tyrosine residues.

We next determined the concentration- and time-dependence of the kinase activity against representatives of our peptide libraries. Peptides **1**–**3** were spotted in decreasing concentrations, and incubated with the kinase for 4 h. The slides were then incubated with the antiphosphotyrosine antibody, washed and scanned as described earlier. It was observed that the intensity of the spots increased with an increasing concentration of substrates used. A plot of the intensities reveals a linear correlation of the concentration of peptides against the fluorescent signal (see Fig. 2). This demonstrates the utility of this method in studying concentration-dependent kinase activity against a wide spectrum of substrates simultaneously in a microarray format. Baseline readouts were obtained for **2**, which was not phosphorylated by the kinase.

The time-dependent kinase activity was also demonstrated using peptides (**1**–**3**). Peptides were spotted at concentrations of 3 mM and separate slides were incubated for varying periods of time with the kinase. It was found that saturation of the target substrates was achieved after approximately 1 h of incubation with the kinase (Fig. 3). In comparing the kinetic profiles of substrates **1** and **3**, not only was the final fluorescence reading of substrate **1** higher in relation to **3**, the rate at which phosphorylation took place was also quicker, indicating that the enzyme has a stronger preference for

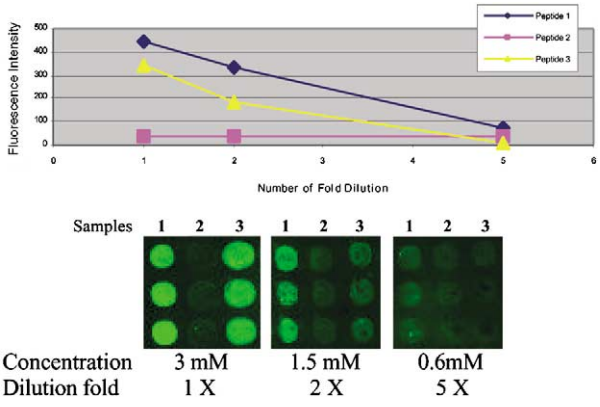

Figure 2. Decreasing concentrations of peptides (**1**–**3**) were arrayed in triplicates on thioester slides and incubated with p60c-src kinase for 4 h. The fluorescence intensities obtained were plotted and shown to vary linearly with substrate concentrations. **2** did not react with the kinase and produced flat baseline results.

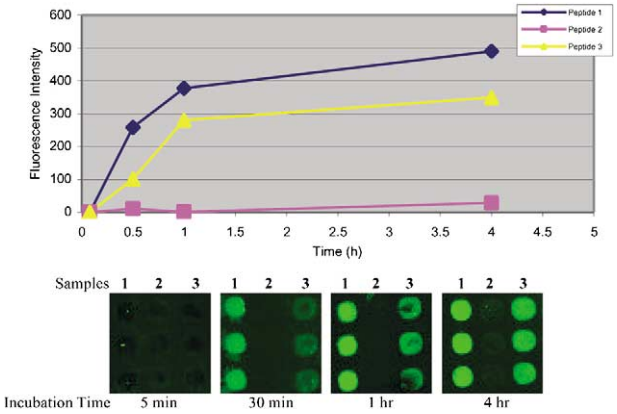

Figure 3. Separate thioester slides spotted with 3 mM of each of the three substrates (**1**–**3**) in triplicates were incubated with the kinase for varying periods of time. The fluorescence intensities obtained after screening with the antibody was graphed. The reaction was estimated to be completed after an h, thereafter the intensities obtained for the positive substrates remained relatively unchanged.

substrate **1**. This strategy may thus be used to establish the substrate preference of the kinase both in terms of the total fluorescence intensity output, as well as the kinetic profiling that is useful in the discovery of strong inhibitors.

In conclusion, we have developed an alternative paradigm for microarray research by demonstrating that libraries of peptides synthesized combinatorially are viable and attractive tools for array applications. Traditional peptide-based arrays have relied on separate preparations and purifications of target molecules.[1] We have demonstrated the utility of this new generation of peptide arrays by successfully identifying critical amino acid residues required in mediating the activity of a tyrosine kinase. The approach described herein is compatible with virtually all peptide array applications. This, together with other emerging technologies in the field of proteomics,[1,14,15] should provide a valuable tool for high-throughput discovery of potential drug targets.

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