

Rapid Affinity-Based Fingerprinting of 14-3-3 Isoforms Using a Combinatorial Peptide Microarray**

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The 14-3-3 proteins are a family of acidic proteins (ca. 30 kDa) expressed in all eukaryotic cells.^[1] By binding as either homo- or heterodimers to a variety of phosphoserine-containing proteins, they regulate important cellular events including cell-cycle progression, DNA damage, apoptosis, protein trafficking, signal transduction, cytoskeletal rearrangements, metabolism, and transcriptional regulation. In humans, there are seven distinct but highly homologous 14-3-3 isoforms: β , ϵ , η , γ , σ , τ , and ζ . Thus far, however, the only isoform directly linked to cancer has been 14-3-3 σ , which is regulated by the major tumor suppressor gene, p53.^[1a] Inactivation of 14-3-3 σ is crucial in tumorigenesis. Consequently, there has been tremendous interest in the determination of the substrate specificity of 14-3-3-phosphopeptide binding as well as the structural basis of this interaction.^[2]

The elegant work of Yaffe et al. is particularly worth noting, in which a degenerate phosphoserine-oriented peptide library was used to examine the sequence requirements for binding to 14-3-3.^[2a] The results confirmed the highly conserved substrate-binding specificity amongst the 14-3-3 isoforms, thus giving rise to a consensus hexapeptide binding motif, RXXpSXP, which binds strongly to all 14-3-3 isoforms tested. In other studies, X-ray crystallography was used to provide detailed structural information of seven human 14-3-3 isoforms bound to a phosphopeptide.^[2b,c] Interestingly, 14-3-3 σ , unlike the other six isoforms, was the only protein shown to preferentially form a homodimer upon substrate binding. Our ongoing interest in the use of 14-3-3 σ as a potential cancer target has prompted us to speculate that it might be possible to identify phosphopeptides that preferentially bind to 14-3-3 σ over other isoforms. Herein, we report the first peptide microarray platform made of fragment-based, combinatorial phosphoserine/phosphothreonine-containing heptapeptide libraries ($P_{-3}P_{-2}P_{-1}p(S/T)-P_{+1}P_{+2}P_{+3}$)

for substrate-specificity determination of seven mammalian 14-3-3 proteins, and the identification of putative 14-3-3 σ -selective motifs (Figure 1).

The peptide microarray is a miniaturized screening platform that enables thousands of individually addressable peptides to be simultaneously interrogated on a glass slide.^[3] It is well-suited for high-throughput (HT) studies of protein-peptide interactions. Compared to other HT peptide-screening approaches, that is, phage display,^[4a] SPOT synthesis,^[4b] and combinatorial peptide libraries (positional scanning (PS),^[4c] degenerate,^[2a] and one-bead-one-compound (OBOC)^[4d]), it offers the unique advantage that every single spot on the slide may be made addressable and quantifiable instantaneously.^[3a]

To obviate the current upper limit of 1000–10000 different features attainable on a microarray platform, we introduced the concept of a fragment-based combinatorial peptide microarray that enables sufficient coverage of all $P_{-3}P_{-2}P_{-1}-p(S/T)-P_{+1}P_{+2}P_{+3}$ sequences with only 1000 different spotting features (500 N- and C-terminal sublibraries each; $P_{+/-}$ represents ten or five individual amino acids (AAs) and $X_{+/-}$ represents an isokinetic mixture of 14 AAs; Figure 1 a).^[5] By “scanning” fragments (that is, the tripeptides flanking $p(S/T)$) rather than positions (for example, in a PS^[4c] or degenerate library^[2a]), we retained the “neighboring-position effect” ignored by PS/degenerate approaches, and ensured a sufficient concentration of peptides was present on the spotted array.^[6] Similar to the case of a PS/degenerate library, the screening results obtained from our fragment-based scanning (Figure 1 b–d) can be reconstituted to give accurate and relevant biological information (Figure 1 e,f). To minimize the number of spots on the array, ten and five representative AAs, instead of twenty, were used at the $P_{1/2}$ and P_3 positions, respectively.

The 1000-member phosphopeptide library was synthesized by the IRORI split-and-pool combinatorial approach in two installments, and subsequently spotted (in duplicate) onto an avidin-coated glass slide to generate the corresponding peptide microarray.^[7] To ensure uniform immobilization, spotted slides were subjected to Pro-Q staining (Figure 1 b); the image indicates that most features (> 99 %) were spotted uniformly and consistently, with minimal spot-to-spot and slide-to-slide variations ($r > 0.95$). We next assessed if this fragment-based combinatorial microarray could be used to obtain substrate specificity of known protein-peptide interactions. Pin1, a well-documented peptidyl prolyl *cis/trans* isomerase, is known to bind to $p(S/T)$ -Pro-containing peptides with exquisite specificity.^[8] As shown in Figure 1 c and e, of the 50 $p(S/T)$ -Pro-containing peptide spots in our 1000-

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[**] Funding was provided by the National University of Singapore (R-143-000-280-112 and R-154-000-281-112) and the Agency for Science, Technology, and Research (A*STAR) of Singapore (R-154-000-274-305).

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/ange.200801395>.

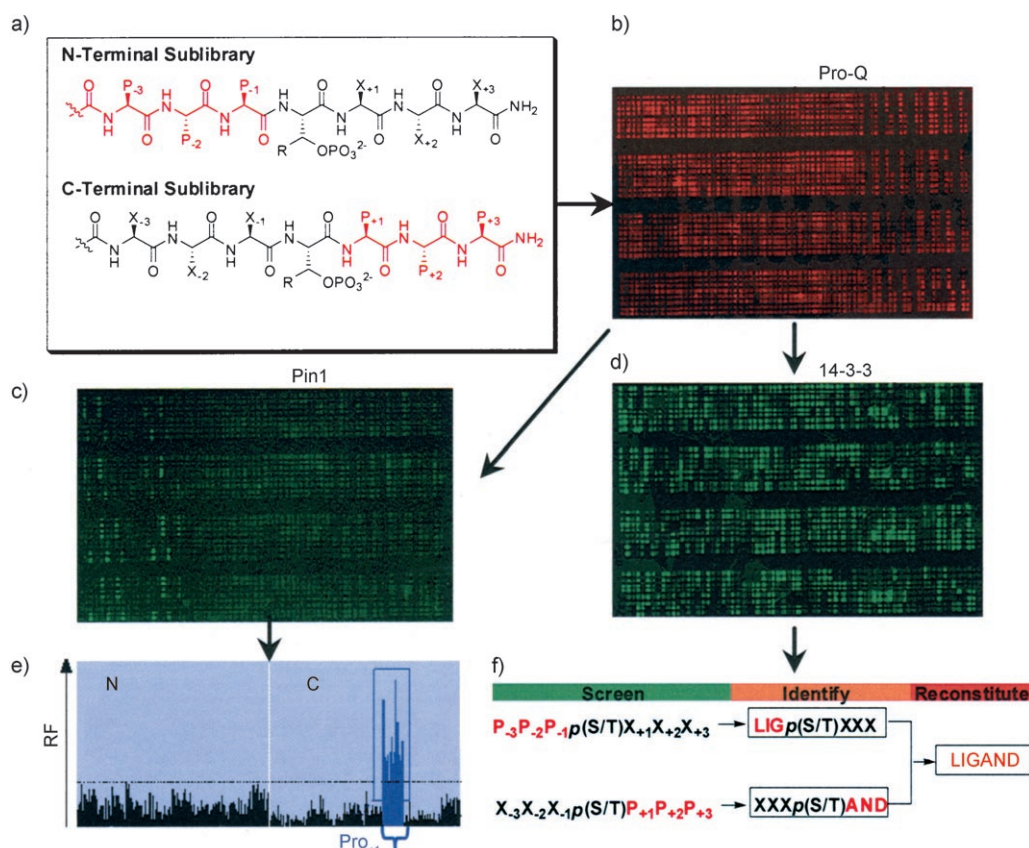


Figure 1. Overall flow of the strategy. a) Chemical structures of the 1000-member fragment-based phosphopeptide library (R = H, Me). Residues were numbered according to their proximity to p(S/T) (S: serine, T: threonine) and were negative if located N-terminal to it. p(S/T) instead of pS was used so that the peptides could probe other phosphopeptide-binding proteins, such as Pin1.^[8] Biotin and a GG linker were introduced into each peptide for microarray immobilization. b) Pro-Q image of the 1000-member peptide microarray. c, d) Microarray images upon screening with Cy3-labeled Pin1 and 14-3-3, respectively. e, f) Data analysis. The 50 p(S/T)-Pro-containing peptides are highlighted in blue in (e). See text and the Supporting Information for details.

member library, 48 bind to Cy3-labeled Pin1 with substantially higher relative fluorescence (RF) values, unambiguously confirming that our fragment-based peptide microarray could be used for HT determination of protein–peptide interactions.

Next, seven mammalian 14-3-3 isoforms were recombinantly expressed, purified, fluorescently labeled, and screened with the peptide microarray (Figure S4 in the Supporting Information). Upon data processing, highly reproducible (as judged from duplicated spots/slides), affinity-based fingerprints were generated and presented as color-heat maps (Figure 2a, left). The results indicate that, although the overall binding profiles of seven isoforms were quite similar as expected (Figure S6 in the Supporting Information; $r > 0.8$ across all isoforms), there were some subtle but distinctive features in the fingerprints that differed from one isoform to another, and could be further explored to identify 14-3-3 σ -specific motifs (Figure 2a, right). This result clearly highlights the advantage of our array-based approach over other mixture-based combinatorial methods where only averaged data are obtained.^[2a, 4c]

Further data analysis was carried out with three different methods: 1) a position-specific scoring matrix to determine the average binding affinity of 14-3-3 across the P_{+/-1}, P_{+/-2},

and P_{+/-3} positions for each AA (Figure S5 in the Supporting Information); 2) the top 50 hits identified from each N- and C-terminal sublibrary against individual 14-3-3 isoforms (Figure S7 in the Supporting Information); and 3) the top 100 hits identified from the 1000-member library (irrespective of N/C terminus) against all seven 14-3-3 isoforms (Figure S8 in the Supporting Information). The results are summarized in Table 1 and indicate that, regardless of how the data were analyzed, the 14-3-3 consensus binding motifs derived from our experiments were in excellent agreement with previous work using degenerate peptide libraries.^[2a]

Table 1: 14-3-3 preferences determined with different methods.^[a]

	Position relative to p(S/T)					
	-3	-2	-1	+1	+2	+3
Yaffe ^[b]	RK	FSYWHA	RKH	WYFLA	PG	X
1 ^[c]	RPLF	AFPRV	RK	AVFL	PA	FLPR
2 ^[c]	R	AFPR	RK	AVFL	PA	FLP
3 ^[c]	R	AFGPRV	RKAF	AVF	P	FL

[a] Standard single-letter abbreviations were used for AAs. [b] Results reproduced from reference [2a]; red AAs not used in our 1000-member fragment-based library. [c] This study; bold AAs coincide with the results of Yaffe et al.

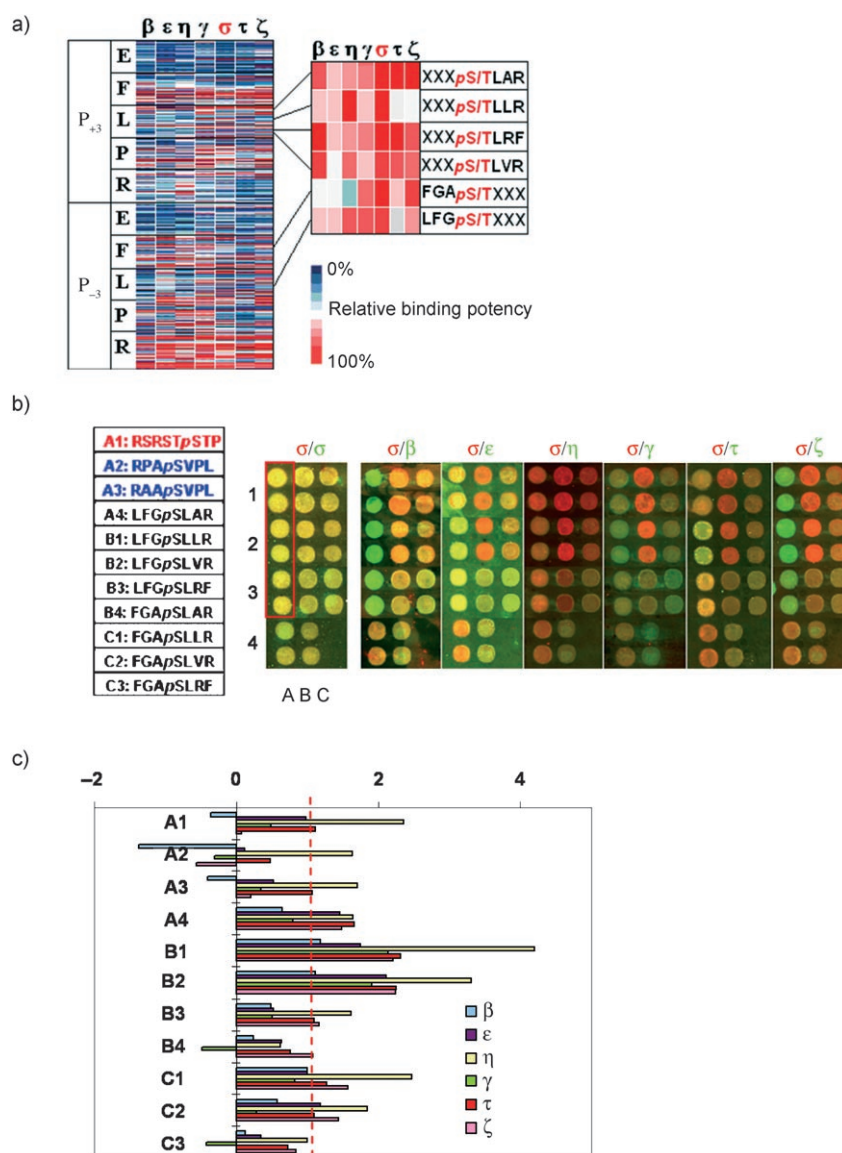


Figure 2. Affinity-based fingerprinting of seven 14-3-3 isoforms. a) Left: color-heat maps displaying binding of the 1000-member peptide library against seven Cy3-labeled 14-3-3 isoforms, sorted in the order of $P_{+3} > P_{+2} > P_{+1}$ AAs. Right: six putative σ -specific peptides were identified. b) Dual-color ratiometric screening using a protein mixture containing Cy5-labeled σ and Cy3-labeled 14-3-3 isoforms. A new microarray was used in which 11 peptides were spotted (in duplicate vertically). As a control (σ/σ), the first slide was treated with an equal amount of Cy5- and Cy3-labeled σ . All spots appeared orange, which indicates equal RF in both channels; σ -specific spots appeared red. c) Bar graph representing the selectivity scores, $\log_2(\text{Cy5 } \sigma/\text{Cy3 isoform})$, of the 11 peptides from (b). Score “0” indicates no selectivity and “+” indicates σ -preferred peptides. An arbitrary threshold of “1” was set (dashed line) to highlight motifs binding to σ at least twice as strongly as other isoforms. See the Supporting Information for details.

In addition to the general RXXpSXP motif, we uncovered more detailed 14-3-3 binding information. For example, Arg/Lys were highly preferred at the -1 position, and mostly nonpolar residues were preferred at the -2 and $+1$ positions. This finding again agrees well with the work of Yaffe et al.^[2a] Interestingly, we also observed some previously undetected preferences of Pro at -2 , Ala at -1 , Val at $+1$, and Phe/Leu at $+3$ positions. To confirm these potentially new 14-3-3 binding motifs, we made reconstituted peptide sequences of

RAApSVPL and RPApSVPL,^[9] followed by microarray-based dissociation constant (K_d) determination and fluorescence polarization experiments (Figure S9 in the Supporting Information). The results showed that both motifs bind to 14-3-3 σ with submicromolar affinity, similar to RLSHpSLPG, a well-known 14-3-3 binding peptide.

As a result of the highly homologous feature of 14-3-3 proteins, it is extremely challenging to identify isoform-specific binding motifs. Given the critical role of 14-3-3 σ in cancer biology,^[1] our aim was to explore the throughput, sensitivity, reliability, and ability to discriminate subtle binding differences of our peptide microarray for HT discovery of 14-3-3 σ -selective motifs. To do this, we identified six selected sequences from the preliminary fingerprinting results (see Figure 2a), and reconstituted and screened them on the microarray by using a dual-color ratio-metric approach developed recently.^[7a,10,11] As shown in Figure 2b and c, with the dual-color approach 14-3-3 σ -selective peptides could be readily and reliably interrogated with another isoform and visually identified (red spots in Figure 2b). A selectivity score of $\log_2(\text{Cy5 } \sigma/\text{Cy3 isoform})$ was obtained for every peptide against the other six 14-3-3 isoforms (Figure 2c).

Of the eleven peptides studied, A1 is RSRSTpSTP (see Figure 2b), a well-known 14-3-3 binding motif,^[8] and A2 and A3 are RAApSVPL and RPApSVPL, respectively, which are strong binders of 14-3-3 isoforms identified in the current study. All of them, as expected, bind to most 14-3-3 isoforms indiscriminately on our microarray. Six out of the other eight reconstituted peptides showed some degree of preference toward 14-3-3 σ binding on the microarray. Among them, B1 (LFGpSLLR) and B2 (LFGpSLVR) preferentially bind to 14-3-3 σ at least twice as strongly as almost all other isoforms. To further confirm these results, we carried out

experiments with surface plasmon resonance (Figure S11 in the Supporting Information). The results confirmed that B1 does indeed bind to 14-3-3 σ preferentially ($K_d = 0.54 \mu\text{M}$) over the other six 14-3-3 isoforms ($K_d > 10 \mu\text{M}$). Work is under way to further study the biological relevance of this finding.

In conclusion, we have developed the first fragment-based combinatorial peptide microarray that enables HT determination of substrate binding specificity and rapid discovery of isoform-selective motifs against the highly homologous 14-3-3

proteins. By introducing the concept of fragment-based scanning in a peptide microarray, our platform was able to cover a large peptide sequence space with a manageable number of spots/peptide libraries, without the loss of detailed and subtle information on protein–peptide interactions. We have uncovered novel putative binding motifs of 14-3-3 σ whose biological relevance needs to be further validated. We predict that this peptide microarray platform could have wide applications in future proteomics research.^[12]

Experimental Section

For peptide microarray screening, protein samples were minimally labeled with either Cy3 or Cy5 *N*-hydroxysuccinimide ester (Amersham) for 1 h on ice, and quenched with a tenfold molar excess of hydroxylamine for a further hour. The excess dye was removed with a Sephadex G-25 spin column (Amersham). The labeled proteins were reconstituted in a final volume of Tris-buffered saline (TBS; 80 μ L, pH 7.4) containing 1% bovine serum albumin. In a standard microarray experiment, the labeled protein (2 μ M; 50 μ L) was applied to the array under a coverslip. In a dose-dependent experiment for K_d measurements, various concentrations of the protein (50 to 5000 nM) were applied to different subarrays on the same slide. For dual-color screening experiments, equal amounts of a Cy3-labeled protein and another Cy5-labeled protein were mixed and applied to the slide. The samples were incubated with the array in a humidified chamber for 2 h at room temperature before repeated rinses with TBS + 0.05% Tween 20, typically 2 $\times$ 10 min washes with gentle shaking. Slides were scanned using an ArrayWoRx microarray scanner installed with the relevant filters (Cy3: $\lambda_{ex/em}$ = 548/595 nm; Cy5: $\lambda_{ex/em}$ = 633/685 nm).

For microarray K_d experiments, selected and/or reconstituted peptides were spotted onto a slide, which accommodated up to eight identical subarrays. Dose-dependent experiments were carried out with different concentrations of the labeled protein. The data generated were extracted and an equation was fitted to them assuming a saturation dynamics relationship (see the Supporting Information).

For the dual-color ratiometric experiment, a new microarray was fabricated with up to eight identical subarrays on the same slide. Equal amounts of Cy5-labeled σ and another Cy3-labeled 14-3-3 isoform were mixed before being applied to the microarray. In total, seven subarrays were screened simultaneously on the same slide. Data were extracted and the selectivity score was obtained from $\log_2(\text{Cy5 } \sigma/\text{Cy3 isoform})$. A positive $\log_2(\text{ratio})$ indicates a higher binding preference for 14-3-3 σ .

Received: March 24, 2008

Revised: June 27, 2008

Published online: August 13, 2008

Keywords: combinatorial chemistry · isoforms · microarrays · peptides · proteins

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