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A rapid method for generation of selective Sox-based chemosensors of Ser/Thr kinases using combinatorial peptide libraries

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ABSTRACT

A novel screening method to identify selective Sox-based fluorescent probes for Ser/Thr kinases has been developed. Peptide libraries were exposed to a kinase of interest and the products of the timed reaction were analyzed by MALDI-TOF. To demonstrate the potential of this methodology, a selective substrate for Aurora A kinase was identified that showed a 7-fold improvement in catalytic efficiency over the best substrate described to date in the literature.

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Phosphorylation is a ubiquitous post-translational modification reaction that is responsible for regulation of protein activity in both eukaryotes and prokaryotes. By catalyzing the transfer of γ -phosphoryl group of ATP to the side chains of serine, threonine, and/or tyrosine (in eukaryotes), protein kinases play an important role in regulation of many aspects of cellular function, including proliferation, the cell cycle, metabolism, transcription, and apoptosis.¹ Protein kinases have also emerged as attractive targets for drug discovery, since many are associated with a wide variety of diseases, from cancer to inflammation.² Thus, tools that allow for simple monitoring of kinase activity are in great demand in both pharmaceutical and academic settings. Recently, our laboratory developed highly versatile sulfonamido-oxine (Sox)-based fluorescent peptides for the continuous assay of Ser/Thr and Tyr kinases.³ The Sox-containing substrate is silent, but upon phosphorylation the chromophore can bind Mg^{2+} and undergoes chelation-enhanced fluorescence (CHEF). Such probes have been used to monitor various kinases both in vitro and in crude cell lysates.⁴

The key challenge in the field of kinase analysis is specificity, particularly with peptide-based substrates, which lack the spatial and temporal control that cellular substrates such as proteins possess. As a result, much effort has been devoted to identification of kinase substrates. Traditional methods to elucidate specificity of kinases include solid-phase phosphorylation screening of either phage display libraries⁵ or synthetic peptides⁶ and the use of degenerate libraries of peptides oriented around the residue to be phosphorylated.⁷ But, these techniques depend on laborious and time-consuming substrate peptide decoding procedures.^{6a,5c}

Furthermore, the detection of phosphate is based on the transfer of ^{32}P from $[\gamma\text{-}^{32}P]ATP$ to target peptides or proteins,⁸ which is a risk to human health and the environment, or on antibodies directed against phosphorylated residues,⁹ which in some cases is problematic due to their low specificity.

Herein, we report the development of a new method for identification of Sox-based probes with improved specificity for serine/threonine kinases. A combinatorial peptide library is first exposed to the desired kinase. Upon chemical modification of the phosphopeptides in the peptide mixture, Matrix-Assisted Laser Desorption Ionization Time-of-Flight mass spectrometry (MALDI-TOF MS) is employed to identify the products. Using this approach, the best sequence for Protein Kinase A (PKA) was found to be the well-established and extensively studied Kemptide,¹⁰ demonstrating that our method is reliable. Moreover, when applied to Aurora A (AurA, Aurora 2), a peptide sequence was identified that exhibited a 7-fold improvement in catalytic efficiency over the best literature substrate when incorporated into a Sox-based probe.

The method to identify probes is summarized in Figure 1. First, a library containing amino acid variations at the site of investigation was generated by Fmoc-based solid-phase peptide synthesis (SPPS). 2-Naphthyl alanine (2-Nal) was incorporated in place of C-Sox (generally placed in the +2 position) due to the tendency of the Sox chromophore to be partially eliminated under the MALDI conditions. The cleaved equimolar peptide mixture was incubated with the desired kinase for varying times. The direct detection of the phosphopeptide product by MALDI-TOF is usually poor due to the inefficient ionization of the negatively charged phosphate group and the tendency of phosphate to be eliminated under the MALDI conditions. Thus, to enhance signal intensities in the MALDI analysis, a previously reported method was em-

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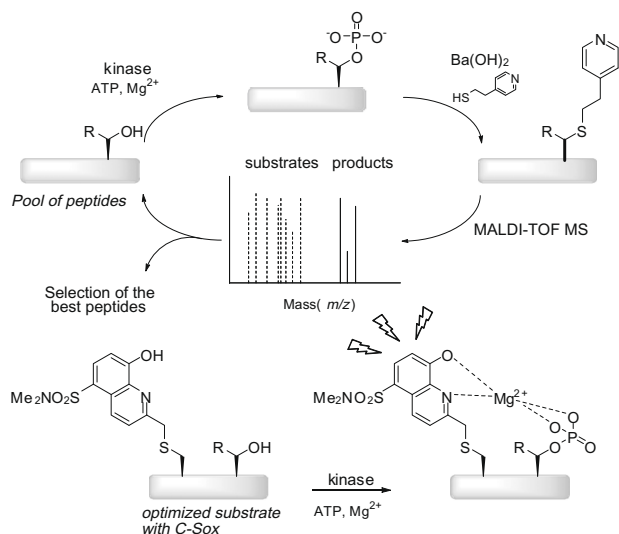


Figure 1. Screening method to design selective substrates for serine/threonine kinases.

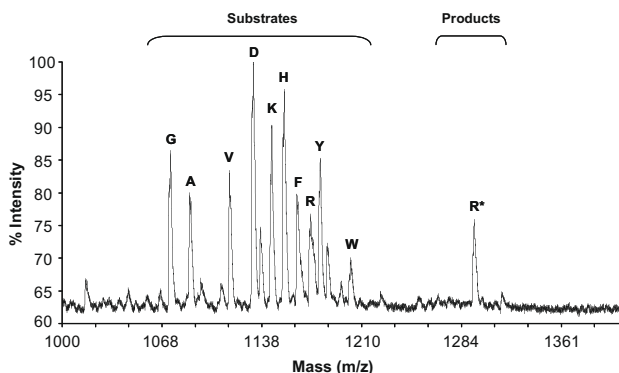


Figure 2. MALDI-TOF spectrum of the peptide library at the –3 position after its incubation with PKA for 1 h and chemical derivatization with Ba(OH)₂/MEP.

ployed.¹¹ Briefly, the kinase reaction products were subjected to base [Ba(OH)₂] which promoted β -elimination of the phosphate moiety, followed by Michael addition of 4-mercaptoethylpyridine (4-MEP). New peaks appearing in the MALDI spectrum (121 g/mol greater than the parent peptide) were interpreted as evidence of phosphorylation. The change in mass after the reaction was due to the loss of phosphate (98 g/mol) during the β -elimination and addition of 4-MEP (139 g/mol). This strategy enabled us to follow the progress of the reaction and to evaluate the kinase activity semiquantitatively by comparing intensities of derivatized peaks with those of the parent peptides in the same spectrum (Fig. 2). Once the best residue was found for a particular position, it was fixed in that place and the method was applied to a different position in an iterative fashion until the best substrate was obtained. Finally, the optimized sequence was synthesized with C-Sox^{3a} instead of 2-Nal, effectively turning the best substrate into a selective kinase reporter and simultaneously enabling determination of the kinetic parameters.

In order to optimize and validate the screening method, we initially focused our efforts on PKA and the corresponding selective substrate, Kemptide (Ac-LRRASLG-CONH₂).¹⁰ Two peptide libraries based on Kemptide were used to assess the preference of PKA for residues at the –1 and –3 positions (Table 1). In all screens, Ser, Thr, Cys, and Met were excluded to avoid side reactions (such as oxidations). Additionally, since there are three groups of amino

Table 1
Peptide libraries for PKA

Entry	Substrate sequences ^a									
	–4	–3	–2	–1	0	+1	+2	+3	+4	
1	Ac	L	X	R	F	S	L	2-Nal	A	A CONH ₂
2	Ac	L	R	R	X	S	L	2-Nal	A	A CONH ₂
Result	L	R	R	G/A	S	L	2-Nal	A	A	

^a X = Asp, Lys, Val, Ala, Arg, Gly, His, Phe, Trp, and Tyr.

Table 2
Peptide libraries for AurA

Entry	Substrate sequences ^a									
	–4	–3	–2	–1	0	+1	+2	+3	+4	
1	Ac	X/Y	R	R	F	S	L	2-Nal	A	A CONH ₂
2	Ac	L	X	R	F	S	L	2-Nal	A	A CONH ₂
3	Ac	L	R	R	X	S	L	2-Nal	A	A CONH ₂
4	Ac	L	R	R	F	S	X	2-Nal	A	A CONH ₂
5	Ac	L	R	R	F	S	L	2-Nal	X/Y	A CONH ₂
6	Ac	L	R	R	F	S	L	2-Nal	A	X CONH ₂
Result	G/A	R	R	F	S	F	2-Nal	G/dA	G	

^a X = Asp, Lys, Val, Ala, Arg, Gly, His, Phe, Trp, and Tyr; Y = DAsp, Dlys, Dval, DAla, DArg, DGly, DHis, DPhe, DTrp, and DTyr.

acids with similar masses (Asp, Leu, Ile, Asn = 131–133 g/mol; Lys, Glu, Gln = 146–147 g/mol; and Val, Pro = 115–117 g/mol), only one from each group was chosen in order to simplify the MALDI analysis. However, if necessary, it is also possible to use all amino acids in the screen by utilizing encoded peptide caps during synthesis, effectively producing a nondegenerate mass ladder for each peptide.¹² A mixture of the following amino acids was selected: Asp, Lys, Val, Ala, Arg, Gly, His, Phe, Trp, and Tyr. Libraries were synthesized on Fmoc-PAL-PEG-PS resin. For positions that were varied, isokinetic mixture of 10 amino acids was created by using a ratio of equivalents of amino acids based on their reported coupling rates.¹³ The MALDI spectra of the peptide libraries before and after incubation with PKA for varying time periods (10 min, 30 min, 1 h, 2 h, and 24 h) followed by chemical derivatization with 4-MEP, showed that there is a preference for Arg at the –3 position (Fig. 2) and for small hydrophobic residues at the –1 position (Table 1). This result is in full agreement with the consensus sequence described for PKA.^{10,14}

To demonstrate the generality of the method, Aurora kinase A (Aurora 2, AurA) was selected. Aurora kinases (A, B, and C) belong to the Ser/Thr protein kinase family and are involved in various aspects of mitosis.¹⁵ AurA, specifically, functions in centrosome maturation, mitotic spindle assembly and it plays a central role in cell cycle progression.¹⁶ Additionally, the gene coding for AurA maps to a region frequently amplified in tumors and its overexpression has been detected in various cancers.¹⁷ However, very little is presently known about its substrates and the mechanism of its activation/deactivation. This is particularly the case with peptide substrates for AurA. The only study so far conducted to determine the preferred residues in the recognition domain surrounding the phosphorylated residue,¹³ proposed the consensus sequence to be RRXSZ (where Z denotes any hydrophobic residue except for Pro and X is a small hydrophobic amino acid) and was based on peptides derived from an extended version of the Kemptide sequence (ALRRASLGAA). The best of these peptides has a *K_M* of ca. 300 μ M (Tables 3 and 4, entry 1). Thus, to make a fluorescent probe that can be used in complex environments to study AurA, it was imperative to first find substrates with enhanced selectivity.

We made six peptide libraries using equimolar mixtures of amino acids at the –1, –3, –4, +1, +3, and +4 positions based on the sequence of Kemptide (Table 2). The results of these libraries after

Table 3
Sequences of Sox-substrates for AurA

Entry	Substrate sequences											
		−5	−4	−3	−2	−1	0	+1	+2	+3	+4	
1	Ac	A	L	R	R	A	S	L	C-Sox	A	A	CONH ₂
2	Ac	A	L	R	R	F	S	L	C-Sox	A	A	CONH ₂
3	Ac	A	L	R	R	F	S	L	C-Sox	G	A	CONH ₂
4	Ac	A	G	R	R	Y	S	L	C-Sox	DAla	A	CONH ₂

Table 4
Kinetics of Sox-substrates with AurA

Entry ^a	K_M^b (μM)	V_{max}^b (μmol mg ^{–1} min ^{–1})	Catalytic efficiency ^c
1	297.8 ± 3.9	1.7 ± 0.3	1
2	152.3 ± 2.7	2.6 ± 0.2	3
3	57.0 ± 8.2	2.2 ± 0.1	7
4	65.0 ± 7.0	1.8 ± 0.1	5

^a Peptides from Table 3.

^b Kinetic parameters (K_M and V_{max}) were obtained from initial slopes and corrected appropriately for substrate and product fluorescence as described in Supporting Information. The values reported are means ± SEM of duplicate experiments as calculated from a direct fit of [S]/v versus [S] plots.

^c Catalytic efficiency of each substrate was calculated as k_{cat}/K_M (min^{–1} μM^{–1}).

incubation with AurA for varying time periods (10 min, 30 min, 1 h, 2 h, and 24 h) followed by chemical derivatization with 4-MEP showed that, first, there was an elevated preference for an aromatic residue, Tyr or Phe, in the –1 position (Table 2, entry 3). For the remaining libraries, Phe was fixed in this position. Second, there was a high preference for Arg in the –3 position, which is in agreement with the consensus sequence described for AurA¹³ (entry 2). Third, in the +1 position, aromatic residues, such as Phe, were favored (entry 4). This result coincides with previous work done by Pinna et al.¹³ that identified Phe, Leu, and Ile in the –1 position (Leu and Ile were excluded from our screen). Fourth, small hydrophobic residues, mainly Gly, were selected for positions –4, +3, and +4 (entries 1, 5, and 6). Lastly, due to the preference for Gly at –4 and +3 positions, we made two additional peptide libraries using D-amino acids (entries 1 and 5). As in the case of the L-peptide libraries, AurA selected Gly in both positions and, interestingly, DAla in +3 position.

Several sequences that were selected by our screen were individually synthesized with C-Sox in place of 2-Nal and evaluated as probes for AurA. Tables 3 and 4 summarize the sequences and the kinetics parameters, respectively, of the best peptides. A 2-fold improved K_M and a 3-fold improved catalytic efficiency were obtained by incorporating a Phe residue at the –1 position (Tables 3 and 4, entry 2). A 6-fold improved K_M and a 5- to 7-fold improved catalytic efficiency compared to Kemptide was obtained with Gly (Tables 3 and 4, entry 3) or DAla (Tables 3 and 4, entry 4) at position +3. These peptides are the best substrates described so far for AurA.

Although chemical methods to detect phosphorylated products using mass spectrometry and fluorescence have been reported, none were able to obtain substrates with improved selectivity for the desired kinase.^{6d,18} Herein, we have presented a new screen that allows identification of substrates for serine/threonine kinases using a chemically modified combinatorial peptide library and MALDI-TOF MS. The strategy was first validated by obtaining Kemptide as the most selective PKA peptide, which is in full agreement with current literature. Moreover, the screen was applied to AurA resulting in a substrate with a 6-fold improvement in K_M and a 7-fold rise in catalytic efficiency with respect to the best

sequence described so far in the literature. Compared with the conventional approaches, this strategy is simple, easy to perform and it does not require complex instrumentation, the use of radioisotopes, or antibodies. The iterative nature of the method and its ability to incorporate unnatural elements (such as D-amino acids) should make searches for substrates of virtually any kinase possible. Lastly, the conversion of the most selective peptides into fluorescent Sox-containing probes should give a specific reporter for any kinase of choice.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.12.090.

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