High-throughput Screening of Peptide Substrates for Tyrosine Kinases Featuring Precisio™ Kinases and PEPscreen® Custom Peptide Library

Danhui Wang, Fan Zhang, Tao Zhao, Fei Zhong, and Keming Song

Sigma-Aldrich Corp.,
St. Louis, MO, 63103, United States

Introduction

Kinases are large group of proteins modulating a wide variety of cellular events, including differentiation, proliferation, metabolism and apoptosis, etc (1). Thus, kinases represent an important target for drug discovery (2). In the human genome, a total of 518 protein kinases are reported that are responsible for phosphorylation of over 30% of all cellular proteins (3). Therefore, better understanding various kinase pathways and their substrates has great impact in both basic research and drug development.

Kinase functions are mediated by a series of signal cascades that involve phosphorylation of specific amino acid residues in target protein substrates (4). Two major classes of protein kinases are: 1) Protein Serine/threonine Kinases (PSKs) and 2) Protein Tyrosine Kinases (PTKs), which are classified based on the amino acid at phosphorylation site in their substrates. The substrates for classical protein kinases vary from large proteins to small peptides to sugars or lipids. Thus, availability of known substrates/phosphorylation sites for a given kinase would be a limiting factor for kinase assay. Here, we report a high-throughput ELISA-based method to identify peptide substrates for class-specific and/or enzyme-specific protein kinases that can be utilized for the detection of kinase activity both *in vivo* and *in vitro*. The amino acid sequences of peptide library containing 13-mer tyrosine peptides were generated by a predicting algorithm developed in-house for all potential protein substrates of protein kinases identified from the public databases. Next, the peptides were synthesized using PEPscreen, a proprietary peptide synthesis platform, and then subjected to a screening of 39 PTKs using an ELISA-based method featuring Precisio Kinases.

Abstract

Protein Tyrosine Kinases (PTKs) play important roles in modulating a wide variety of cellular events, including differentiation, proliferation, metabolism and apoptosis. Those regulations are mediated by a series of signal cascades that involve phosphorylation of tyrosine residues in target proteins. Therefore, identification of the substrates for PTKs is very important for the studies in both basic research and drug development environment. Here, we report a high-throughput ELISA-based method to identify peptide substrates for class-specific and/or enzyme-specific PTKs that can be utilized for the detection of kinase activity both *in vivo* and *in vitro*. The amino acid sequences of peptide library containing 13-mer tyrosine peptides were generated by a predicting algorithm developed in-house for all potential protein substrates of PTKs identified from the public databases. Next, the peptides were synthesized using PEPscreen, a proprietary peptide synthesis platform, and then subjected to a screening of 39 PTKs using an ELISA-based method. The number of peptides selected for each PTK ranges from 2 to 71 with an average of 25 from a total of 376 peptides tested. The 173 sequences selected were classified into different groups with the reactivity to single, multiple, or all PTKs. The reactivity and specificity of the selected peptide substrates were further validated. The validation results indicated that this screening method has a very high sensitivity and reproducibility. Thus, the combination of our algorithm for selecting peptide sequences, the PEPscreen peptide synthesis platform, and ELISA-based assay, using Precisio Kinases, provide a successful high-throughput system for the screening of peptide substrates for many PTKs.

Materials and Methods

Unless otherwise indicated, all reagents and materials used in this work were obtained from Sigma-Aldrich (St. Louis, MO). The biotinylated peptides were synthesized on 96-well format (Sigma). PTKs were Precisio Kinases, obtained from Sigma-Aldrich (St. Louis, MO). Phosphorylated and unphosphorylated tyrosine peptides (Biotin-RRLIEDAEYAARG) were obtained from AnaSpec, Inc. (San Jose, CA). SigmaScreen™ Streptavidin-coated 384-well plate **(S8686)** was used to anchor biotinylated peptides. Anti-phosphotyrosine monoclonal antibody **(P5872)**, anti-mouse IgG alkaline phosphatase conjugate **(A3562)**, and p-nitrophenyl phosphate liquid substrate system **(p-NPP, N7653)** were used to detect phosphorylated tyrosine peptides. The optical density was obtained using SpectraMax Plus[384] microplate spectrophotometer (Molecular Device, Sunnyvale, CA).

Selection of tyrosine peptides

The potential substrates of identified pharmaceutical important kinases were obtained by searching public databases (Phospho.ELM, and PhosphoSite). The FASTA sequence file including all the amino acid sequences of the substrates were then compiled and loaded onto KinasePhos database **(http://kinasephos.mbc.nctu.edu.tw/index.html)** searching for the potential phosphorylation sites of each substrate. The output files were then modified by a computer program to identify the 13-mer peptides covering the potential phosphorylation sites. The commercially available peptide substrates for tyrosine kinases were used as the positive controls for screening.

Preparation of peptide library for screening

The peptides were reconstituted with 50% Acetonitrile at the final concentration of 5 mM. Four 96-well plates of peptides were reformatted into one 384-well plate. The 5 mM peptides were diluted to 0.5 mM with Tris-buffered saline (TBS) as working solutions for screening.
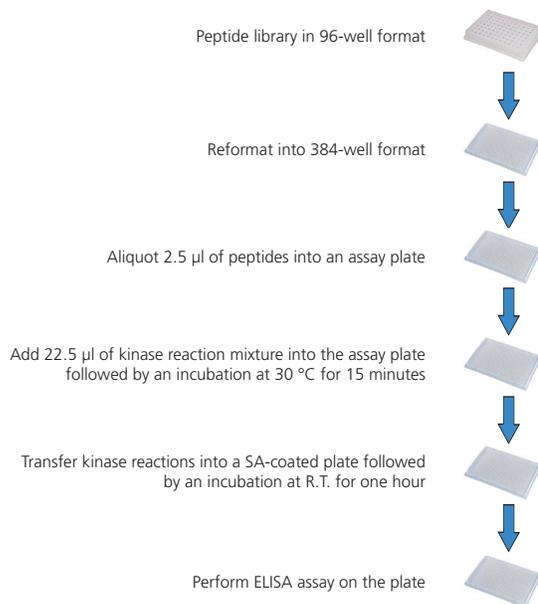
Kinase reaction

The kinase reaction buffer, the amounts of kinase and ATP for each reaction were used as indicated in the product information from the vendor. 2.5 µl of each 0.5 mM peptide was aliquoted into each well of a 384-well plate followed by adding 22.5 µl of kinase reaction mixture including kinase reaction buffer, ATP, and kinase. The plate was then incubated at 30 °C for 15 minutes with constant shaking of 120 rpm. 6 µl of 0.5 M EDTA was then added into each well to stop the kinase reaction.

ELISA assay

The kinase reaction solutions were transferred into a 384-well Streptavidin-coated plate followed by an incubation of one hour at room temperature with shaking. The solutions were then removed followed by three washes with 50 µl of Tris-buffered saline with TWEEN-20 (TBST). 40 µl of mouse anti-phosphotyrosine antibody (1:2,000 dilution) was added into each well followed by an incubation of one hour at room temperature with shaking. The solutions were then removed followed by four washes with 50 µl of TBST. 40 µl of anti-mouse IgG conjugated alkaline phosphatase (1:30,000 dilution) was added into each well followed by an incubation of one hour at room temperature with shaking. The solutions were then removed followed by four washes with 50 µl of TBST. 40 µl of p-NPP was then added into each well followed by an incubation of 30 minutes at room temperature with shaking. 8 µl of 3 M sodium hydroxide was then added into each well to stop the enzymatic reaction. The optical absorptions at 405 nm were obtained by placing the plate into the plate reader.

Overview of Screening Procedure

Peptide library in 96-well format

Reformat into 384-well format

Aliquot 2.5 µl of peptides into an assay plate

Add 22.5 µl of kinase reaction mixture into the assay plate followed by an incubation at 30 °C for 15 minutes

Transfer kinase reactions into a SA-coated plate followed by an incubation at R.T. for one hour

Perform ELISA assay on the plate

SIGMA-ALDRICH®

Results – Establishment and Optimization of Screening Platform

## ELISA-based assay offers both sensitivity and selectivity for the screening of peptide substrates
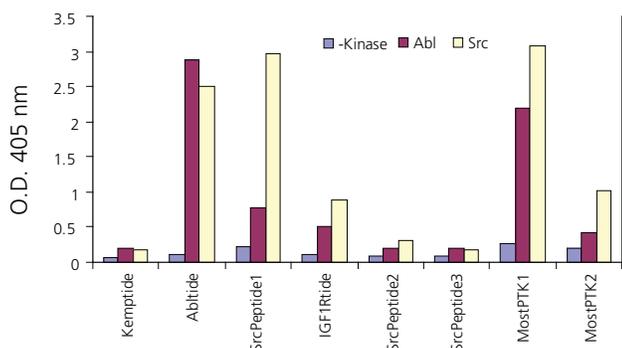
Figure 1: A total of nine biotinylated peptides were synthesized and the kinase assays were performed with Abl or Src kinase as described in Materials and Methods. The phosphorylated tyrosine residue was then detected by an ELISA-based assay using anti-phosphotyrosine antibody. The results shown in the graph are the averages of two replicates. Kemptide: Biotin-LRRASLG-OH (a serine peptide as negative control); Abltide: Biotin-EAIYAAPFAKKK-OH; Src peptide1: Biotin-KVEKIGEGTYGVVYK-OH; IGF1Rtide: Biotin-KKKSPGEYVNIEFG-OH; Src peptide2: Biotin-IYGEF-OH: Src peptide3: Biotin-TSTEPQYQPGENL-OH: Most PTK1: Biotin-KKKGPWLEEEEEAYGWLDF-OH; Most PTK2: Biotin-RRLIEDAEYAARG-OH.

Optimization of ELISA-based assay platform
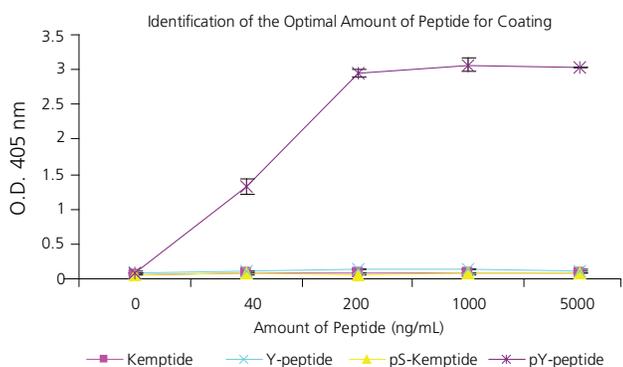
Figure 2a: A total of four biotinylated peptides were synthesized and then coated on the Streptavidin plate at 5 µg/mL, 1 µg/mL, 0.2 µg/mL, and 40 ng/mL. The ELISA-based assay was performed as described in Materials and Methods. Kemptide: Biotin-LRRASLG-OH; pS-Kemptide: Biotin-LRRApSLG-OH; Y-peptide: Biotin-RRLIEDAEYAARG-NH2; pY-peptide: Biotin-RRLIEDAEpYAARG-NH2.
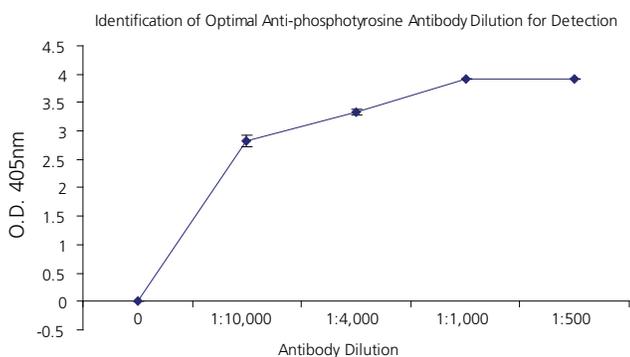
Figure 2b: 50 µl of pY-peptide (200 ng/mL) was used to coat Streptavidin plate. The phosphorylated tyrosine was detected by a series dilution of anti-phosphotyrosine antibody following the protocol described in Materials and Methods.

Results – Screening of Peptide Substrates for Thirty-nine Tyrosine Kinases

Primary Screening Results

Kinase	Number of Peptides Screened	Number of Positive Hits	Range of Fold / Background	Cut-off Value for Selection
AXL	384	0	1-2.3	2.5
EphA1	384	9	1-10.6	2.5
EphA2	384	20	1-11.4	3
EphB1	384	49	1-15.3	4
EphB2	384	48	1-15.3	2.5
EGFR	384	71	1-11.8	4
ERBB2	384	8	1-7.5	2.5
ERBB4	384	13	1-10.7	2.5
FGFR1	384	34	1-10	3
FGFR2	384	55	1-12	4.5
FGFR3	384	5	1-11	4
FGFR4	384	3	1-11.8	2.5
Met	384	12	1-8	3
IR	384	3	1-7.6	2.5
IGFI-R	384	15	1-19.5	3
TRKA	384	18	1-13.2	4
TRKB	384	56	1-9.3	3
PDGFα-R	384	13	1-6.3	3
PDGFβ-R	384	15	1-5.9	3
KIT	384	7	1-4.8	2.5
KDR	384	60	1-10.8	3
FLT3	384	30	1-10.7	3
Abl	384	12	1-12	6
BTK	384	8	1-6.6	2.5
CSK	384	9	1-7.3	3
FAK	384	10	1-5.6	2.5
FES	384	25	1-13	4.5
JAK2	384	62	1-11.6	7
ZAP70	384	1	1-3	2.5
Src	384	28	1-15	7
BLK	384	54	1-12.2	3.5
FGR	384	14	1-5.5	2.5
FYN	384	19	1-15.6	4
HCK	384	35	1-19.9	6.5
LCK	384	13	1-15.9	4
LynA	384	40	1-13.9	3.5
LynB	384	13	1-13.8	3
YES	384	44	1-11.7	4
FRK	384	20	1-10.7	4

Table 1

Validation of Selected Peptides from Primary Screening
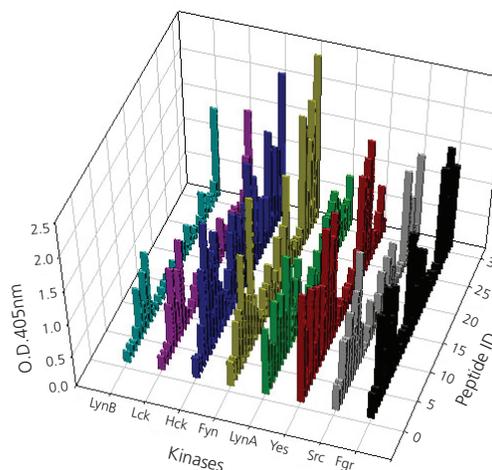
Figure 3: A total of 170 peptide substrates, divided in three groups based on different tyrosine protein kinases, have been selected after primary screening. Validation of peptide candidates was performed by ELISA assay with three repeats for each enzymatic reaction. This figure shows the validation results of activity assay for Tyrosine kinases of Src family against selected candidate peptides. The validation data are consistent and positively correlate more than 80% of results obtained in primary screening.

Screening Summary of Tyrosine Peptide Library Showed Reactivity with Thirty-nine PTKs

	Number of Peptides
Single PTK	47
Non-receptor PTKs	14
Receptor PTKs	17
Non-receptor or Receptor PTKs	96

Table 2

Reactivity of Selected Seven Peptides with Thirty-nine PTKs

Kinase	58	125	174	363	97	102	103
AXL							
EphA1					x	x	x
EphA2			x		x	x	x
EphB1			x		x	x	x
EphB2			x			x	x
EGFR			x			x	x
ERBB2					x		
ERBB4							x
FGFR1			x				x
FGFR2			x	x	x	x	x
FGFR3					x		
FGFR4							x
Met					x		
IR							
IGFI-R			x	x			x
TRKA					x		x
TRKB			x	x	x		x
PDGFα-R					x		
PDGFβ-R					x		
KIT							x
KDR			x			x	x
FLT3						x	x
Abl							x
BTK	x						x
CSK						x	x
FAK					x		x
FES	x					x	x
JAK2							
ZAP70							
Src	x	x				x	x
BLK	x	x				x	x
FGR					x		
FYN	x	x				x	x
HCK	x	x				x	x
LCK	x				x	x	x
LynA	x	x				x	x
LynB	x				x	x	x
YES	x	x				x	x
FRK					x	x	x

X: positive reactivity.

Table 3

Results

With Sigma-Aldrich's PEPscreen Custom Peptide Synthesis capability, we have made one peptide library with a total of 384 peptides for substrate screening. All peptides were lyophilized in a 96-well plate format. The successful rate for peptide synthesis is over 99%, only less than 1% of peptide synthesis failed. Upon reconstitution of peptides in suitable solution (50% acetonitrile), peptides were subjected to high-throughput ELISA screening for kinase activity with each given protein kinase in 384-well plate.

Before the screening for PTK peptide substrates, we synthesized a set of biotinylated peptide substrates specific for kinase Abl, Src, IGF1R, or non-specific peptide substrates for most PTKs to test the binding selectivity of SA-coated plate and antibody specificity for phosphorylated tyrosine peptides by an ELISA-based assay using anti-phosphotyrosine antibody. The results shown in Figure 1 indicated that the ELISA-based assay offered both sensitivity and selectivity for peptide screening with specific detection of phosphorylated tyrosine peptides. Next, the optimal amount of biotinylated peptide for binding to SA-coated plate was identified to be 10 ng in a total of 50 µl reaction (Figure 2A). Also, the optimal dilution of a Sigma's Anti-phosphotyrosine monoclonal antibody (P5872) was 1:1000 (Figure 2B).

Primary screen was conducted with 384 peptides from the PTK library against each of 39 PTKs using Precisio Kinases. The number of positive peptide substrates screened for each PTK was summarized in Table 1. Based on the primary screening results a total of 170 peptides were selected as potential substrate candidates for single or multiple PTKs and validated by a second round assays with three replicates for each peptide. Figure 3 shows an example of the validation results for Tyrosine kinases of Src family against selected candidate peptides from primary screening. The validation data are consistent and positively correlate more than 80% of results obtained in primary screening.

Among those validated peptides, some were specific to single kinases, some were for group of non-receptors or receptors, and some were for both receptors and non-receptors (Table 2). Finally, a total of seven peptides were selected and tested for their reactivity to each of the 39 PTKs selected (Table 3).

Conclusions

- The Algorithm developed in house for selecting peptide sequences works well as 50% of the peptides selected showed reactivity to single or multiple kinase.
- The synthesis of peptide library using PEPscreen method is very successful as only 1% of a total of 384 peptides failed.
- The ELISA-based assay offers both sensitivity and selectivity for the screening of peptide substrates for Tyrosine kinases.
- Precisio Kinases provide highly active recombinant protein kinases for high throughput kinase assays with consistent and reliable results.

Acknowledgements

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References

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3. Manning aet al. (2002) Trends in Biochem. Sci. 27, 514-20.
4. Hunter, T. (1987) Cell 50, 823-29.

Related Products

Product	Prod.No.
PEPscreen® Custom Peptide Library	Custom Order
SigmaScreen™ Streptavidin-coated 384-well Plate	S8686
Anti-phosphotyrosine Monoclonal Antibody	P5872
Anti-mouse IgG Alkaline Phosphatase Conjugate	A3562
p-Nitrophenyl Phosphate Liquid Substrate System	N7653
Tris Buffered Saline (TBS)	T6664
Tris Buffered Saline with Tween 20 (TBST)	T9039
AXL (473-end), Active, Human, His tagged	A4736
EphA1, Human recombinant	E7902
EPHA2 (561-end), Active, Human, GST tagged	E7284
EPHB1 (591-end), Active, Mouse, GST tagged	E7032
EPHB2 (570-end), Active, Human, GST tagged	E7157
Epidermal Growth Factor Receptor From Human	E3641
HER2 (676-end), Active, Human, GST tagged	H3040
HER4 (682-993), Active, Human, GST tagged	H3165
FGFR1 (FLT2) (399-822), Active, Human, GST tagged	F5055
Fibroblast Growth Factor receptor 2β (iiib)/Fc Chimera from mouse	F1927
FGFR3 (397-end), Active, Human, GST tagged	F7930
MET (956-end), Active, Human, GST tagged	M0574
InsR (1011-end), Active, Human, GST tagged	I2535
IGF1R (960-end), Active, Human, His tagged	I0786
TRKA (440-end), Active, Human, GST tagged	T4202
TRKB (455-end), Active, Human, GST tagged	T2080
FYN A, Active, Human, GST tagged	F6557
HCK (230-497), Active, Human, GST tagged	H2915
LCK, Active, Human, GST tagged	L2792
LYN B, Active, Human, GST tagged	L2670
FRK (208-end), Active, Human, GST tagged	F8305
PDGFRα (550-end), Active, Human, GST tagged	D0946
PDGFRβ (557-end), Active, Human, GST tagged	G8671
c-KIT (544-end), Active, Human, GST tagged	C0624
KDR (789-end), Active, Human, GST tagged	K2643
FLT3 (571-993), Active, Human, GST tagged	F6432
ABL1 (27-end), Active, Mouse, His tagged	A0608
BTK, Active, Human, His tagged	B4312
CSK, Active, Human, GST tagged	C1495
FAK (393-698), Active, Human, His tagged	F7680
FES, Active, Human, GST tagged	F4930
ZAP-70 Tyrosine Kinase,His tagged	Z2126