Analytical Biochemistry 413 (2011) 30–35

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Antibody-free peptide substrate screening of serine/threonine kinase (protein kinase A) with a biotinylated detection probe

Mira Kim [a], Yong-Sun Park [a], Dong-Sik Shin [a], Jaehi Kim [a], Byung-Gee Kim [a,b], Yoon-Sik Lee [a,*]

[a] School of Chemical and Biological Engineering, Seoul National University, San 56-1, Shilim-dong, Kwnak-gu, Seoul 151-744, South Korea
[b] Interdisciplinary Program for Biochemical Engineering and Biotechnology, Seoul National University, San 56-1, Shilim-dong, Kwnak-gu, Seoul 151-744, South Korea

ARTICLE INFO

Article history:
Received 25 October 2010
Received in revised form 7 January 2011
Accepted 2 February 2011
Available online 3 March 2011

Keywords:
Ser/Thr kinase
Combinatorial chemistry
Peptide library
Phospho-peptide
β-Elimination

ABSTRACT

Being different from anti-phosphotyrosine antibodies, anti-phosphoserine- or anti-phosphothreonine-specific antibodies with high affinity for the detection of serine/threonine kinase substrates are not readily available. Therefore, chemical modification methods were developed for the detection of phosphoserine or threonine in the screening of protein kinase substrates based on β-elimination and Michael addition. We have developed a biotin-based detection probe for identification of the phosphorylated serine or threonine residue. A biotin derivative induced a color reaction using alkaline phosphate-conjugated streptavidin that amplified the signal. It was effective for the detection and separation of the target peptide on the resin. The detection probe was successfully used in identifying PKA substrates from peptide libraries on resin beads. The peptide library was prepared as a ladder-type, such that the active peptides on the colored resin beads were readily sequenced with the truncated peptide fragments by MALDI-TOF/MS analysis after releasing the peptides from the resin bead through photolysis.

Protein kinases catalyze the phosphorylation of Ser, Thr, and Tyr residues in peptides and proteins with adenosine-triphosphate (ATP)[1] [1–3], which regulates cellular functions, such as cell-cycle regulation, apoptosis, and metabolism [4]. Moreover, protein kinases are involved in diseases including cancer and inflammation. Identifying phosphorylation in specific peptide sequences of substrates plays a crucial role for developing kinase inhibitors for drug discovery [5–7]. Various approaches for detecting phosphoryl residue in peptides have been investigated with the goal of determining the primary sequence of the protein kinase phosphorylation site [8–10].

Radiolabeling of target peptides with [γ-^{32}P]ATP is a traditional method for the detection of phosphorylation [11,12]. However, it requires long analysis time and special handling procedures for radioactivity measurements with X-ray instrumentation. In addition, a fluorophore-conjugated metal ion complex was used to capture the phosphorylated peptide and to detect the phosphorylation event [13–15]. Despite versatility and ease of use, the chelating reaction has a disadvantage because of competition reactions of a metal ion complex on a phosphate group with other anionic compounds including ATP and negatively charged amino acid sequences.

Alternatively, an antibody-based detection method has been developed to identify phosphorylated proteins [16,17]. Recently, our group reported a screening method for Tyr kinase substrates using a ladder-type peptide library. The substrate specificities of protein Tyr kinases, such as p60$^{c\text{-}src}$, ZAP-70, and Brk, were identified using an anti-phospho-Tyr antibody [18,19]. Although anti-phospho-Tyr antibodies have shown good affinity, selectivity, and availability, only a few antibodies that specifically recognize a certain substrate of a Ser/Thr kinase-mediated phosphorylation have been discovered [20–22].

As an alternative detection heuristic, chemical modification methods based on β-elimination and Michael addition were developed to detect phospho-Ser/Thr residues and to avoid nonspecific interactions with immune-affinity and metal-affinity systems [23–27]. Here, we report a new chemical modification method for the detection of phospho-Ser/Thr, which is suitable for the screening of protein kinase substrates, in lieu of an antibody-based method.

A thiol group containing a biotin derivative was designed as a detection probe for phospho-Ser/Thr. After phosphorylation of the Ser/Thr-containing ladder-type peptides on resin beads, the

* Corresponding author. Fax: +82 2 876 9625.
E-mail addresses: redcat00@snu.ac.kr1 (M. Kim), gastight@hotmail.com (Y.-S. Park), dongss2@snu.ac.kr (D.-S. Shin), susia45@yahoo.co.kr (J. Kim), byungkim@snu.ac.kr (B.-G. Kim), yslee@snu.ac.kr (Y.-S. Lee).

[1] *Abbreviations used:* Fmoc, *N*-(9-fluorenylmethoxycarbonyl; DMF, dimethylformamide; NMP, *N*-methylpyrrolidone; DIEA, *N*,*N*-diisopropylethylamine; NHS, *N*-hydroxysuccinimide; DCC, dicyclohexylcarbodiimide; PS, polystyrene; BOP, benzotriazol-1-yloxy-tris(dimethylamino) phosphonium hexafluorophosphate; HOBt,1-hydroxy-benzotriazole; Tricine, *N*-(tri(hydroxymethyl) methyl)glycine; MALDI-TOF/MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; BCIP, 5-bromo-4-chloro-3′-indolyl phosphate p-toluidine salt; NBT, nitroblue tetrazolium chloride; TEG, tetraethyleneglycol; AP, alkaline phosphatase; SA, succinic acid.

0003-2697/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2011.02.005

phosphorylated peptides were converted by β-elimination to the α, β-unsaturated peptide residues which could be captured by the thiol group-containing biotin probe and recognized by alkaline phosphatase (AP)-conjugated streptavidin-derived colorimetric reaction. Consequently, the active peptide substrates on the colored resin bead could be sequenced by MALDI-TOF/MS after release from the bead (Fig. 1).

Materials and methods

Synthesis of biotin–TEG–Cys–NH_2

For biotin–TEG (tetraethyleneglycol)–Cys–NH_2 synthesis, Cys, TEG, and biotin were coupled to a Rink-amide resin using the solid-phase BOP/HOBt-coupling method and Fmoc chemistry (Scheme 1).

Fmoc–TEG–succinic acid (SA), used as a building block, was prepared separately in solution-phase synthesis [28,29]. 4,7,10-Trioxa-1,13-tridecanediamine (10 mmol) was reacted with succinic anhydride (10 mmol) in acetonitrile to yield H_2N–TEG–SA. Then, Fmoc–TEG–SA was prepared from the reaction of H_2N–TEG–SA and Fmoc–OSu (11 mmol) with DIEA in acetonitrile. The purity of the product was 93%, and the yield was 59%.

Biotin-NHS was synthesized from biotin (5 mmol), NHS (5 mmol), and DCC (5 mmol) in DMF.

Fmoc–Cys(Trt)-OH (2 eq) and Fmoc–TEG–succinic acid were sequentially coupled to a Rink-amide MBHA resin (0.6 NH_2 mmol/g) in the presence of BOP (2 eq), HOBt (2 eq), and DIEA (4 eq). The Fmoc group was removed from the resin in a 20% piperidine/DMF solution. Biotin–NHS (2 eq) was coupled to the NH_2–TEG–Cys–Rink amide resin in the presence of DIEA (2 eq). Biotin–TEG–Cys–NH_2 and the Trt protecting group on the Cys residue were cleaved from the resin by Reagent K (TFA:phenol:H_2O:thioanisole:1,2-ethanedithiol = 82.5:5:5:5:2.5). The purity of biotin-TEG–Cys–NH_2 was confirmed by MADLI-TOF/MS and HPLC (66% purity and 71% yield based on the amount of Cys).

Ladder-type peptide synthesis on the resin

Kemptide (LRRASLG) and the random peptide library were synthesized on a HiCore resin (core–shell-type PEGylated PS resin). HiCore resin was prepared from chloromethyl (CM) PS resin using a method previously reported in the literature [30]. For introduction of the linker and spacer to the HiCore resin, the photolabile linker [31,32] and spacer, βAla-εACA-βAla-εACA ($\beta\varepsilon\beta\varepsilon$, where β is β-alanine and ε is ε-aminocaproic acid), were coupled to the resin with the Fmoc strategy of solid-phase peptide synthesis.

To synthesize Kemptide on the resin in a ladder-type configuration, each of the corresponding Fmoc amino acids (4 eq) was coupled in series to the NH_2–$\beta\varepsilon\beta\varepsilon$–PLL–resin in the presence of Ac-Gly (0.2 eq), BOP (4.2 eq), HOBt (4.2 eq), and DIEA (8.4 eq). The product, Ac-Gly-L-R-R-A-S-L-G-$\beta\varepsilon\beta\varepsilon$–PLL–resin, was confirmed by sequencing with MALDI-TOF/MS analysis after UV photocleavage (360 nm).

For a ladder-type random peptide library to be used in a Ser/Thr kinase assay, the spacer and linker-conjugated resin (1.6 g) was divided into 16 equal parts, which corresponded to 16 amino acids (the natural amino acids except for Ser, Thr, Tyr, and Cys). The ladder-type peptide library was prepared via the split-and-mix method. At every coupling steps, each resin portion was reacted with Fmoc amino acid (4 eq), Ac-Gly (0.2 eq), BOP (4.2 eq), HOBt (4.2 eq), and DIEA (8.4 eq) in NMP. After coupling, the resins were combined in a reaction tube, washed, and dried under reduced pressure.

For quantification of amino groups, ca. 100 mg of resin was treated with a 20% piperidine/DMF solution and filtered, and the filtrate solution was titrated by UV (290 nm) absorbance to calculate the amount of available amine groups after partial capping with Ac-Gly. After removing the Fmoc group, the resin was divided into 16 equal parts and reacted with Fmoc amino acid. These steps were repeated, and the side-chain protecting groups were deprotected by cleavage cocktail (Reagent K) at the final step.

Kinase assay and detection

Kemptide-coupled resin (5 mg) was reacted with 1000 U PKA (protein kinase A) and 1 mM ATP in an assay buffer solution containing Tricine (0.9% w/v), $MgSO_4$ (0.12% w/v), DTT (0.04% w/v), EDTA (0.0016% w/v), and BSA (0.1% w/v) at 37 °C for 30 min. As a control reaction, Kemptide-coupled resin was reacted with the same buffer solution without PKA.

β-Elimination of phosphorylated peptide resin was accomplished under alkaline conditions with 0.5 N NaOH:DMSO:*i*-PrOH

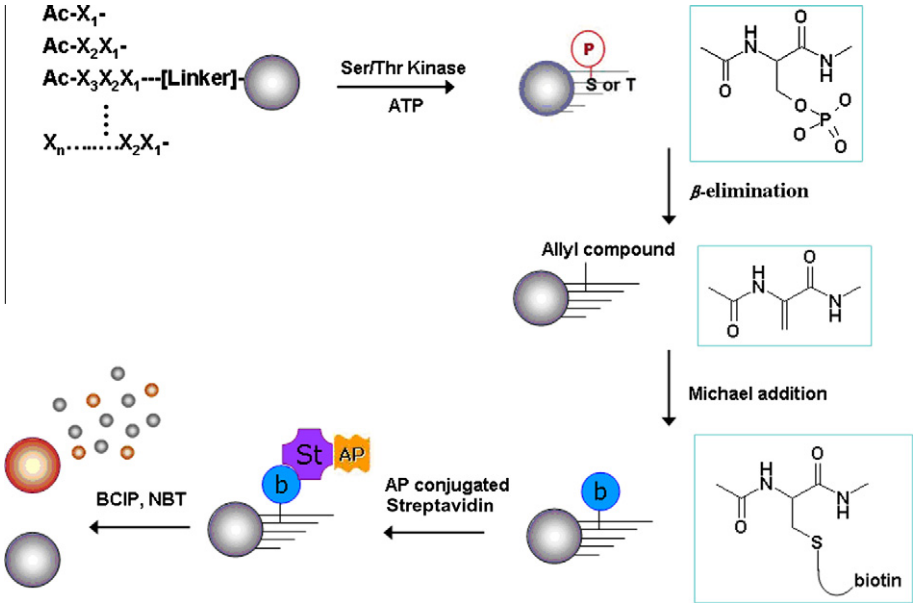
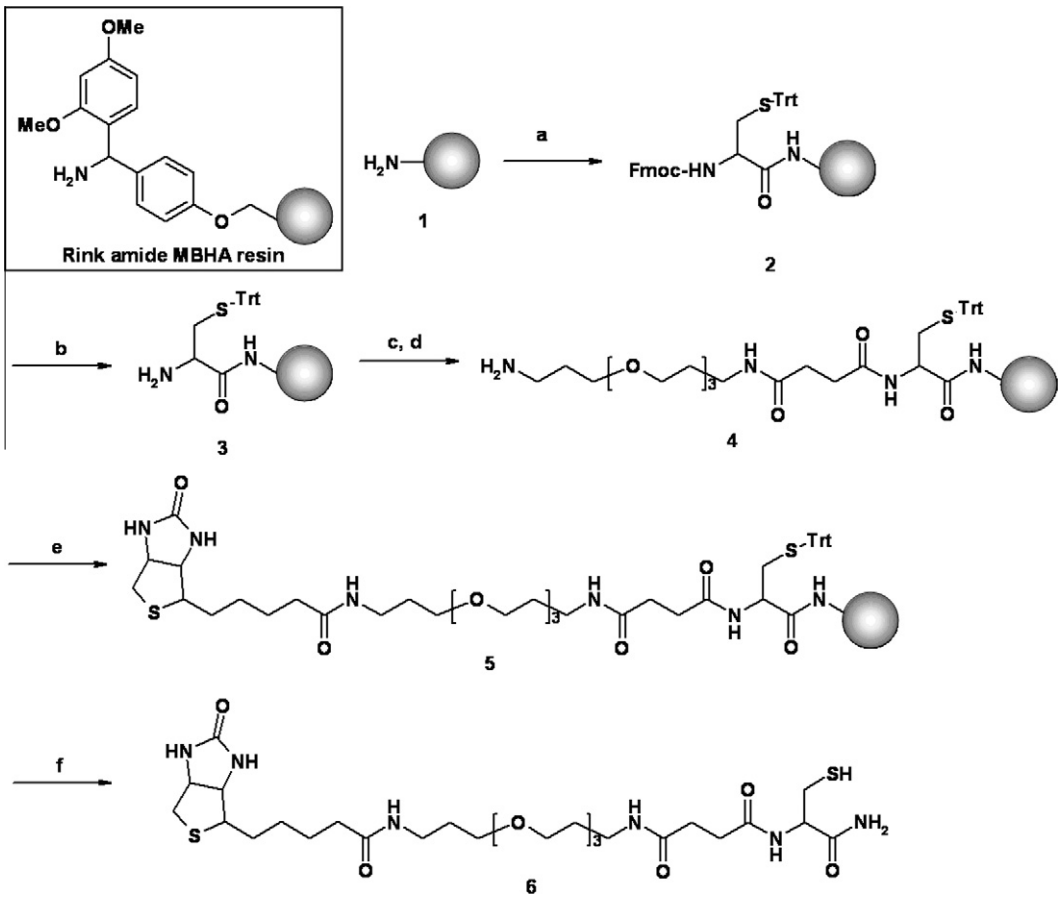


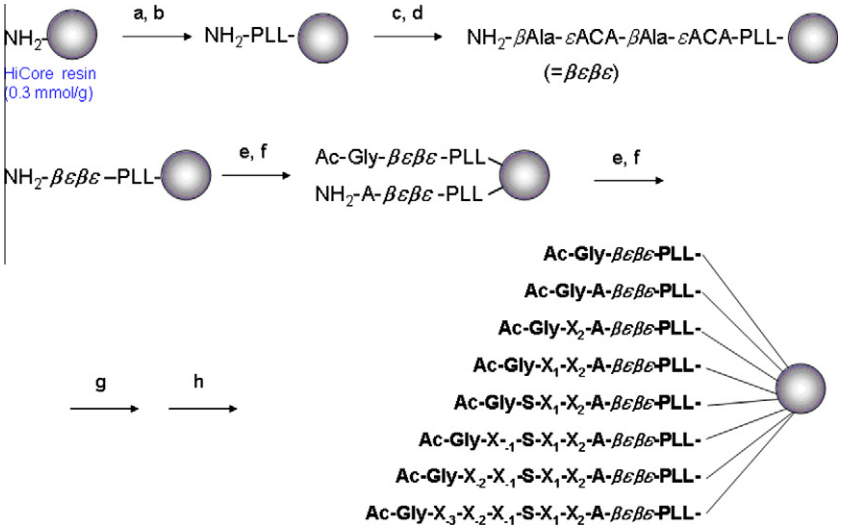
Fig.1. Outline of the detection method of phospho-Ser/Thr. (AP, alkaline phosphatase; NBT, nitroblue tetrazolium; BCIP, bromo-chloroindolyl phosphate).



Scheme 1. Preparation of biotin-TEG–Cys–NH_2: (a) Fmoc–Cys(Trt), BOP, HOBt, DIEA, NMP; (b) 20% piperidine/DMF; (c) Fmoc–TEG–COOH, BOP, HOBt, DIEA, NMP; (d) 20% piperidine/DMF; (e) biotin–NHS, DIEA, DMF; and (f) Reagent K (TFA, phenol, H_2O, thioanisole, 1,2-ethandithiol).

(3:5.5:1.5) at 50 °C for 4 h. Michael addition was performed with biotin–TEG–Cys–NH_2 (2 mM) in 0.05 N NaOH:DMSO:*i*-PrOH (3:5.5:1.5). Then each sample was treated with alkaline phosphatase-conjugated streptavidin (AP-ST, 1 U/mL) for 1 h. The AP on the resin promoted a color reaction (purple) with 5-bromo-4-chloro-3′-indolyl phosphate *p*-toluidine salt (BCIP) and nitroblue tetrazolium chloride (NBT) in Tris buffer (pH 9.5). After the color reaction, the reaction was quenched by adding 1 N HCl solution.

PKA substrate screening was performed on the peptide library-conjugated resin (25 mg) using 2500 U PKA and 1 mM ATP in an assay buffer solution. After β-elimination and Michael addition with biotin–TEG–Cys–NH_2, color reaction was performed through



Scheme 2. Preparation of the ladder-type peptide library: (a) Fmoc-photolabile linker (PLL), BOP, HOBt, DIEA, NMP; (b) 20% piperidine/DMF; (c) Fmoc-ε-aminocaproic acid or Fmoc-β-alanine, BOP, HOBt, DIEA, NMP; (d) 20% piperidine/DMF; (e) Fmoc-Ala (4 eq), Ac-Gly (0.2 eq), BOP, HOBt, DIEA, NMP; (f) 20% piperidine/DMF; (g) Fmoc-amino acid, AcGly (2 eq), BOP, HOBt, DIEA, NMP; and (h) Reagent K (TFA, phenol, H_2O, thioanisole, 1,2-ethandithiol).

AP-ST treatment and BCIP/NBT reaction. One hundred reddish-brown colored beads were picked during a fourfold assay (4×25 mg resin), and each peptide together with its ladder-type fragments was released from the resin by UV irradiation. The peptide sequences were identified by MALDI-TOF/MS analysis and 88 beads were sequenced successfully. Detailed conditions of each experimental step are described in the Supplementary Material.

Results and discussion

Synthesis of biotin–TEG–Cys–NH_2

Biotin–TEG–Cys–NH_2 was designed as a thiol-containing biotin probe. Cys was added because the thiol group could act as a nucleophile in the Michael addition step. Biotin at the end of the probe served as a binding ligand of streptavidin for a colorimetric reaction. TEG was added to the probe for water compatibility. The detection probe was prepared using the Fmoc strategy for solid-phase peptide synthesis. Fmoc–Cys, Fmoc–TEG-–SA, and biotin were successively coupled to the Rink-amide resin. Then, biotin-*N*-hydroxysuccinimide (biotin–NHS) was coupled to the TEG–Cys residue on the resin. After cleavage from the resin, the biotin–TEG–Cys–NH_2 was obtained as a sticky solid.

Ladder- type peptide library synthesis on the resin

A photolabile linker and spacer ($\beta\varepsilon\beta\varepsilon$) were successfully introduced to the resin bead. The $\beta\varepsilon\beta\varepsilon$ was added to avoid the overlap of peaks in mass analysis and to increase the accessibility of biomolecules.

Then, the ladder-type peptide was constructed on the resin using a partial capping method. To prepare the ladder-type peptide library, Ac-Gly was chosen as a capping reagent instead of acetic acid (as in our previous work) because capping was easier to control than the use of acetic acid. To produce a longer and more random peptide library, precise control of capping is necessary to perform the reliable bioassay with full sequence peptides and, at the same time, to properly interpret sequencing information.

First, Ac-Gly-L-R-R-A-S-L-G-$\beta\varepsilon\beta\varepsilon$-PLL-resin was prepared for the model reaction with PKA and we confirmed that the ladder-type peptide was successfully synthesized through MALDI-TOF/MS analysis after UV photocleavage.

Next, a random peptide library was synthesized on the spacer-conjugated resin using the split-and-mix synthesis method in a one-bead one-compound (OBOC) approach (Scheme 2). Quantitative analysis of a truncated peptide library pool showed that the amount of available free amine groups for coupling on the resin gradually decreased over the course of the synthesis. The amount of each ladder peptide fragment equals the difference of available free amine groups before and after coupling (Fig. 2).

Model test with Kemptide-coupled resin and PKA

To verify the detection system, Kemptide, a well-known peptide substrate of protein kinase A [33,34], was synthesized on a HiCore resin (PEGylated PS resin) as a ladder-type peptide by partial capping with *N*-acetylglycine (Ac-Gly) at each coupling step. After phosphorylation, the Kemptide-coupled resin was treated under alkaline conditions for β-elimination [35,36]. Then, biotin–TEG–Cys–NH_2 was added to the resulting resin for Michael addition. We determined that the optimal conditions for β-elimination were 50 °C for 2 h and for Michael addition were 25 °C for 1 h. Kemptide-coupled resin, when treated with ATP and PKA, turned to a brown color, while a control sample, which was treated without PKA, remained yellow after AP-conjugated strepatavidin addition and the NBT/BCIP colorimetric reaction. We could clearly distinguish the color difference between the phosphorylated sample and the control sample.

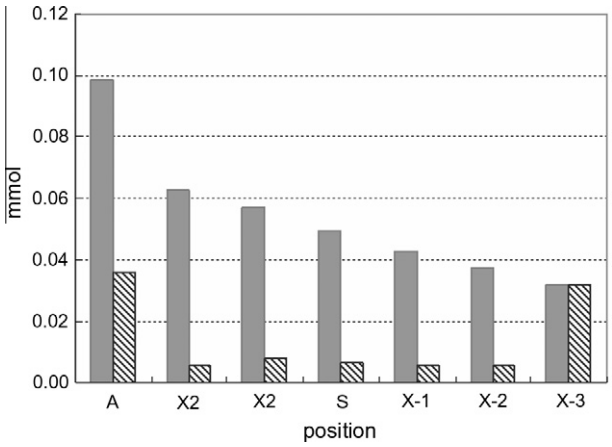


Fig.2. Fmoc titration of peptide resin at each coupling step: the gray solid bar represents the free amine group at each coupling step, and the striped bar represents truncated peptide.

PKA substrate screening with the random peptide library

The ladder-type peptide library on the resin was incubated with PKA and ATP and treated with an alkaline solution for β-elimination followed by the addition of biotin–TEG–Cys–NH_2. After the colorimetric reaction, dark brown resin beads were hand-picked under a microscope (Fig. 3). The full-length peptide and its subpeptides containing truncated sequences were released from the resin by UV photocleavage, and the sequence of the peptide was easily identified by mass analysis.

The PKA substrate profiling results are summarized in Fig. 4, and the priorities of amino acid sequences are listed in Table 1. Arg specificity at X_{-3} (57%) and hydrophobic residue specificity (73%) (e.g., Val, Ala at the X_1 position) were clearly observed in

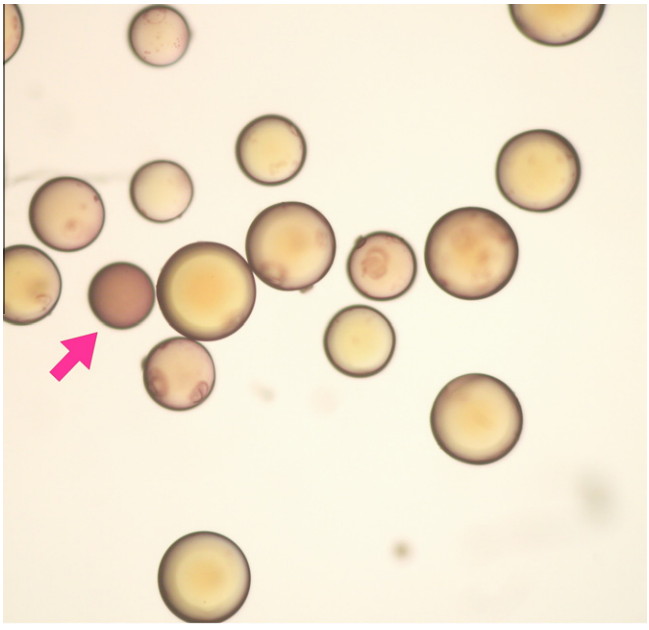


Fig.3. Optical microscope images of ladder-type peptide libraries on resin beads after PKA assay and colorimetric reaction.

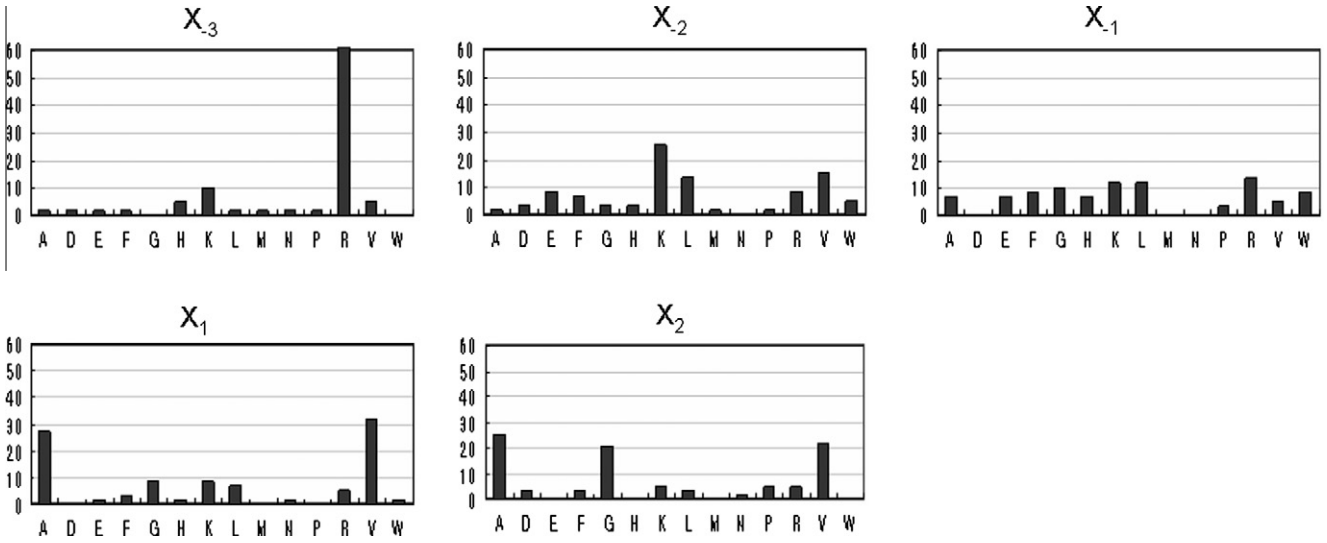


Fig.4. PKA substrate peptide profiling results.

Table 1
Sequence frequency at each position of the PKA substrates.

Occurrence frequency	X_{-3}	X_{-2}	X_{-1}	X_1	X_2
Most	R (57%)	K/Q (23%)[a]	R (14%)	V (36%)	A (25%)
Second most	K/Q (14%)[a]	V (15%)	L/I (12%)[b]	A (21%)	V (23%)

[a] K(Lys) and Q(Gln) have similar molecular weights.
[b] L(Leu) and I(Ile) have identical molecular weights.

Table 2
Proteins matched to the screened substrate sequence of PKA (NetworKIN) [40].

Screened peptide sequence	Real protein sequence	Protein substrate of PKA
RKLSVA	RKLSVA	ATP-binding cassette subfamily A member 1
RKVSVA	RKVSLA	Cystic fibrosis transmembrane conductance regulator

the PKA assay. Lys (or Gln; Lys and Gln have identical same molecular weights) was frequently found at the X_{-3} (15%) and X_{-2} (23%) positions. These results are in good agreement with the previous PKA assay results, which revealed that PKA has two Glu residues as anionic binding sites and a hydrophobic site [37–39]. The anionic sites correspond to the X_{-3} and X_{-2} positions, and the hydrophobic site corresponds to the X_1 position. Therefore, the motif of the phosphorylation site is Arg or Lys at the X_{-3} and X_{-2} position and a hydrophobic residue at the X_1 position. We found that some peptide sequences are identical or similar to the substrate protein sequences through a kinase substrate data base search (NetworKIN) (Table 2) [40].

The detailed results of the synthesis and PKA assay of Kemptide-coupled resin and additional peptide sequences in substrate screening are described in the Supplementary Material.

Conclusion

We developed a thiol-containing biotin compound as a detection probe of phospho-Ser/Thr in a protein kinase assay. Our detection system played a key role in the screening of the peptide substrate specificity of PKA. Resin beads containing phosphorylated peptides after PKA treatment were easily recognized by color change that could be distinguished by the naked eye, and were easily separated from the resin bead mixture. We expect that our screening tool can be applied to other Ser/Thr kinase substrate screening systems without the use of an anti-phospho-antibody.

Acknowledgments

This work was supported by the Korea Biotech R&D Group of the Next-Generation Growth Engine Project (F104AB010005-07A0201-00510) and by the Seoul R&BD program (10538).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2011.02.005.

References

[1] K. Helmbrecht, E. Zeise, L. Rensing, Chaperones in cell cycle regulation and mitogenic signal transduction: a review, Cell. Prolif. 33 (2000) 341–365.
[2] T.B. Kuhn, P.J. Meberg, M.D. Brown, B.W. Bernstein, L.S. Minamide, J.R. Jensen, K. Okada, E.A. Soda, J.R. Bamburg, Regulating actin dynamics in neuronal growth cones by ADF/cofilin and rho family GTPases, J. Neurobiol. 44 (2000) 126–144.
[3] J. Herrington, L.S. Smit, J. Schwartz, C. Carter-Su, The role of STAT proteins in growth hormone signaling, Oncogene 19 (2000) 2585–2597.
[4] S. K Hanks, T. Hunter, Protein kinases 6 the eukaryotic protein kinase superfamily: kinase (catalytic) domain structure classification, FASEB J. 9 (1995) 576–596.
[5] A.J. Bridges, Chemical inhibitors of protein kinases, Chem. Rev. 101 (2001) 2541–2572.
[6] P. Cohen, Protein kinases – the major drug targets of the twenty-first century?, Nat Rev. Drug. Discov. 1 (2002) 309–315.
[7] M.E.M. Noble, J.A. Endicott, L.N. Johnson, Protein kinase inhibitors: insights into drug design from structure, Science 303 (2004) 1800–1805.
[8] G.W. Tessmer, J.R. Skuster, L.B. Tabatabai, D.J. Graves, Studies on the specificity of phosphorylase kinase using peptide substrates, J. Biol. Chem. 252 (1977) 5666–5671.
[9] Z. Songyang, K.L. Carraway, M.J. Eck, S.C. Harrison, R.A. Feldman, M. Mohammadi, J. Schlessinger, S.R. Hubbard, D.P. Smith, E. Eng, M.J. Lorenzo, B.A.J. Ponder, B.J. Mayer, L.C. Cantley, Catalytic specificity of protein–tyrosine kinases is critical for selective signaling, Nature 373 (1995) 536–539.
[10] M Kim, D.S. Shin, J. Kim, Y.S. Lee, Substrate screening of protein kinases: detection methods and combinatorial peptide libraries, Biopolym. Pept. Sci. 94 (2010) 753–762.
[11] J.J. Witt, R. Roskoski Jr., Rapid protein kinase assay using phosphocellulose-paper absorption, Anal. Biochem. 66 (1975) 253–258.
[12] P. Geer, T. Hunter, Phosphopeptide mapping and phosphoamino acid analysis by electrophoresis and chromatography on thin-layer cellulose plates, Electrophoresis 15 (1994) 544–554.

[13] E. Hough, L.K. Hansen, B. Birknes, High-resolution (1.5 A) crystal structure of phospholipase C from Bacillus cereus, Nature 338 (1989) 357–360.
[14] C.A. Chen, R.H. Yeh, D.S. Lawrence, Design and synthesis of a fluorescent reporter of protein kinase activity, J. Am. Chem. Soc. 124 (2002) 3840–3841.
[15] S. Aoki, M. Zulkefeli, M. Shiro, M. Kohsako, K. Takeda, A luminescence sensor of inositol 14 5-triphosphate its model compound by ruthenium-templated assembly of a bis (Zn^{2+} – cyclen) complex having a 2 2′-bipyridyl linker (cyclen = 1,4,7,10-tetraazacyclododecane), J. Am. Chem. Soc. 127 (2005) 9129–9139.
[16] A. Pandey, A.V. Podtelejnikov, B. Blagoev, X.R. Bustelo, M. Mann, H.F. Lodish, Analysis of receptor signaling pathways by mass spectrometry: identification of vav-2 as a substrate of the epidermal and platelet-derived growth factor receptors, Proc. Natl. Acad. Sci. USA 97 (2000) 179–184.
[17] D.L. Foletti, R. Lin, M.A.F. Finley, R.H. Scheller, Phosphorylated syntaxin 1 is localized to discrete domains along a subset of axons, J. Neurosci. 20 (2000) 4535–4544.
[18] Y.G. Kim, D.S. Shin, E.M. Kim, H.Y. Park, C.S. Lee, J.H. Kim, B.S. Lee, Y.S. Lee, B.G. Kim, High-throughput identification of substrate specificity for protein kinase by using an improved one-bead-one-compound library approach, Angew. Chem. Int. Ed. 46 (2007) 5408–5411.
[19] D.S. Shin, Y.G. Kim, E.M. Kim, M. Kim, H.Y. Park, J.H. Kim, B.S. Lee, B.G. Kim, Y.S. Lee, Solid-phase peptide library synthesis on HiCore resin for screening substrate specificity of Brk protein tyrosine kinase, J. Comb. Chem. 10 (2008) 20–23.
[20] S. Kane, H. Sano, S.C. Liu, J.M. Asara, W.S. Lane, C.C. Garner, G.E. Lienhard, A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain, J. Biol. Chem. 277 (2002) 22115–22118.
[21] M. Gronborg, T.Z. Kristianzen, A. Stensballe, J.S. Andersen, O. Ohara, M. Mann, O.N. Jensen, A. Pandey, A mass spectrometry-based proteomic approach for identification of serine/threonine-phosphorylated proteins by enrichment with phospho-specific antibodies: identification of a novel protein Frigg as a protein kinase A substrate, Mol. Cell. Proteomics 1 (2002) 517–527.
[22] G. Redlich, U.M. Zanger, S. Riedmaier, N. Bache, A.B.M. Giessing, M. Eisenacher, C. Stephan, H.E. Meyer, O.N. Jensen, K. Marcus, Distinction between human cytochrome P450 (CYP) isoforms and identification of new phosphorylation sites by mass spectrometry, J. Proteome. Res. 7 (2008) 4678–4688.
[23] G. Moorhead, R.W. MacKintosh, N. Morrice, T. Gallagher, C. MacKintosh, Purification of type 1 protein (serine/threonine) phosphatases by microcystin-Sepharose affinity chromatography, FEBS Lett. 356 (1994) 46–50.
[24] P. Fadden, T.A. Haystead, Quantitative and selective fluorophore labeling of phosphoserine on peptides and proteins: characterization at the attomole level by capillary electrophoresis and laser-induced fluorescence, Anal. Biochem. 225 (1995) 81–88.
[25] H. Jaffe, Veeranna, H.C. Pant, Characterization of serine and threonine phosphorylation sites in β-elimination/ethanethiol addition-modified proteins by electrospray tandem mass spectrometry and database searching, Biochemistry 37 (1998) 16211–16224.
[26] S. Akita, N. Umezawa, N. Kato, T. Higuchi, Array-based fluorescence assay for serine/threonine kinases using specific chemical reaction, Bioorgan. Med. Chem. 16 (2008) 7788–7794.
[27] S. Akita, N. Umezawa, T. Higuchi, On-bead fluorescence assay for serine/threonine kinases, Org. Lett. 7 (2005) 5565–5568.
[28] A. Song, X. Wang, J. Zhang, J. Marik, C.B. Lebrilla, K.S. Lam, Synthesis of hydrophilic and flexible linkers for peptide derivatization in solid phase, Bioorg. Med. Chem. Lett. 14 (2004) 161–165.
[29] Z.G. Zhao, J.S. Im, K.S. Lam, D.F. Lake, Site-specific modification of a single-chain antibody using a novel glyoxylyl-based labeling reagent, Bioconjugate Chem. 10 (1999) 424–430.
[30] H.Y. Kim, J.K. Cho, W.J. Chung, Y.-S. Lee, Core–shell-type resins for solid-phase peptide synthesis: comparison with gel-type resins in solid-phase photolytic cleavage reaction, Org. Lett. 6 (2004) 3273–3276.
[31] C.P. Holmes, D.G. Jones, Reagents for combinatorial organic synthesis: development of a new *O*-nitrobenzyl photolabile linker for solid phase synthesis, J. Org. Chem. 60 (1995) 2318–2319.
[32] R. Wieboldt, D. Ramesh, E. Jabri, P.A. Karplus, B.K. Carpenter, G.P. Hess, Synthesis and characterization of photolabile *O*-nitrobenzyl derivatives of urea, J. Org. Chem. 67 (2002) 8827–8831.
[33] B.E. Kemp, R.B. Pearson, Protein kinase recognition sequence motifs, Trends. Biochem. Sci. 15 (1990) 342–346.
[34] R.B. Pearson, B.E. Kemp, Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations, Methods. Enzymol. 200 (1991) 62–81.
[35] W. Li, R.A. Boykins, P.S. Backlund, G. Wang, H.C. Chen, Identification of phosphoserine and phosphothreonine as cysteic acid and β-methylcysteic acid residues in peptides by tandem mass spectrometric sequencing, Anal. Chem. 74 (2002) 5701–5710.
[36] F. Thaler, B. Valsasina, R. Baldi, J. Xie, A. Stewart, A. Isacchi, H.M. Kalisz, L. Rusconi, A new approach to phosphoserine phosphothreonine analysis in peptides proteins: chemical modification enrichment via solid-phase reversible binding and analysis by mass spectrometry, Anal. Bioanal. Chem. 376 (2003) 366–373.
[37] Z. Songyang, S. Blechner, N. Hoagland, M.F. Hoekstra, H. Piwnica-Worms, L.C. Cantley, Use of an oriented peptide library to determine the optimal substrates of protein kinases, Curr. Biol. 4 (1994) 973–982.
[38] P.T. Tuazon, W.C. Spanos, E.L. Gump, C.A. Monnig, J.A. Traugh, Determinants for substrate phosphorylation by p21-activated protein kinase (γ-PAK), Biochemistry 36 (1997) 16059–16064.
[39] J.A. Ubersax, J.E. Ferrell Jr., Mechanisms of specificity in protein phosphorylation, Nat. Rev. Mol. Cell. Biol. 8 (2007) 530–541.
[40] R. Linding, L.J. Jensen, G.J. Ostheimer, M.A. van Vugt, C. Jørgensen, I.M. Miron, F. Diella, K. Colwill, L. Taylor, K. Elder, P. Metalnikov, V. Nguyen, A. Pasculescu, J. Jin, J.G. Park, L.D. Samson, J.R. Woodgett, R.B. Russell, P. Bork, M.B. Yaffe, T. Pawson, NetworKIN: a resource for exploring cellular phosphorylation networks, Cell 129 (2007) 1415–1426.