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Development of high-throughput phosphorylation profiling method for identification of Ser/Thr kinase specificity‡

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Identification of substrate specificity of kinases is crucial to understand the roles of the kinases in cellular signal transduction pathways. Here, we present an approach applicable for the discovery of substrate specificity of Ser/Thr kinases. The method, which is named as the 'high-throughput phosphorylation profiling (HTPP)' method was developed on the basis of a fully randomized one-bead one-compound (OBOC) combinatorial ladder type peptide library and MALDI-TOF MS. The OBOC ladder peptide library was constructed by the 'split and pool' method on a HiCore resin. The peptide library sequence was Ac-Ala-X-X-X-Ser-X-X-Ala-BEBE-PLL resin. The substrate specificity of murine PKA (cAMP-dependent protein kinase A) and yeast Yak1 kinase was identified using this method. On the basis of the result, we identified Ifh1, which is a co-activator for the transcription of ribosomal protein genes, as a novel substrate of Yak1 kinase. The putative Yak1-dependent phosphorylation site of Ifh1 was verified by *in vitro* kinase assay. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Ser/Thr kinase; substrate specificity; high-throughput screening; peptide library; MS

Introduction

Protein phosphorylation by protein kinase is one of the key mechanisms in intracellular signal transduction pathways [1]. Identification of the substrate specificity of protein kinases is important not only to understand the signaling pathways but also to develop drugs against protein kinases involved in human diseases. In nature, the occurrence of phosphoserine and phosphothreonine is about 2000 times more than that of phosphotyrosine. Therefore, screening of the substrate specificity of Ser/Thr kinases is important to understand their biological functions [2,3]. Several approaches such as combinatorial peptide microarrays and positional-scanning peptide libraries have been introduced to determine the substrate specificity of kinases [4–8]. Especially, positional-scanning peptide library is a powerful high-throughput approach to determine the consensus phosphorylation site for kinases [9]. However, these methods have difficulties in identifying the complete substrate peptide sequences of kinases because they focus on single-site randomization to optimize the amino acid sequences adjacent to the phosphorylation site. To overcome this disadvantage, we developed a method for high-throughput identification of substrate specificity of Ser/Thr kinases using a fully-randomized one-bead one-compound (OBOC) combinatorial ladder type peptide library and MALDI-TOF MS, which is named as 'high-throughput phosphorylation profiling (HTPP)' method [10,11]. Through HTPP method, the complete substrate sequence is obtained from the ladder peptide library, which is sufficient to cover all the variations of amino acids at each randomized position. Moreover, only one reaction using the target kinase is enough to obtain the potential substrates.

Material and Methods

Materials

Murine PKA (cAMP-dependent protein kinase A) catalytic domain expressed in *Escherichia coli* was purchased from New England BioLabs (Ipswich, MA, USA). Biotin-conjugated antiphosphoserine antibody was purchased from Abcam (Cambridge, MA, USA) and alkaline phosphatase (AP) conjugated neutravidin (neuravidin-AP) was purchased from PIERCE (Rockford, IL, USA). All the chemicals and reagents required for a kinase reaction and peptide synthesis were obtained from Sigma (St. Louis, MO, USA) or Aldrich Chemicals (Milwaukee, WI, USA). Fmoc-photolabile linker (Fmoc protected 4-[4-(1-aminoethyl)-2-methoxy-5-nitrophenoxy] butyric acid) was purchased from Advanced ChemTech (Louisville, KY, USA). Fmoc-amino acid and its derivatives were obtained from BeadTech. Inc. (Seoul, Republic of Korea). *N*-methyl pyrrolidone, dichloromethane and methanol were purchased from Junsei

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Chemical Co. (Tokyo, Japan). HiCore resin was prepared according to a previously described method [12] and is commercially available from BeadTech. Inc. (Seoul, Republic of Korea).

Peptide Library Synthesis

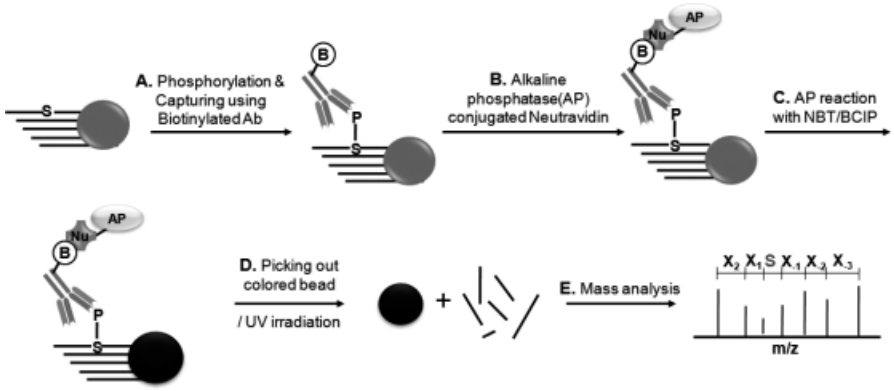
The peptide library, as a ladder type, was synthesized by the 'split and mix' method on the PEGylated core-shell type HiCore resin (0.34 mmol/g, <100 μm). First, 1.8 g of HiCore resin was swollen in *N*-methyl pyrrolidone (NMP) and then added to a mixture of Fmoc-photolabile linker (Fmoc-PLL; 2 equiv.), BOP (2 equiv.), HOBt (2 equiv.) and DIPEA (4 equiv.). The resin and the mixture were stirred at 25 °C for 2 h in a shaking incubator. The excess reagents in the mixture were filtered out after the reaction was completed, and the resin was washed with NMP, DCM and methanol (MeOH) about three times each. The completion of the coupling reaction was confirmed through Kaiser's ninhydrin test. After the confirmation of a perfect coupling reaction, the Fmoc group was removed by using a 20% of piperidine/NMP solution for 30 min. Fmoc-ε-aminocaproic acid (Fmoc-ε-ACA) and Fmoc-β-alanine (Fmoc-β-Ala) were alternately coupled on the resin in the same way as Fmoc-PLL. The resin was distributed into 17 reaction tubes with same quantities (~100 mg), after BEBE-PLL was bounded to the resin. Each resin was swollen in NMP, and then Fmoc-amino acids (all of them were of L-form, except Cys, Ser and Thr; 2 equiv.), acetic acid (0.2 equiv.), BOP (2.2 equiv.), HOBt (2.2 equiv.) and DIPEA (4.4 equiv.) were added to the resin. All tubes were stirred at 25 °C for 2 h in a shaking incubator, the mixture containing an excess amount of reagents was filtered and the remaining resin was washed with NMP, DCM and MeOH about three times each. After the washing, all the resin was put into one large reaction tube. By using the Fmoc-titration method (Fmoc quantitation), the loading level of the resin was calculated and its Fmoc groups were removed. This 'split and mix' method was repeated until the octapeptide libraries containing 'Ala-X-X-X-S-X-X-A-BEBE-PLL' sequence (where X is one of the L-amino acids except Cys, Ser and Thr) was produced. After all the libraries were synthesized, all the resin was put into one large reaction tube, was swollen in NMP and then added to the mixture of AcOH (2 equiv.), BOP (2 equiv.), HOBt (2 equiv.) and DIPEA (4 equiv.). The reaction tube was stirred at 25 °C for 2 h in a shaking incubator, and the mixture containing excess reagent was filtered out after the reaction. The remaining resins were washed with NMP, DCM and MeOH three times each. For the deprotection of the side chains of the peptides, Reagent K ((TFA) 82.5%, phenol 5%, thioanisol 5%, H_2O 5% and ethandithiol (EDT) 2.5%) was added to a reaction tube and the tube was stirred at 25 °C for 1 ~ 2 h in a shaking incubator. After the reaction, Reagent K was filtered out and the remained resin was washed carefully with TFA, DCM and MeOH until the odor of EDT disappeared. With these steps, the peptide libraries having a sequence of 'Ac-A-X_{-3}-X_{-2}-X_{-1}-S-X_1-X_2-A-BEBE-PLL' were synthesized on the HiCore resin.

Substrate Specificty Screening of Kinases

Before the screening for substrate specificity, control experiments were performed to determine the appropriate concentration of biotin conjugated antiphosphoserine antibody and neutravidin-AP not producing false-positives. To determine the optimum concentration, the serial dilution of antibody and neutravidin-AP followed by reaction was performed with bare beads and peptide-containing beads. Optimum concentration of antibody or neutravidin-AP was determined to be 5 or 12 μg/ml, respectively. A quantity of 150 mg of the ladder peptide library, which contained over 1 500 000 randomized sequences and was sufficient to cover all the variations of amino acids at the five positions, was used to identify the substrate specificity of the Ser/Thr kinase. In the screening of Ser/Thr kinase substrate specificities, each 2 μg serine kinase (PKA, Yak1) was applied to the serine kinase reaction buffer (100 μM ATP, 50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$) and incubated with 150 mg peptide coupled resin beads for 4 h at 30 °C with gentle shaking. After the kinase reaction, the beads were mixed with biotin-conjugated antiphosphoserine antibody (5 μg/ml) and were incubated for 1.5 h. The beads were mixed with neutravidin-AP (12 μg/ml) solution and were incubated for 20 min. Finally, a standard AP substrate solution (NBT, nitro-blue tetrazolium chloride/BCIP, 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt) in 50 mM phosphate bufferd saline (PBS) was added to the beads and the reaction solution was incubated for 30 min. After each step, the reacted beads were washed with PBS. Phosphorylated beads were identified by the change in color to dark-red after incubation with the AP substrate solution. Dark-red color beads were selected using a low magnification microscope (X40). The selected beads were irradiated using ultraviolet (UV) radiation (spot light source, 365 nm, Lightningcure 2000, hamamatsum Photonic K) for 30 min to cleave the photocleavable linker. The peptide sequences of the selected beads were analyzed by MALDI-TOF MS. MALDI-TOF MS analysis was performed with a Bruker Datonics Biflex IV TOF MS (Bruker, Bremen, Germany). Each spectrum was the result of an average of 100 laser shots and the average value of measurements was acquired from Bruker X-TOF 5.1.1 and Biotools 2.1 programs (Bruker, Germany).

In vitro Phosphorylation Kinase Assay

pGEX4T-1vector (GE Healthcare, Piscataway, NJ) was used for the cloning of Ifh1 (1–400), Ifh1 (401–800), Ifh1(401–800)^{S522A} and Yak1 from *Saccharomyces cerevisiae*. The expression vector for Yak1 was described previously [13]. Ifh1 (1–400) was amplified by PCR using forward (5′-GCGCCCGGGTGCAGGCAAAAAAAGTCCTC-3′; underline indicates a restriction site) and reverse (5′-GCG-CTCGAGTCATTCTTGATTGTATTCATT-3′) primers. Ifh1 (401–800) was amplified by PCR using forward (5′-GCGCCCGGGTAACGGA-TATGATGAAGAAG-3′) and reverse (5′-GCGCTCGAGTCAGTAA-TTGTTCTCCATATC-3′) primers. PCR products were digested with SmaI and XhoI (Koschem, Seoul, Republic of Korea), and cloned into the pGEX4T-1 vector. For the site-directed mutagenesis, Ifh1 (S522A) was amplified by overlapping PCR using mutagenic primers, 5′-AGGCAGGCATAAAGCGGGCAAAAGTCATAT-3′, and 5′-ATATGACTTTTGCCCGCTTTATGCCTGCCT-3′, and forward and reverse primers for Ifh1(401–800). Three proteins were designed to be expressed in glutathione *S*-transferase (GST) fusion form. Expression vectors encoding target proteins were transformed into *E.coli* Rosetta gami 2 strain (DE3, pLysS, EMD Bioscience, Darmstadt, Germany). Proteins were induced with 1 mM IPTG for 3 h and purified using glutathione-agarose (Novagen, Darmstadt, Germany). Kinase assays were performed by incubating 1 μg of GST-Yak1 with 3 μg of GST-Ifh1 (1–400), GST-Ifh1 (401–800) and GST-Ifh1(S522A) in 20 μl reaction buffer containing 25 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 50 μM ATP and 5 μCi of [γ-^{32}P]-ATP at room temperature for 90 min. Reactions were terminated by boiling in SDS-PAGE sample buffer. Reaction samples were separated by 8% SDS-PAGE and the phosphorylated proteins were detected using phosphorimager analysis.



Scheme 1. HTPP procedure to determine Ser/Thr kinase substrate specificity. (A) The phosphorylation and biotinylation step. PKA and Yak1 kinase reaction is performed using peptide library beads. After the phosphorylation reaction, phosphoserine peptides were incubated with biotin-conjugated antiphosphoserine Ab. (B) Capturing step of the biotinylated peptide beads containing phosphoserine. AP-conjugated Neutravidin is added and reaction mixture is further incubated. (C) Detection step. After the reaction of AP, the color of phosphorylated beads changes to dark-red. (D) Picking up step. Color-changed beads are selected manually using microscope and eluted from the bead using UV irradiation. (E) Analysis step. Seven ladder peptides eluted from one bead were analyzed by MALDI-TOF. The peptide was sequenced by mass difference of each peptide peak [19]. (Ab, antibody; Nu, neutravidin; AP, alkaline phosphatase; B, biotin).

Results and Discussion

HTPP Method and Peptide Synthesis

The HTPP method for the identification of Ser/Thr kinase substrate specificity is summarized in Scheme 1. Phosphorylation reaction using Ser/Thr kinase was performed on the peptide library bound to beads (150 mg), which is followed by capturing biotin-conjugated highly selective antiphosphoserine antibody. It has been shown that antiphosphothreonine and antiphosphoserine antibody are not completely independent of the sequence adjacent to the phosphorylated residue. In order to minimize the sequence-dependent bias, we used the recently developed highly selective antiphosphoserine antibody and optimized the concentration of the antibody to reduce false-positive results. Phosphorylated beads were detected by adding neutravidin-AP and then reacted with the AP substrate solution. Antiphosphoserine antibody and neutravidin-AP did not show nonspecific binding to nonphosphorylated peptides or to the bead surface (Figure 1(A)).The phosphorylation reaction generated dark-red colored beads. One hundred dark-red phosphorylated beads were picked out under an inverted microscope and separated into individual microtubes. And then the ladder peptides were eluted from the beads by UV irradiation and sequenced using MALDI-TOF MS.

The OBOC ladder peptide library was constructed by 'split and pool' method on a HiCore resin. As phosphorylation of the target substrate by kinases is strongly affected by the amino acid sequences that are adjacent to a phosphorylation site, the peptide library sequence was designed in the form of Ac-Ala-X_{-3}-X_{-2}-X_{-1}-Ser-X_1-X_2-Ala-BEBE-PLL-resin (X = degenerate position; 17 amino acids can be incorporated except Ser, Thr and Cys; B = β-alanine; E = ε-aminocaproic acid; PLL = photo labile linker). Three amino acids at the *N*-terminal and two amino acids at the *C*-terminal except a fixed serine residue were randomized with 17 natural amino acids. In the study of substrate specificity of Ser/Thr kinases, five positions (three in *N*-terminal and two in *C*-terminal) surrounding the phosphorylation site were most influential for substrate recognition by kinase. In other previous research, specificities of residues around the phosphorylation site were examined but only the five positions mentioned above were revealed to have distinguishable specificity [6,7,9]. Ala was inserted between the terminal sequence and the BEBE spacer to prevent steric hindrance in the kinase reaction. In addition, to avoid peak overlap with the matrix peaks in MALDI-TOF analysis, the insertion of the BEBE spacer, which shifts the mass peak toward the high mass range, was designed and included in the peptide synthesis. The PLL was introduced to efficiently collect the peptides from beads [14,15].

Substrate Specificity of PKA

Prior to the screening of Yak1 kinase substrate specificity, murine PKA (cAMP-dependent protein kinase A), which is a previously well-examined kinase, was employed as a model kinase to verify the feasibility of the HTPP method [16]. Subsequent to the use of the HTPP method, 98 peptide sequences out of 100 red-colored beads (98%) could be identified from MALDI-TOF MS spectra. The eluted ladder peptides from one bead could be sequenced by calculating mass differences corresponding to each amino acid (Figure 1(B)). However, the isobaric amino acids (e.g. Ile and Leu or Gln and Lys) could not be determined as we reported previously [15]. Therefore, it was assumed that the isobaric amino acids appear with equal frequency. On the basis of the peptide sequencing results, the distributions of amino acids at each position are statistically summarized (Figure (2), PKA). In agreement with the known PKA consensus sequence, RR/KXS, our result showed strong specificity of Arg at P (−3). Also, the frequency of Arg or Lys at the P (−2) was two times higher than average frequency (5.76). However, we also observed enrichment of Arg at P (+1) and P (+2), which has not been detected in previous studies on PKA specificity. The reason for this discrepancy is not clear yet, although it could be partly explained by the potential sequence preference of the antiphosphoserine antibody we used. Among the obtained peptide sequences, peptides containing Arg at P (−3) and Arg/Lys at P (−2) were compared with the NetworKIN database. The four peptides of RRDSLK (RRDSHK, phosphorylation motif, Ser194 in prolactin precursor), RRKSNK (RRKSGK, Ser303 in tumor-associated calcium signal transducer 2 precursor), RRNSKF (RRNSEF, Ser58 in tryptophan 5-hydroxylase) and RKGSWV (RKGSVV, Ser900 in mitogen-activated protein kinase 4) matched well with sequences in potential substrate proteins of PKA.

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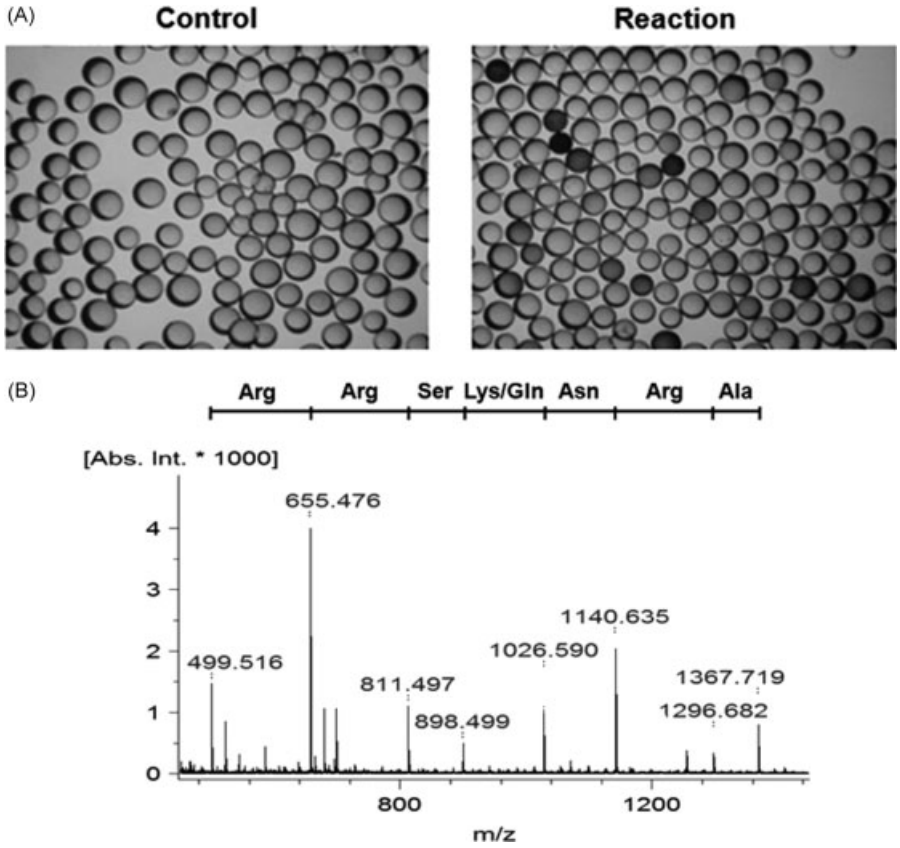

Figure 1. Color change in peptides through kinase reaction and ladder sequence peptide analysis using MALDI-TOF. (A) 'Control' shows the nonphosphorylated beads and 'Reaction' shows the beads phosphorylated by kinase reaction. (B) Method for the determination of peptide sequence of one bead using MALDI-TOF analysis.

Substrate Specificty of Yak1

Next, we applied the HTPP method to identify substrate specificity of *S. cerevisiae* Yak1 kinase, which is a member of an evolutionarily conserved family of Ser/Thr protein kinases known as dual-specificity tyrosine-regulated kinase. Yak1 was first identified as a functional antagonist of the Ras/PKA signaling pathway and has been characterized as a growth inhibitor. The known substrates of the Yak1 kinase include Pop2 regulating mRNA deadenylation, Msn2 and Hsf1 transcription factors for stress response genes, and Crf1 co-repressor for the expression of ribosomal protein genes [13,17,18]. However, its substrate specificity has not yet been completely characterized. After phosphorylation by Yak1 kinase, the phosphorylated peptides were sequenced by MALDI-TOF MS with 97% of efficiency. Substrate identification profile of Yak1 is presented in Figure 2. Arg at P (−3) is the only dominant residue identified by our screening. Therefore, the substrate motif of Yak1 kinase can be determined to be RXXSXX. While we are preparing this manuscript, substrate specificity of Yak1 was also determined by positional-scanning peptide library [9]. In agreement with our result, Yak1 is classified as the less substrate specificity group with Arg specificity at P (−3), although slight enrichment of Pro at P (+1) was also observed.

Identification of Ifh1 as a New Substrate of Yak1

Among the obtained peptide sequences, peptides having Arg residue at P (−3) were compared with the *S. cerevisiae* database using the BLAST search and the peptide that matched perfectly was selected. The RHKSGK peptide sequence perfectly matched with amino acid residues 519–524 of Ifh1, which is a co-activator for the transcription of ribosomal protein genes. Ifh1 competes with Crf1 as co-repressor in binding to Fhl1. It has been shown previously that Crf1 is phosphorylated by Yak1 [17].

We further attempted to confirm the newly identified Yak1 substrate protein, Ifh1, by *in vitro* kinase assay. Ifh1 (401–800) containing the potential phosphorylation site (RHK**S**GK), but not Ifh1 (1–400), was phosphorylated by Yak1 (Figure 3(A)). To confirm the phosphorylation site, Ser522 of Ifh1 (401–800) was replaced with Ala. In agreement with the prediction, the S522A mutant showed reduced phosphorylation by Yak1 (Figure 3(B)).

These results indicate that substrate specificity identified by our method can be applied to identify novel kinase targets. Although we used the peptide as the substrate of the kinase, phosphorylation reaction can occur regardless of the secondary structures in substrate proteins. Therefore, our HTPP method could serve as a useful tool to understand the substrate specificity and function of Ser/Thr kinase.

Conclusions

We have developed a high-throughput method for the substrate identification of Ser/Thr kinase using fully randomized OBOC ladder type peptide library and MS, which is named as 'high-throughput phosphorylation profiling (HTPP)' method. The ladder peptides of the sequence Ala-X-X-X-S-X-X-Ala-BEBE-PLL-bead were synthesized and used to determine the Ser/Thr kinase

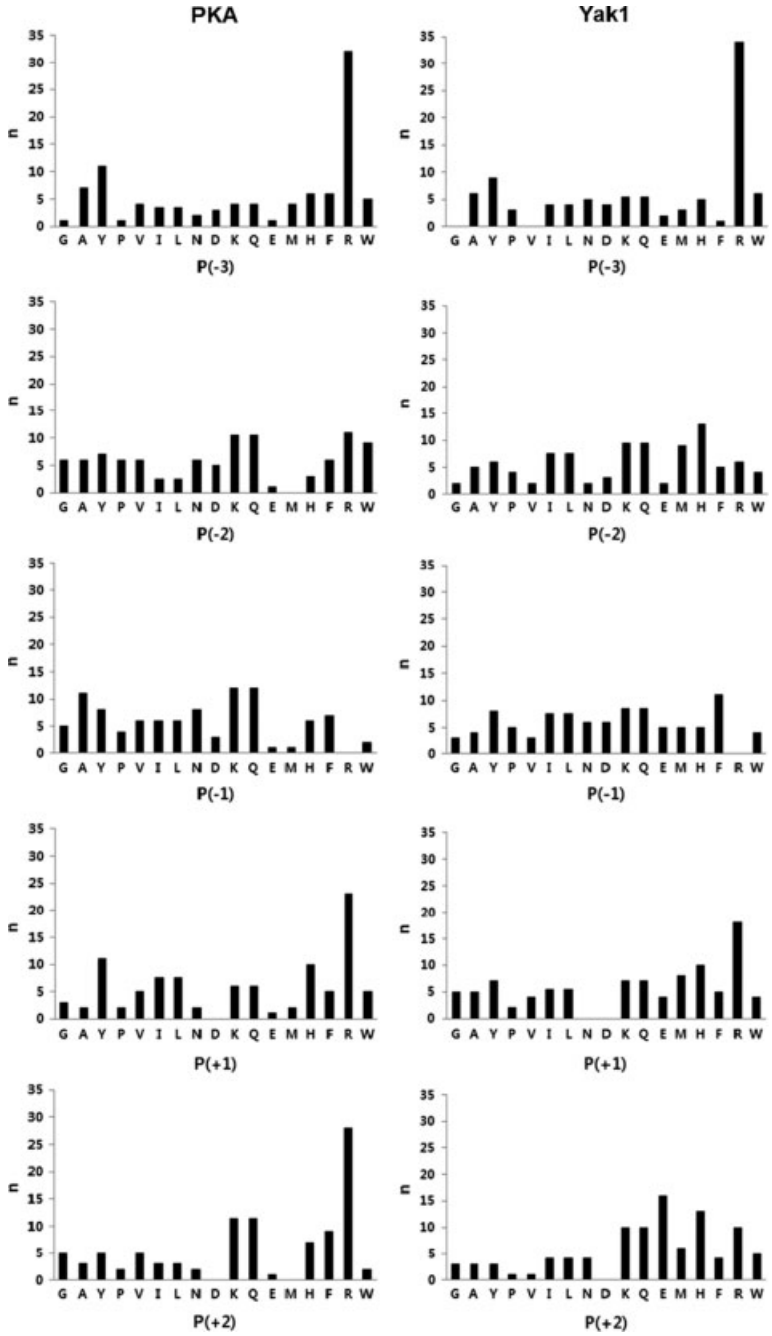

Figure 2. Substrate specificities of the PKA and Yak1 Ser/Thr kinases. The *x*-axis indicates the amino acid position and *y*-axis indicates the number of appearances of a particular amino acid at a position (+, *C*-terminal residue; −, *N*-terminal residue; *n*, number of appearances). The isobaric amino acids (Ile and Leu or Gln and Lys) are assumed to appear with equal frequency.

substrate specificity. The native activity and substrate specificities of PKA and Yak1 kinases could be successfully phosphorylated with peptide library. The substrate specificity of PKA was successfully reproduced by our method, although additional, yet undefined, specificity was detected by our method. Furthermore, we could identify Ifh1 as a novel substrate of Yak1 kinase. This is the first report identifying Ser/Thr kinase substrate specificity utilizing a fully randomized peptide library and actual kinase reaction. Our method can reduce the search space for the identification of the substrate of a given kinase. Moreover, an important feature of our screening method is that the *in vivo* phosphorylation site of the substrate can be predicted by database search using a native phosphorylated peptide sequence. We have shown that our method was effective and robust in the high-throughput screening of Ser/Thr kinase substrate motif and putative target proteins. Therefore, the HTPP method might contribute to the study of Ser/Thr kinases after further optimization.

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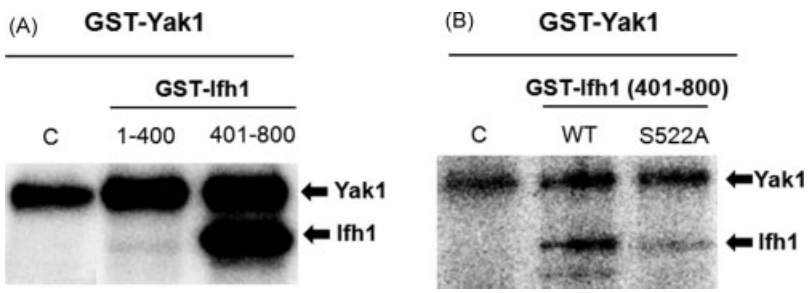

Figure 3. Yak1-dependent phosphorylation of Ifh1 by Yak1 *in vitro*. (A) GST-Yak1 was purified in *E. coli* and *in vitro* kinase assays were performed using GST-Ifh1 (1–400) and GST-Ifh1 (401–800). Autophosphorylation of Yak1 and phosphorylation of proteins were detected by SDS-PAGE followed by autoradiography. (B) GST-Ifh1 (401–800) wild type and S522A mutant were phosphorylated by GST-Yak1 *in vitro*.

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