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Supporting Information

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High-throughput identification of substrate specificity for protein kinase using improved one-bead-one-compound library approach

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Materials

Protein tyrosine kinase (PTK) $p60^{c\text{-}src}$ expressed by baculovirus in Sf9 insect cells and ZAP-70 expressed by baculovirus in Sf21 insect cells were purchased from Upstate Biotechnology (Lake Placid, NY) and used as received. Anti-phosphotyrosine antibody conjugated alkaline phosphatase was purchased from Oncogene Research Products (Cambridge, MA). All other chemicals and peptide synthesis reagents were obtained from Sigma (St. Louis, MO) or Aldrich Chemicals (Milwaukee, WI) and used without further purification. Fmoc-photolabile linker (Fmoc protected 4-[4-(1-aminoethyl)-2-methoxy-5-nitrophenoxy] butyric acid) was purchased from Advanced ChemTech (Louisville, KY). Fmoc amino acid and their derivatives were purchased from BeadTech (Seoul, Korea). NMP (N-methyl pyrrolidone), DCM (dichloromethane) and methanol were purchased from Junsei Chemical Co. (Tokyo, Japan). HiCore® resin was prepared as described [1].

Peptide Library Synthesis

The peptide library was synthesized by the "split and pool" method on the HiCore® resin as a ladder type introduced by a photolabile linker. Firstly, a certain amount of the HiCore® resin (0.3-0.5 mmol g^{-1}) was swollen in NMP, and then added to mixture of 2 eq. of Fmoc-photolabile linker, BOP (benzotriazol-1-yloxytris(dimethylamino) phosphonium hexafluorophosphate), HOBt (1-hydroxybenzotriazole), and 4 eq. of DIEA (diisopropylethylamine) [1]. The mixture was stirred at 25 °C for 2 hrs in a shaking incubator, and the solution containing excess reagents was filtered out with nitrogen gas. The resin was washed with NMP, DCM and MeOH. Fmoc was removed at each cycle with 20% piperidine/NMP (3∓17 min). Successively, Fmoc-ε-aminocaproic acid (Fmoc-ε-ACA) and Fmoc-β-alanine (Fmoc-β-Ala) were alternately coupled on the Fmoc-photolabile linker in the same manner. The completion of each coupling was confirmed through Kaiser's ninhydrin test [2]. Photolabile linker and spacer bound resin prepared was separated into eighteen reaction tubes with the same quantities. Each resin was swollen in NMP, and then 2 eq. of Fmoc amino acids (all naturally occurring L-amino acids except Cys and Tyr), 0.2 eq. of

acetic acid, 2.2 eq. of BOP, HOBt, and 4.4 eq. of DIEA were added. The reaction mixture was stirred at 25 °C for 2 hrs in a shaking incubator and then the solution which contained excess reagents was filtered out with nitrogen gas. The resin was washed with NMP, DCM and MeOH. Next, the resin was pooled into one reaction vessel, and then Fmoc groups were removed. This process was repeated to produce the heptapeptide libraries. The remaining amino groups were quantified after each coupling step by Fmoc quantitation [3]. For side chain deprotection, Reagent K (TFA 85%, Phenol 5%, Thioanisol 5%, Ethandithiol, 2.5%, H_2O 2.5%) was used. The amount of full-length peptide containing Ala-X-X-Tyr-X-X-Ala-BEBE-NH_2 (where X = 18 naturally occurring amino acids except Cys and Tyr) sequence was about ca. 30%, and a series of corresponding terminated products indicating the peptide sequence was qualified by MALDI-TOF-MS.

Screening

One hundred thousand beads were screened for appropriating substrates using PTK $p60^{c\text{-}src}$ and ZAP-70. The peptide library beads were washed with 50 mM phosphate buffered saline (PBS, pH 7.4) and incubated with 3% (w/v) BSA in PBS containing 0.05 mM Tween20 at 25 °C for 2 hrs to block protein nonspecific binding. After the blocking step, the beads were washed with 1% (w/v) BSA in PBS. The solid-phase peptide phosphorylation was performed in 25 mM Tris-HCl buffer (pH 7.4, 15 mM $MnCl_2$, 7 mM $MgCl_2$ and 0.5 mM EGTA) adding 100 μM ATP and PTK $p60^{c\text{-}src}$ or ZAP-70 at 30 °C for 4 hrs with gently shaking [4]. Next, the beads were washed with 25 mM Tris-HCl buffer and distilled water. After the phosphorylation reaction, the beads were incubated with 100 ng mL^{-1} anti-phosphotyrosine antibody conjugated alkaline phosphatase in 50 mM PBS (pH 7.4). The supernatant was discarded, and then the beads were washed with PBS. A standard mixture of BCIP (5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt) and NBT (nitro-blue tetrazolium chloride) in 50 mM PBS (pH 7.4) was added to the beads. The vivid red colored beads were picked by forceps under an inverted microscope (Olympus CK2, Tokyo, Japan). The selected bead was put into a tube (0.2 ml) and suspended in water (5 μL) and irradiated for 15 min under a spot light source (365 nm, Lightningcure 2000, Hamamatsu Photonics K.) at a measured intensity of 50 mW cm^{-2}. The eluted solution, which contained peptide product, was evaporated with vacuum evaporator and the remaining product was dissolved in matrix solution (1 μL, 30 mg ml^{-1} 2,5-dihydroxybenzoic acid in MeCN:H_2O = 7:3) for MALDI-TOF-MS analysis. MALDI-TOF mass spectrometric analysis was performed with a Bruker Datonics Biflex IV time of flight mass spectrometry (Bruker, Germany) with delayed extraction condition, operating with a pulsed N_2 laser at 337 nm. Positive ion mass spectra were acquired using reflector mode with an accelerating voltage of 20.0 kV. MALDI-TOF spectra were acquired from *m*/*z* 0 to 2000 with an average of 100 shots and the average values of the measurements were acquired from Bruker X-TOF 5.1.1 and Biotools 2.0 programs (Bruker, Bremen, Germany)

Molecular modeling of p60$^{c\text{-}src}$-substrate and ZAP-70-substrate complex

Molecular modeling studies between protein tyrosine kinases and optimal substrates were performed in order to identify their biological activity differences using the molecules implemented in the molecular modeling software package SYBYL 7.1 (Tripos Inc., St. Louis, MO). The p60$^{c\text{-}src}$ (PDB code 1Y57) and ZAP-70 (PDB code 1U59) protein tyrosine kinase used for the docking is available from the Protein Data Bank [5,6]. Flexible docking was performed using FlexX method to predict X-ray crystallographic structures of the enzyme-substrate complexes [7]. The final structures of the kinases-optimal substrates were minimized using the Tripos force field [8] until RMS gradient was less than 0.05 kcal mol^{-1}·Å laying all residues within 15 Å around the fixed active sites.

A subsequent three-dimensional docking study of the optimal substrates at the active site of p60$^{c\text{-}src}$ well supported the experimental results (Figure S1a). For the most optimal substrate determined by the statistical analysis, Ile-Tyr-Glu-Glu, Ile located at the -1 position electrostatically interacts with Gly406 in p60$^{c\text{-}src}$, and the hydroxyl group of Tyr very closely protrudes toward the phosphate group of ATP (P-O distance: 4.16 Å) and can make hydrogen bonds with Arg388 and Asp286 in the active site. The docking energy (ΔG) of the optimal substrate was -15.76 kcal/mol. Replacement of Ile with the suboptimal amino acid Glu also showed similar docking energy (ΔG=-11.22 kcal mol^{-1}) and P-O distance (4.34 Å) at the reaction center. Interestingly, if the Glu at the +1 and +2 positions were changed to other amino acids, the substrate peptides could not be even inserted into the active site pocket of kinase p60$^{c\text{-}src}$. Consequently, these results again confirmed that the identified substrate specificity of the kinase was in agreement with previous reports [9], and that this method is generally applicable to the identification of kinase-specific substrates.

In addition, the preference for Glu at the -1, +1 and +2 positions was also demonstrated in the ZAP-70 tyrosine kinase-substrate modeling study (Figure S1b). Glu at the -1 position makes contact with Ser497 coordinating at the edge of the binding pocket, and Glu at the +1 position is positioned to form a hydrogen bond with Lys500. The hydroxyl group of Tyr makes hydrogen bonds simultaneously with the polar residues, Asp479 and Asp461, in the active site. For the optimal substrate, the docking energy was -28.17 kcal mol^{-1}, and the P-O distance with the reaction center was 3.53 Å. When Glu at the -1 position is replaced with Asp, the hydroxyl group of Tyr only interacts with Asp479 and the Tyr residue is apart from the active site of the enzyme with a longer P-O distance (4.44 Å).

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Table S1. p60$^{c\text{-}src}$ tyrosine kinase phosphorylation profiles

NH_2 - Ala – $X_{(-2)}$ – $X_{(-1)}$ - Tyr – $X_{(+1)}$ – $X_{(+2)}$ - Ala - linker - bead				
ZGYBE	AEYVE	NFYEE	EDYZD	WNYEP
GZYDE	BNYEE	EMYBE	GEYBD	NWYBH
VDYZE	NPYBE	DBYTE	BPYMD	NVYET
HRYHE	BNYGE	RHYSE	PSYED	PNYZM
BEYPE	GEYZE	VBYEE	ZGYPD	EBYPN
ARYPE	ZAYZE	FZYZE	ZZYBD	DMYDT
NBYVE	ZTYBE	EBYME	BHYND	WZYES
MTYHE	ENYAE	ASYWE	HZYND	DEYPN
SWYGE	BAYGE	VSYWE	EBYWD	EZYBN
PDYEE	SMYDE	DDYBE	DBYET	ERYEG
AVYDE	NZYVE	GEYVE	BAYEA	NNYEM
BDYNE	ZWYDE	EFYZE	SSYGG	EDYNH
AWYDE	MBYNE	REYVE	NEYEB	MBYNF
AHYEE	ZPYPE	HEYTD	NBYGZ	
AZYDE	TWYHE	TDYED	VWYVN	
GAYBE	ABYEE	GDYED	BRYZT	
SEYME	GZYDE	SSYED	WSYMA	

*B = I/L, Z = Q/K

Table S2. ZAP-70 tyrosine kinase phosphorylation profiles

NH_2 - Ala – $X_{(-2)}$ – $X_{(-1)}$ - Tyr – $X_{(+1)}$ – $X_{(+2)}$ - Ala - linker - bead				
MNYDE	ZNYDE	FVYBE	WTYED	MEYGP
RVYTE	BZYWE	BFYBE	HTYED	MBYET
SEYWE	WWYEE	ZVYDE	SAYWD	AEYBB
DTYDE	RZYHE	GFYDE	HNYED	HGYEF
NDYDE	MFYZE	ZNYNE	EDYZD	HTYEM
BBYDE	GFYNE	ZEYNE	TDYZD	AEYDB
TSYNE	ZSYHE	TNYRE	EEYMD	FHYSG
DBYBE	VDYZE	GFYED	ENYGD	GNYEM
EGYGE	HPYAE	GWYSD	NEYSD	PGYEV
ZBYDE	FZYAE	EBYED	EDYVD	HDYEW
AEYWE	FSYNE	EGYHD	RTYED	BGYDB
BMYHE	BNYNE	DDYHD	TNYGD	MEYEB
DDYNE	MMYME	TFYED	SDYBD	BGYES
SPYDE	WAYSE	NEYFD	ZEYND	PEYES
SHYPE	ZAYME	WSYZD	DDYHD	DPYEN
VVYNE	DDYSE	RHYSD	AAYZT	ZZYEZ
EDYSE	AAYWE	HEYHD	ADYBZ	
TSYME	ABYAE	BTYZD	ZEYZR	
SDYWE	RBYME	ZAYDD	EZYDM	

*B = I/L, Z = Q/K

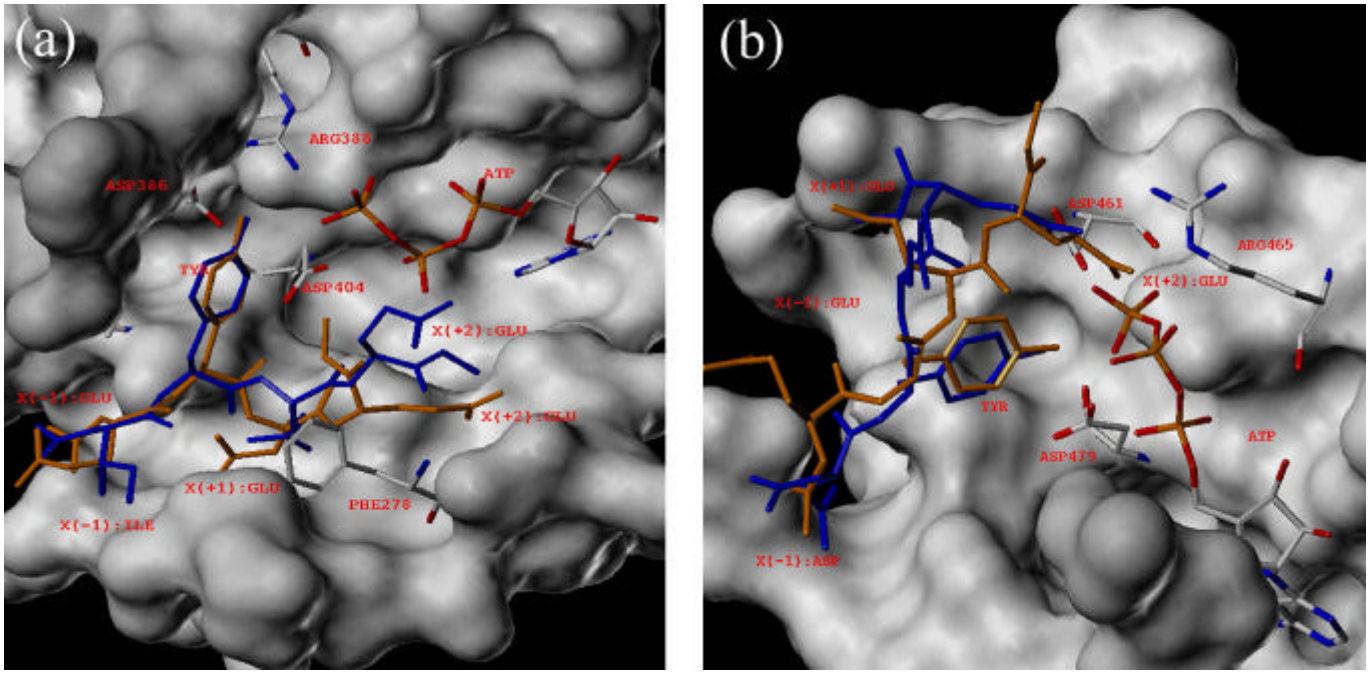

Figure S1. (a) Three-dimensional model of $p60^{c\text{-}src}$ tyrosine kinase bound to the tetrapeptide substrates Ile-Tyr-Glu-Glu (blue color) and Glu-Tyr-Glu-Glu (dark yellow color). **(b)** Three-dimensional model of ZAP-70 tyrosine kinase bound to the tetrapeptide substrates Glu-Tyr-Glu-Glu (dark yellow color) and Asp-Tyr-Glu-Glu (blue color).

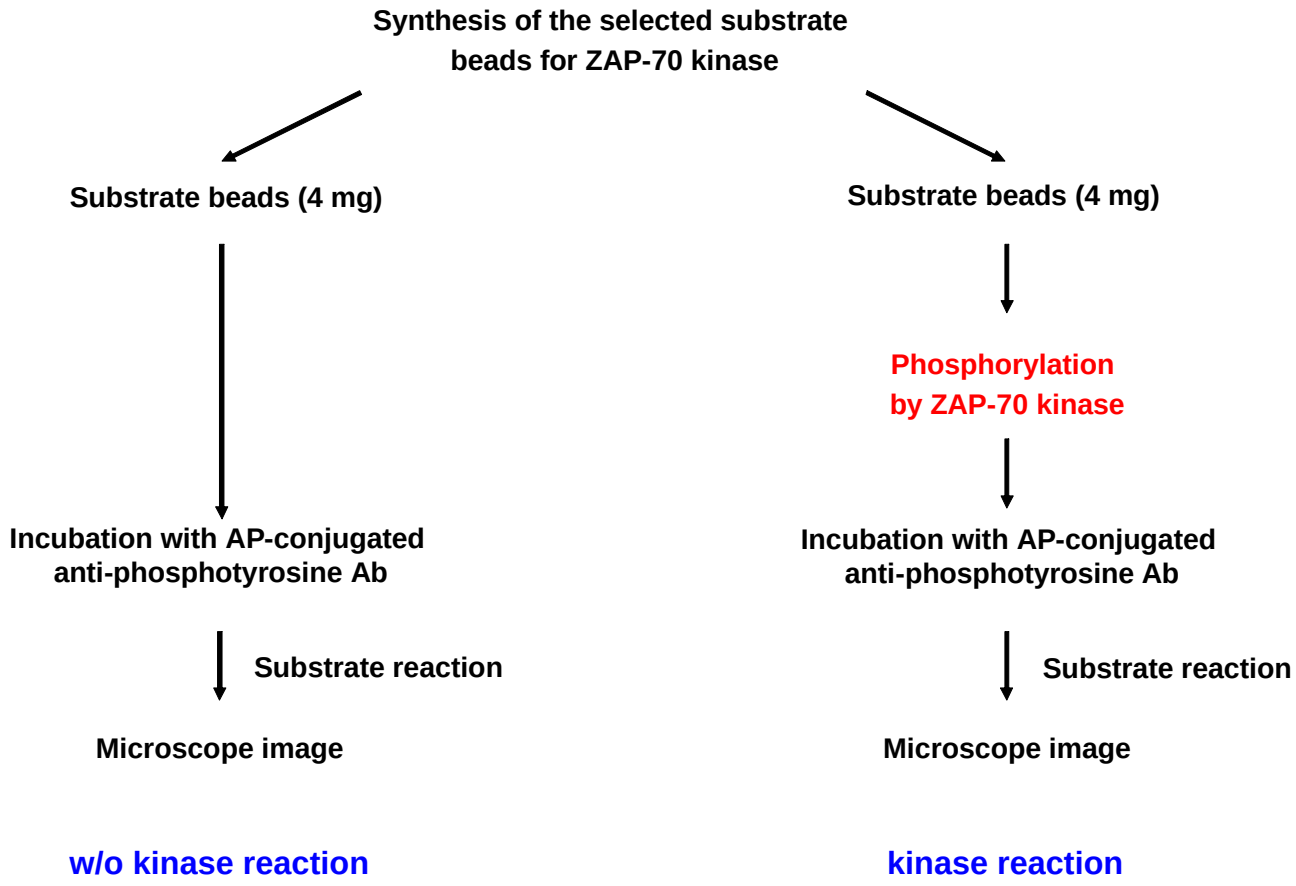

Figure S2. Overall scheme of the qualification for ZAP-70 kinase substrate.