A Phos-tag-based fluorescence resonance energy transfer system for the analysis of the dephosphorylation of phosphopeptides

Kei Takiyama, Eiji Kinoshita*, Emiko Kinoshita-Kikuta, Yoshitake Fujioka, Yusuke Kubo, and Tohru Koike*

Department of Functional Molecular Science, Graduate School of Biomedical Sciences, Hiroshima University, Kasumi 1-2-3, Hiroshima 734-8553, Japan

Short title: A Phos-tag-based FRET system

* Correspondence authors. Fax: +81 82 257 5336.
E-mail address: kinoeiji@hiroshima-u.ac.jp (E. Kinoshita); tkoike@hiroshima-u.ac.jp (T. Koike).

[1] *Abbreviations used:* ELISA, enzyme-linked immunosorbent assay; FRET, fluorescence resonance energy transfer; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; AMCA, 7-amino-4-methylcoumarin-3-acetic acid; FAM, carboxyfluorescein; PTP1B, protein-tyrosine phosphatase 1B; PKA, protein kinase A; MAPK2, mitogen-activated protein kinase 2; MOPS, 3-morpholinopropanesulfonic acid; TFA, trifluoroacetic acid.

Abstract

Fluorescence resonance energy transfer (FRET) is a distance-dependent interaction between the electronic excited states of two dye molecules. Here, we introduce a novel FRET system for the detection of phosphopeptides using a phosphate-binding tag molecule, Zn^{2+}−Phos-tag (1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato dizinc(II) complex) attached with a 7-amino-4-methylcoumarin-3-acetic acid (AMCA). Carboxyfluorescein (FAM)-labeled phospho- and nonphosphopeptides were prepared as the target molecules for the FRET system. A set of FAM (a fluorescent acceptor, λ_{em} 520 nm) and AMCA (a fluorescent donor, λ_{ex} 345 nm) is frequently used for a FRET system. The AMCA-labeled Zn^{2+}−Phos-tag captured specifically the FAM-labeled phosphopeptide to form a stable 1:1 complex, resulting in efficient FRET. After the FAM-labeled phosphopeptide was dephosphorylated with alkaline phosphatase, the FRET disappeared. Using this FRET system, we demonstrated the detection of the time-dependent dephosphorylation of the FAM-labeled protein-tyrosine phosphatase 1B substrate.

Key words: FRET; Phos-tag; Phosphorylation; Dephosphorylation; Phosphopeptide

Introduction

Protein kinases and phosphatases are implicated in a variety of cellular processes, such as proliferation, differentiation, and apoptosis. An estimated 20% of all proteins in humans are phosphorylated, and the families of protein kinases and phosphatases represent up to 5% of the human genome [1,2]. These enzymes increase (or suppress) the activity of other enzymes, mark proteins for destruction, allow proteins to move from one subcellular compartment to another, or enhance (or impede) protein-protein interactions. Any change in the expression level, activity, or localization of these enzymes greatly influences the regulation of key processes. Because of the critical roles of protein kinases and phosphatases in cellular functions, they represent important drug targets [3,4]. The existing methods to detect phosphorylation and dephosphorylation include the radiometric assay [5], enzyme-linked immunosorbent assay (ELISA)[1] [6], ATP consumption assay [7], and several fluorescence-based assays, such as the time-resolved fluorescence [8], fluorescence polarization [9,10], fluorescence resonance energy transfer (FRET) [11], and fluorescence quench assays [12]. Although the radiometric method can be applied to almost all kinase and phosphatase targets with high sensitivity, radioisotopes are inconvenient to handle regarding certain safety and disposal. The ELISA method requires specific antibody-based detection reagents. Unfortunately, the specificity of antibodies is problematic in some cases. Fluorescence-based methods do not necessarily require a radioactive or immunoactive label and are thus attractive as convenient and high-quality approaches for the analysis of the phosphorylation status of targeted substrates.

Recently, we reported original methods for detecting phosphorylated compounds, such as phosphopeptides, phosphoproteins, and phospholipids, using a phosphate-binding tag molecule (Phos-tag), which is a dinuclear metal(II) complex of 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olate [13]. The procedures for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for the enhanced analysis of the phosphorylated compounds [14−16], phosphate affinity

chromatography for the separation of phosphopeptides and phosphoproteins [17,18], surface plasmon resonance analysis for reversible peptide phosphorylation [19,20], Western blot analysis for the detection of phosphoproteins on a blotting membrane [21,22], and phosphate affinity electrophoresis for the mobility shift detection of phosphoproteins on an SDS-PAGE gel [23−28] have been established and put to practical use.

Herein, we describe a novel FRET system using Zn^{2+}−Phos-tag for the real-time analysis of peptide phosphorylation status. FRET is a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor one without emission of a photon. For this FRET system, a Phos-tag derivative as a fluorescent donor attached with a 7-amino-4-methylcoumarin-3-acetic acid (AMCA) and carboxyfluorescein (FAM)-labeled peptides as fluorescent acceptors were prepared. As the first practical example using the AMCA-labeled Zn^{2+}−Phos-tag, we demonstrated the detection of the time-dependent dephosphorylation of the FAM-labeled protein-tyrosine phosphatase 1B (PTP1B) substrate.

Materials and methods

Materials

Bovine intestinal mucosa alkaline phosphatase, L-serine, *O*-phospho-L-serine (phosphoserine), *O*-phospho-L-tyrosine (phosphotyrosine), ATP, glycerol 2-phosphate disodium salt hydrate, and boric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thin-layer and silica gel column chromatographies were performed using a Merck silica gel TLC plate, numbers 5554 and 5567 (Darmstadt, Germany), and Fuji Silysia Chemical NH-DM 1020 silica gel (Kasugai, Japan), respectively. DIAION HP-20 was purchased from Mitsubishi Kagaku (Tokyo, Japan). 2,4,6-Trihydroxyacetophenone was purchased from Aldrich (Milwaukee, WI, USA). A recombinant mouse protein kinase A (PKA) catalytic subunit and sodium orthovanadate were purchased from Calbiochem (La Jolla, CA, USA). Recombinant mouse mitogen-activated protein kinase 2 (MAPK2), and recombinant Abl were purchased from Upstate Biotechnology (Lake Placid, NY, USA). 6-Carboxyfluorescein (6-FAM) succinimidyl ester and 6-([7-amino-4-methylcoumarin-3-acetyl]amino)hexanoic acid succinimidyl ester were purchased from Invitrogen (Carlsbad, CA, USA). 5-FAM-labeled Kemptide, 5-FAM-labeled EGF receptor 661–681, 5-FAM-labeled Abltide, a 5-FAM-labeled phosphorylated PTP1B substrate, and a 5-FAM-labeled phosphorylated histone H1-derived peptide were purchased from Anaspec (San Jose, CA, USA). Microcon YM10 filter units were purchased from Millipore (Bedford, MA, USA). NANOSEP MF GHP (45 μm) centrifugal devices were purchased from Pall (Ann Arbor, MI, USA). All reagents and solvents used were of the highest commercial quality. All aqueous solutions were prepared using deionized and distilled water.

Apparatus

UV and visible spectra were recorded on a Jasco spectrophotometer V-630 (Tokyo, Japan) at 25.0 ± 0.1 ˚C. Fluorescence spectra were obtained with a Hitachi F-2500 fluorescence

spectrophotometer (Tokyo, Japan) at 25.0 ± 0.1 ˚C. The IR spectrum was recorded on a Horiba FT-710 infrared spectrometer (Kyoto, Japan) with a KCl pellet (Real Crystal IR Card) at 20 ± 2 ˚C. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra at 25.0 ± 0.1 ˚C were recorded on a JEOL LA500 spectrometer (Tokyo, Japan). Tetramethylsilane (in $CDCl_3$) (Merck) was used as an internal reference for the NMR measurements. MALDI-TOF MS spectra (positive reflector mode) were obtained on a Voyager RP-3 BioSpectrometry Workstation (PerSeptive Biosystems, Path Framingham, MA, USA) equipped with a nitrogen laser (337 nm, 3-ns pulse). Time-to-mass conversion was achieved by external calibrations using the peaks for angiotensin I (*m/z* 1296.7 for $M + H^+$) and a peptide, ACTH (clip 18-39) (*m/z* 2465.2 for $M + H^+$). The pH measurement was conducted with a Horiba F-12 pH meter and a combination pH electrode (Horiba-6378), which was calibrated using pH standard buffers (pH 4.01 and 6.86) at 25 ˚C. High-performance liquid chromatography (HPLC) was performed using an HPLC system Jasco LCSS-905 with a column oven CO-2060 (at 40 ˚C), a UV-detector UV-2070 (at 480 nm), and a reversed phase column (Shiseido CAPCELL PAK C18 UG80, 4.6 × 150 mm, Yokohama, Japan). All samples were separated under the proper gradient conditions (eluent: 0.1% [v/v] TFA/H_2O and 0.1% [v/v] TFA/CH_3CN) for 20 min at a flow speed of 1.0 mL/min.

Synthesis of the AMCA-labeled Phos-tag ligand

An amino-pendant Phos-tag ligand (*N*-[5-(2-aminoethylcarbamoyl)pyridine-2-ylmethyl] -*N,N',N'*-tris[pyridin-2-ylmethyl]-1,3-diaminopropan-2-ol) was synthesized as described previously [17]. A solution of 6-([7-amino-4-methylcoumarin-3-acetyl]amino)hexanoic acid succinimidyl ester (9.7 mg, 21.9 μmol) in 1 mL of *N,N*-dimethylformamide was added dropwise to a solution of the amino-pendant Phos-tag ligand (10.5 mg, 19.4 μmol) in 5 mL CH_3CN at room temperature. After the reaction mixture was stirred for 1 h at room temperature, the solvent was evaporated. The residue was purified by silica gel column chromatography (eluent: $CHCl_3$/MeOH = 50:0 to 50:1, silica gel: NH-DM1020) to obtain the AMCA-labeled Phos-tag ligand, *N*-(5-[2-(*N*-6-[(7-amino-4-methylcoumarin-3-acetyl)amino] hexyl)aminoethylcarbamoyl]pyridine-2-ylmethyl)-*N,N',N'*-tris[pyridin-2-ylmethyl]-1,3-

diaminopropan-2-ol) as a pale-yellow oil (16.2 mg, 18.6 μmol, 96% yield). TLC (Merck 5567, eluent: $CHCl_3$/MeOH/28% (v/v) aqueous NH_3 = 10:2:0.5) R_f = 0.6. IR: 3339, 3226, 3063, 2930, 2854, 1648, 1598, 1553, 1475, 1436, 1390, 1367, 1249, 1172, 1150, 1035, 846, 765, 472, 408 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.20−1.28 (2H, m, CCH_2CCC), 1.42 (2H, quin, J = 7.0 Hz, $CCCH_2CC$), 1.58 (2H, quin, J = 7.3 Hz, $CCCCH_2C$), 2.15 (2H, t, J = 7.1 Hz, CH_2CCCC), 2.43 (3H, s, CH_3), 2.54−2.68 (4H, m, $NCCCH_2N$), 3.15 (2H, dd, $CCCCCH_2$, J = 12.6, 6.4 Hz), 3.49 (2H, s, $NCOCH_2$), 3.51−3.65 (4H, m, $NCCH_2N$), 3.80−3.96 (9H, m, NCCHCN and $PyCH_2N$), 4.40 (2H, bs, NH_2), 6.49 (1H, d, J = 2.1 Hz, COOCCHC), 6.54−6.58 (2H, m, COOCCCCHC, and CONH), 6.66 (1H, CONH), 7.10−7.12 (3H, m, PyH), 7.33 (3H, d, J = 7.8 Hz, PyH), 7.37 (1H, d, J = 8.7 Hz, COOCCCCCHC), 7.42 (1H, d, J = 8.0 Hz, PyH), 7.56−7.60 (3H, m, PyH), 7.87 (1H, CONH), 8.02 (1H, dd, PyH, J = 8.1 and 2.2 Hz), 8.49 (3H, m, PyH), 8.95 (1H, d, J = 1.6 Hz, PyH). ^{13}C NMR ($CDCl_3$) δ 11.6, 15.5, 24.9, 26.1, 28.8, 35.7, 36.4, 39.1, 39.4, 41.6, 59.2, 60.8, 61.0, 67.3, 100.6, 111.9, 112.3, 115.1, 122.1, 122.7, 123.2, 126.4, 127.1, 128.0, 128.3, 135.4, 136.6, 145.0, 149.0, 150.8, 154.2, 158.2, 159.3, 159.4, 163.1, 163.5, 163.8, 164.1, 165.7, 166.3, 166.6, 170.3, 175.0, 176.8. MALDI-TOF MS: AMCA-labeled Phos-tag ligand in a 50% (v/v) CH_3CN solution containing 2,4,6-trihydroxyacetophenone (5 mg/mL), *m/z* 869.4. 1:1 phosphate-bound dizinc(II) complex (AMCA-labeled Zn^{2+}−Phos-tag–$HOPO_3^{2-}$) in a 25% (v/v) CH_3CN solution containing 2,4,6-trihydroxyacetophenone (2.5 mg/mL), 20 μM $ZnCl_2$, 10 μM AMCA-labeled Phos-tag ligand, and 20 μM $NaHPO_4$-NaOH (pH 7.4), *m/z* 1090.7.

Preparation of 6-FAM-labeled serine, phosphoserine, and phosphotyrosine

A solution of 6-FAM succinimidyl ester (5.5 mg, 12.4 μmol) in 230 μL of dimethyl sulfoxide was added to a solution of the amino acid (serine, phosphoserine, or phosphotyrosine) in 2.3 mL of 0.20 M $NaHCO_3$-NaOH (pH 8.4) at room temperature. After stirring overnight at room temperature, the reaction mixtures were diluted to 7.3 mL with distilled water and acidified by the addition of aqueous 6.0 M HCl to pH 3. A lipophilic resin, DIAION HP-20 (6.0 g) was added to the reaction mixtures. The resin was washed with 20 mL

of aqueous 1.0 mM HCl and the adsorbed compound was eluted with methanol. After the solvent was evaporated, the residue was lyophilized to obtain 6-FAM-labeled serine, phosphoserine, and phosphotyrosine as orange-colored powders. The purity of each powder was determined by HPLC. As for 6-FAM-labeled serine, TLC (Merck 5554, eluent: $CHCl_3$/MeOH/0.10 M CH_3COOH*aq* = 5:6:2): R_f = 0.7. HPLC (eluent: 0.1% [v/v] TFA/H_2O from 90% to 40% (v/v) and 0.1% [v/v] TFA/CH_3CN from 10% to 60% [v/v], 20 min): Retention time = 11.5 min, purity 90%. MALDI-TOF MS: 6-FAM-labeled serine (100 μM) dissolved in 50% CH_3CN solution containing 2,4,6-trihydroxyacetophenone (5.0 mg/mL) and 0.1% (v/v) TFA, *m/z* 464.0. As for 6-FAM-labeled phosphoserine, TLC (Merck 5554, eluent: $CHCl_3$/MeOH/0.1 M CH_3COOH*aq* = 5:6:2): R_f = 0.3. HPLC (eluent: 0.1% [v/v] TFA/H_2O from 90% to 40% (v/v) and 0.1% [v/v] TFA/CH_3CN from 10% to 60% [v/v], 20 min): Retention time = 9.0 min, purity 77%. MALDI-TOF MS: 6-FAM-labeled phosphoserine (100 μM) dissolved in a 50% CH_3CN solution containing 2,4,6-trihydroxyacetophenone (5.0 mg/mL) and 0.1% (v/v) TFA, *m/z* 544.1. As for 6-FAM-labeled phosphotyrosine, TLC (Merck 5554, eluent: $CHCl_3$/MeOH/0.1 M CH_3COOH*aq* = 5:6:2): R_f = 0.4. HPLC (eluent: 0.1% [v/v] TFA/H_2O from 90% to 40% (v/v) and 0.1% [v/v] TFA/CH_3CN from 10% to 60% [v/v], 20 min): Retention time = 10.9 min, purity 82%. MALDI-TOF MS: 6-FAM-labeled serine (100 μM) dissolved in a 50% (v/v) CH_3CN solution containing 2,4,6-trihydroxyacetophenone (5.0 mg/mL) and 0.1% (v/v) TFA, *m/z* 620.1.

Preparation of 5-FAM-labeled peptides and phosphopeptides, and 6-FAM-labeled tyrosine

Kinase reactions were conducted at 30 ˚C. For phosphorylation of 40 nmol of 5-FAM-labeled Kemptide (5-FAM-LRRA-S-LG-OH), EGF receptor 661–681 (5-FAM-KRELVEPL-T-PSGEAPNQALLR-NH_2), and Abltide (5-FAM-KKGEAI-Y-AAPFA-NH_2) by PKA, MAPK2, and Abl, respectively, a reaction buffer (200 μL) containing 0.10 M MOPS-NaOH (pH 7.2), 25 mM dithiothreitol, 50 mM EGTA, 50 mM glycerol 2-phosphate, 50 mM sodium orthovanadate, 20 mM $MgCl_2$, and 300 μM ATP was used. The amino acids between two hyphens are phosphorylated by each kinase.

The amount of each kinase in the buffer was 2,500 units of PKA, 0.4 μg of MAPK2, and 0.16 μg of Abl. For the dephosphorylation of the 5-FAM-labeled phosphorylated PTP1B substrate (5-FAM-DADE-*p*Y-LIPQQG-OH), the 5-FAM-labeled phosphorylated histone H1-derived peptide (5-FAM-GGGPA-*p*T-PKKAKKL-OH), and 6-FAM-labeled phosphotyrosine by alkaline phosphatase (4.3 units, 86 units, and 4.3 units, respectively), a reaction buffer (200 μL) containing 50 mM Tris-HCl (pH 9.0) and 1.0 mM $MgCl_2$ was used. These phosphatase reactions were conducted overnight at 37 ˚C. To remove the enzymes, the reaction mixture was put into a Microcon YM10 filter unit and centrifuged at 14,000 × *g* for 10 min. One micro-liter of aqueous 6.0 M HCl and 0.2 g of DIAION HP-20 were added to the filtrates containing FAM-labeled compounds. After the solutions were stirred, the resins were applied in sample reservoirs (Centrifugal Devices, NANOSEP MF GHP) and centrifuged at 2,000 × *g* for 20 s, and the filtered buffers were discarded. To wash the resin, a 1.0 mM HCl solution (300 μL) was added to the sample reservoir. The filter units were centrifuged at 2,000 × *g* for 20 s, and the filtered buffers were discarded. The washing operation was repeated 5 times. To elute the adsorbed FAM-labeled compounds, 300 μL of methanol was added to the sample reservoir. The filter units were centrifuged at 2,000 × *g* for 20 s, and the filtrates were collected. The eluting operation was repeated 3 times. The eluting fractions were combined, evaporated, and lyophilized. The residues were dissolved in 100 μL of a 10 mM Tris-HCl buffer (pH 8.8). The washing and eluting operation was also carried out for 5-FAM-labeled Kemptide, EGF receptor 661–681, Abltide, the phosphorylated PTP1B substrate, and the phosphorylated histone H1-derived peptide, which were not treated by kinase or phosphatase. The purities of all prepared compounds were determined by HPLC. As for 6-FAM-labeled tyrosine, HPLC (eluent: 0.1% [v/v] TFA/H_2O from 90% to 40% [v/v] and 0.1% [v/v] TFA/CH_3CN from 10% to 60% [v/v], 20 min): Retention time = 14.2 min, purity 83%. MALDI-TOF MS: 6-FAM-labeled tyrosine (100 μM) dissolved in a 50% (v/v) CH_3CN solution containing 2,4,6-trihydroxyacetophenone (5.0 mg/mL) and 0.1% (v/v) TFA, *m/z* 540.1. As for all prepared peptides, the data of purity and MALDI-TOF MS were shown in supplementary material.

MALDI-TOF MS analysis of a complex of the FAM-labeled phosphorylated compound and AMCA-labeled Zn^{2+}−Phos-tag

Six aqueous solutions (pH 8, 1 mM Tris-HCl) containing AMCA-labeled Zn^{2+}−Phos-tag (10 μM), a FAM-labeled phosphorylated (20 μM) compound, and its nonphosphorylated counterpart (30 μM) were prepared. The FAM-labeled substituent groups were serine, tyrosine, Kemptide, the PTP1B substrate, the histone H1-derived peptide, and EGF receptor 661–681. An aqueous solution containing AMCA-labeled Zn^{2+}−Phos-tag (10 μM), 5-FAM-labeled phosphorylated Abltide (27 μM), and its nonphosphorylated counterpart (33 μM) was prepared. Each solution (0.50 μL) was mixed with 0.50 μL of a CH_3CN solution containing 2,4,6-trihydroxyacetophenone (10 mg/mL). The predicted Phos-tag complexes with phosphorylated compounds in the mixed solutions were determined by MALDI-TOF MS analyses (positive mode).

Fluorescence analysis

All fluorescence measurements were performed in duplicate at 25.0 ± 0.1 ˚C with 5-nm slit width. The quantum yield of AMCA-labeled Zn^{2+}−Phos-tag was determined by comparison of the integrated corrected emission spectrum of a chemical standard (quinine). Excitation at 345 nm was used for quinine in 0.10 M H_2SO_4, and its quantum yield was assumed to be 0.54. In all fluorescence analyses, a HEPES buffer (a 10 mM HEPES-NaOH buffer [pH 7.4] containing 0.50 M NaCl, 2.0 μM $ZnCl_2$, and 20% [v/v] ethanol) was used. For the excitation and emission spectra measurements of AMCA-labeled Zn^{2+}−Phos-tag, the emission and excitation wavelengths were 445 nm and 345 nm, respectively. The fluorescence spectra of the solutions containing the FAM-labeled compound (0–2 μM) and AMCA-labeled Zn^{2+}−Phos-tag (0.80 μM) excited at 345 nm were measured.

Results and discussion

Preparation of AMCA-labeled Zn^{2+}−Phos-tag

The fluorescent Phos-tag ligand was synthesized by a coupling reaction of an active ester of AMCA (a coumarin derivative) and an amino-pendant Phos-tag derivative in 96% yield (see Materials and methods). The AMCA group emits visible light of wavelength 440−460 nm on irradiation of UV light (*ca.* 350 nm). The Stokes shift of 100 nm allows easy filter discrimination of exciting and emitting radiation. The coumarin derivative is frequently used as a fluorescent donor of a coumarin-fluorescein-based FRET pair [29−31]. The structure of a dinuclear zinc(II) complex of the AMCA-labeled Phos-tag ligand (AMCA-labeled Zn^{2+}−Phos-tag) is illustrated in Fig. 1a. The UV absorption and fluorescence spectra of AMCA-labeled Zn^{2+}−Phos-tag in a buffer solution (pH 7.4) containing 10 mM HEPES-NaOH, 0.50 M NaCl, and 20% (v/v) ethanol at 25 ˚C are shown in Figs. 1b and c, respectively. The absorption maximum (λ_{max}) is 350 nm and the fluorescence peak appears at 445 nm. The quantum yield of AMCA-labeled Zn^{2+}−Phos-tag in the HEPES buffer was 0.38. We confirmed that the fluorescence intensity of AMCA-labeled Phos-tag is proportional to its concentration up to 5 μM.

Determination of the 1:1 complex formation by MALDI-TOF MS

The Phos-tag ligand forms a stable dinuclear zinc(II) complex in the presence of two equivalents of zinc(II) ions at physiological pH [13,14]. In the presence of a phosphate dianion such as $HOPO_3^{2-}$ and phosphorylated peptides, the Zn^{2+}−Phos-tag molecule captures an equimolar phosphate to give a phosphate-bound complex (see Fig. 1a). The phosphate-bound AMCA-labeled Zn^{2+}−Phos-tag complex was confirmed by MALDI-TOF MS (e.g., *m/z* 1090.7 for the AMCA-labeled Zn^{2+}−Phos-tag complex with $HOPO_3^{2-}$). Similarly, we performed MALDI-TOF MS analyses using all pairs of the prepared FAM-labeled phosphopeptides and their nonphosphorylated counterparts. Figures 2a, b, and c show typical mass spectra for the 5-FAM-labeled phosphorylated histone H1-derived peptide

(*m/z* 1690.2), the nonphosphorylated counterpart (*m/z* 1610.1), and the phosphopeptide-bound Zn^{2+}−Phos-tag complex, respectively. The sample for Fig. 2c was prepared by mixing of AMCA-labeled Zn^{2+}−Phos-tag (50 pmol), 5-FAM-labeled phosphopeptide (100 pmol), and 5-FAM-labeled nonphosphopeptide (150 pmol) at pH 8. The MS signal of the phosphopeptide-bound Zn^{2+}−Phos-tag complex appeared at *m/z* 2682.6. While the signals for the phosphopeptide and nonphosphopeptide were also detected in the same spectrum (Fig. 2c), there was no signal for the nonphosphopeptide-bound Zn^{2+}−Phos-tag complex. These results demonstrate that the AMCA-labeled Zn^{2+}−Phos-tag preferentially captures FAM-labeled phosphorylated peptides to form the 1:1 complexes.

Determination of the Phos-tag-based FRET system

We conducted the FRET analysis using AMCA-labeled Zn^{2+}−Phos-tag (a fluorescent donor: λ_{ex} 345 nm, λ_{em} 445 nm) and FAM-labeled phosphorylated compounds (a fluorescent acceptor: λ_{ex} 490 nm, λ_{em} 520 nm) in an aqueous solution (pH 7.4) at 25 ˚C. The test solution contains AMCA-labeled Zn^{2+}−Phos-tag (0.80 μM) and a FAM-labeled phosphorylated compound (0−2.0 μM). The reference experiments were performed using the nonphosphorylated counterparts (0−2.0 μM) under the same conditions. The spectra of 6-FAM-labeled phosphoserine, 5-FAM-labeled phosphorylated Kemptide, the 5-FAM-labeled phosphorylated PTP1B substrate, and 5-FAM-labeled phosphorylated EGF receptor 661–681 are shown in Figs. 3a–d, respectively. The left panels are the spectra using the phosphorylated compounds, and the right panels are those using the nonphosphorylated counterparts. As shown in the left panels, the fluorescence intensity at 445 nm, which is derived from the AMCA-labeled Zn^{2+}−Phos-tag, decreased with an increase in the concentration of the FAM-labeled phosphorylated compounds. Among the phosphorylated compounds tested, the efficiency of FRET varied widely, which is possibly due to the differences in the length (5–9 amino acid sequences) between the AMCA and FAM groups and the dissociation constants K_d for the Zn^{2+}−Phos-tag complexes with the phosphorylated compound (see below). On the other hand (see right panels), the fluorescence spectra using the nonphosphorylated

compounds showed almost no change in intensity at 445 nm. Furthermore, the increases of the emission intensity at 520 nm, which is derived from the FAM group, using the nonphosphorylated compounds were smaller than those using the phosphorylated counterparts. Similar changes in the fluorescence spectra were observed using 6-FAM-labeled phosphotyrosine, the 5-FAM-labeled phosphorylated histone H1-derived peptide, and 5-FAM-labeled phosphorylated Abltide (see supplemental Fig. S1 in supplementary material). Thus, the combination of AMCA-labeled Zn^{2+}−Phos-tag and a FAM-labeled phosphorylated compound provides a novel FRET system for quantitative analysis.

From the changes in the fluorescence intensity at 445 nm as shown in Fig. 3 and supplemental Fig. S1 (see supplementary material), the dissociation constants K_d (μM) of the 1:1 complexes of AMCA-labeled Zn^{2+}−Phos-tag and FAM-labeled phosphorylated compounds (K_d = [AMCA-labeled Zn^{2+}−Phos-tag] [FAM-labeled phosphorylated compound]/[1:1 complex]) were estimated [32]: 0.04 μM for 6-FAM-labeled phosphotyrosine, 0.07 μM for 6-FAM-labeled phosphoserine, 0.06 μM for the 5-FAM-labelded phosphorylated PTP1B substrate, 0.07 μM for 5-FAM-labeled phosphorylated Abltide, 0.6 μM for 5-FAM-labeled phosphorylated Kemptide, 0.6 μM for phosphorylated EGF receptor 661−681, and 0.7 μM for the 5-FAM-labeled phosphorylated histone H1-derived peptide. Although there is a small difference in the K_d values of phosphotyrosine and phosphoserine, the values of the phosphoserine- and phosphothreonine-containing peptides are over 10 times larger than those of the phosphotyrosine-containing peptides. Similarly, the FRET efficiencies determined from the changes in intensity at 445 nm varied as follows: 84% for 6-FAM-labeled phosphotyrosine, 84% for 6-FAM-labeled phosphoserine, 86% for the 5-FAM-labelded phosphorylated PTP1B substrate, 84% for 5-FAM-labeled phosphorylated Abltide, 59% for 5-FAM-labeled phosphorylated Kemptide, 55% for phosphorylated EGF receptor 661−681, and 47% for the 5-FAM-labeled phosphorylated histone H1-derived peptide. These facts suggest that the steric hindrance around the phosphate groups of the phosphoserine- and phosphothreonine-containing peptides would be larger than that of the phosphotyrosine-containing peptides. The other factors responsible for the difference in the

phosphopeptides might be the charge and hydrophobicity of the amino acid sequence context. The donor-to-acceptor distance for an efficient FRET system should be in a range of 1−10 nm [33]. The distances between the FAM and AMCA groups in the phosphate-bound Phos-tag complexes used are all in the range, which is shown by the general molecular models.

Kinetic study of the dephosphorylation of a phosphopeptide

For the kinetic study of the dephosphorylation of a phosphopeptide using alkaline phosphatase, we applied this FRET system. Real-time analyses of the dephosphorylation of 5-FAM-labeled phosphoserine, phosphorylated PTP1B substrate, and phosphorylated histone H1-derived peptide were performed using a 10-mm quartz cell under stirring at 25 ˚C. The reaction mixture (3 mL) contains AMCA-labeled Zn^{2+}−Phos-tag (0.80 μM), a FAM-labeled phosphorylated compound (0.80 or 1.0 μM), 1.0 mM $MgCl_2$, and 10 mM HEPES-NaOH (pH 7.4). The dephosphorylation reaction was initiated by addition of an appropriate amount of alkaline phosphatase. Immediately, the fluorescence intensity at 445 nm increased time-dependently. The rate of the dephosphorylation increased with an increase in the concentration of alkaline phosphatase. Typical fluorescence changes using 6-FAM-labeled phosphoserine in the presence of five concentrations of alkaline phosphatase (0.05, 0.10, 0.20, 0.40, and 0.81 μg/mL) are shown in Fig. 4a. The fluorescence spectrum change for the 5-FAM-labeled phosphorylated PTP1B substrate (1.0 μM) in the presence of alkaline phosphatase (0.60 μg) is displayed in Fig. 4b, which shows the fluorescence increase at 445 nm and the decrease at 520 nm. The plots of the intensities at 445 and 520 nm against the reaction times (0–60 min) are shown in Fig. 4c. At 60 min, the fluorescence spectrum was the same as that for a solution (pH 7.4) containing AMCA-labeled Zn^{2+}−Phos-tag (0.80 μM) and the 5-FAM-labeled nonphosphorylated PTP1B substrate (1.0 μM). The dephosphorylation reaction progressed according to a pseudo-first-order rate kinetics until 20 min. Thus, the fluorescence change is consistent with the time-dependent dephosphorylation of the phosphopeptide to produce the dephosphorylated PTP1B substrate and the phosphopeptide-unbound the AMCA-labeled Zn^{2+}−Phos-tag. On the other hand, there was no

change in the fluorescence spectrum using another substrate, the 5-FAM-labeled phosphorylated histone H1-derived peptide (data not shown). This result indicates the substrate specificity of alkaline phosphatase under the experimental conditions. Consequently, this FRET system would be useful to determine the profile of various phosphatases at physiological pH.

Conclusions

In this report, we introduced a novel FRET system to analyze the dephosphorylation status of phosphorylated compounds. For this system, a novel fluorescent phosphate-binding molecule, AMCA-labeled Zn^{2+}−Phos-tag was synthesized. The Zn^{2+}−Phos-tag derivative selectively captures a FAM-labeled phosphorylated compound, resulting in efficient FRET. Using this FRET system, the real-time analysis of the dephosphorylation reaction of the FAM-labeled phosphopeptide using alkaline phosphatase was demonstrated. The Phos-tag-based FRET system has the following major advantages: i) The real-time analysis of the dephosphorylation reaction is possible without multiple samplings, ii) the analysis requires a simple procedure just using two solutions of AMCA-labeled Phos-tag and a FAM-labeled phosphorylated compound, and iii) the system would be useful for the reliable and comprehensive dephosphorylation assays for various phosphopeptides containing phosphoserine, phosphothreonine, or phosphotyrosine, *in vitro*. Thus, the principle of this system would be applied to a high-throughput phosphatase profiling such as an array-format screening involved in the investigation of substrate specificity, the measurement of enzyme activity, and the determination of an activator (or an inhibitor).

Acknowledgments

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Figure legends

Figure 1.

(a) Structures of AMCA-labeled Zn^{2+}−Phos-tag and its 1:1 complex with phosphomonoester dianion (*R*-OPO_3^{2-}). (b) Absorption spectrum of AMCA-labeled Zn^{2+}−Phos-tag [50 μM] at 25 ˚C and pH 7.4 (10 mM HEPES-NaOH, 0.50 M NaCl, and 20% [v/v] ethanol): λ_{max} = 350 nm and ε = 1.32 × 10^4 L/mol·cm. (c) Emission spectrum of AMCA-labeled Zn^{2+}−Phos-tag (1.0 μM) by 345-nm excitation at 25 ˚C and pH 7.4 (10 mM HEPES-NaOH, 0.50 M NaCl, and 20% [v/v] ethanol): λ_{max} = 445 nm.

Figure 2.

MALDI-TOF MS spectra: (a) the 5-FAM-labeled phosphorylated histone H1-derived peptides, 5-FAM-GGGPA-*p*T-PKKAKKL-OH (100 pmol), (b) the nonphosphorylated counterpart, 5-FAM-GGGPA-T-PKKAKKL-OH (150 pmol), (c) a mixture of 5-FAM-GGGPA-*p*T-PKKAKKL-OH (100 pmol), 5-FAM-GGGPA-T-PKKAKKL-OH (150 pmol), and AMCA-labeled Zn^{2+}−Phos-tag (50 pmol).

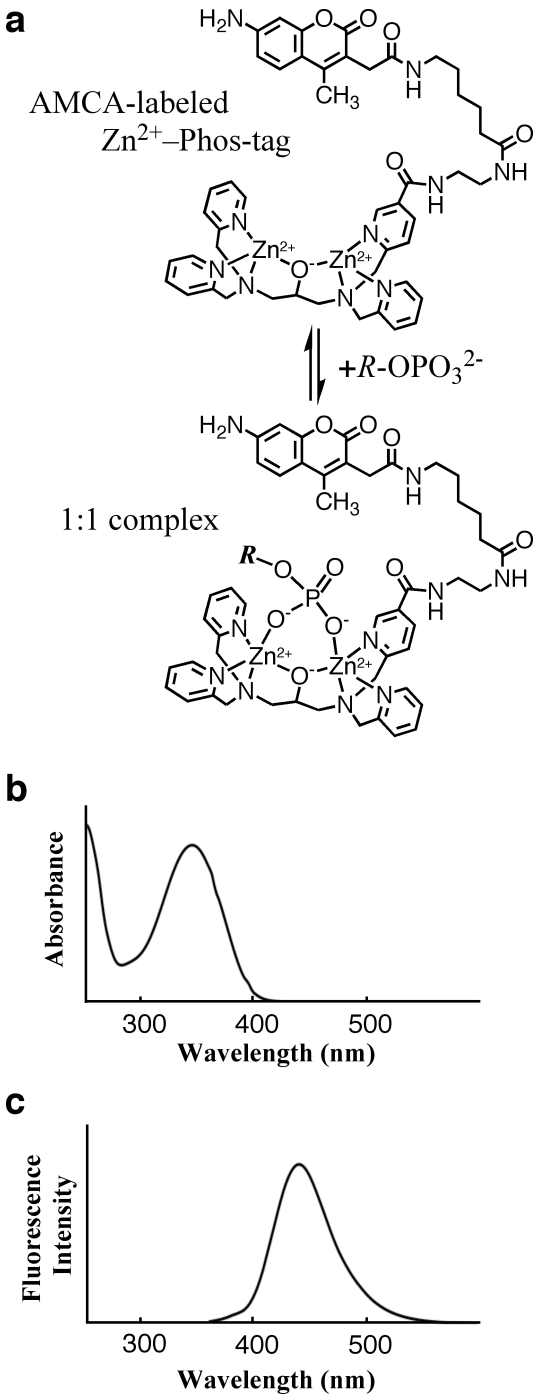
Figure 3.

Overlaid emission spectra (λ_{ex} 345 nm, 25 ˚C, pH 7.4) of the mixtures of AMCA-labeled Zn^{2+}−Phos-tag (0.80 μM) with FAM-labeled phosphorylated (left panels) and nonphosphorylated compounds (right panels): (a) 0 (solid line), 0.2 (circle), 0.4 (triangle), 0.8 (square), and 1.6 μM (cross) of 6-FAM-labeled serine, (b) 0 (solid line), 0.2 (circle), 0.5 (triangle), 0.9 (square), and 1.8 μM (cross) of Kemptide (5-FAM-LRRA-[*p*]S-LG-OH), (c) 0 (solid line), 0.3 (circle), 0.5 (triangle), 1.0 (square), and 2.0 μM (cross) of the PTP1B substrate (5-FAM-DADE-[*p*]Y-LIPQQG-OH), (d) 0 (solid line), 0.2 (circle), 0.5 (triangle), 0.9 (square), and 1.8 μM (cross) of EGF receptor 661–681 (5-FAM-KRELVEPL-[*p*]T-PSGEAPNQALLR-NH_2).

Figure 4.

(a) Time-course changes of the fluorescence intensity at 445 nm of the solutions containing AMCA-labeled Zn^{2+}−Phos-tag (0.80 μM) and of 6-FAM-labeled phosphoserine (0.80 μM) in the presence of alkaline phosphatase (0.05 [black circle], 0.10 [triangle], 0.20 [open circle], 0.40 [cross], and 0.81 μg/mL [square]). (b) Overlaid emission spectra of a mixture of AMCA-labeled Zn^{2+}−Phos-tag (0.80 μM) and the 5-FAM-labeled phosphorylated PTP1B substrate (1.0 μM) at 0, 5, 10, 20, 30, 40, 50, and 60 min after the addition of alkaline phosphatase (0.60 μg). (c) Time-course changes of the fluorescence intensity taken from the spectra of Fig. 4b at 445 nm (open circle) and 520 nm (triangle).

Fig. 1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Wavelength (nm)
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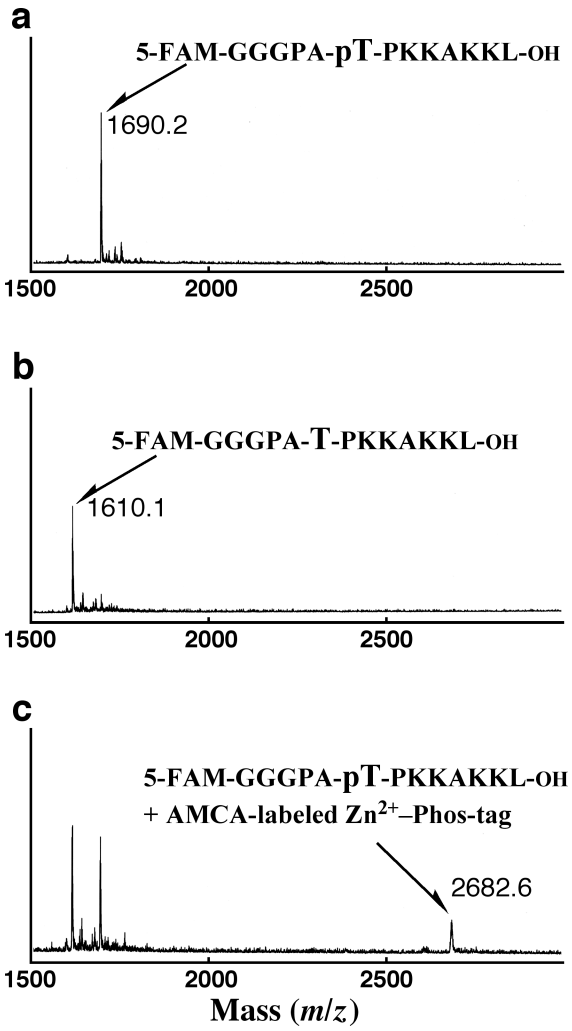
Fluorescence Intensity
300
400
500
Wavelength (nm)

Fig. 2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1500
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2500
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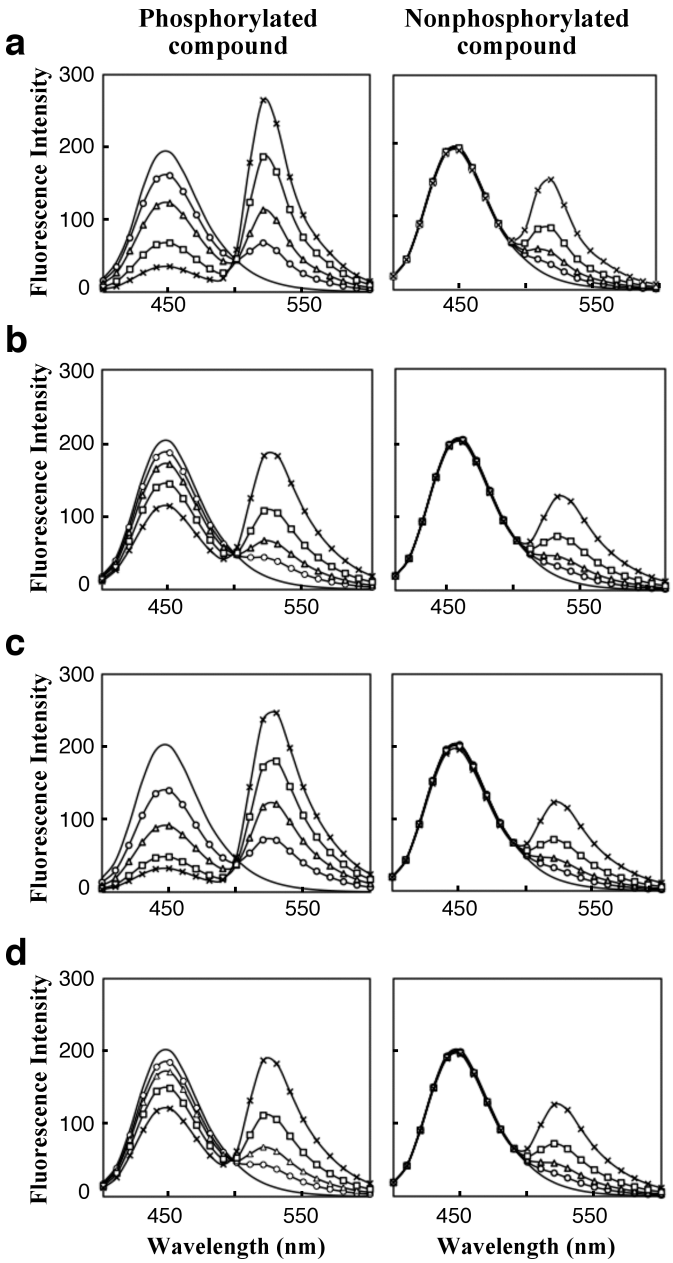
Mass (m/z)

Fig. 3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b
c
d
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100
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450
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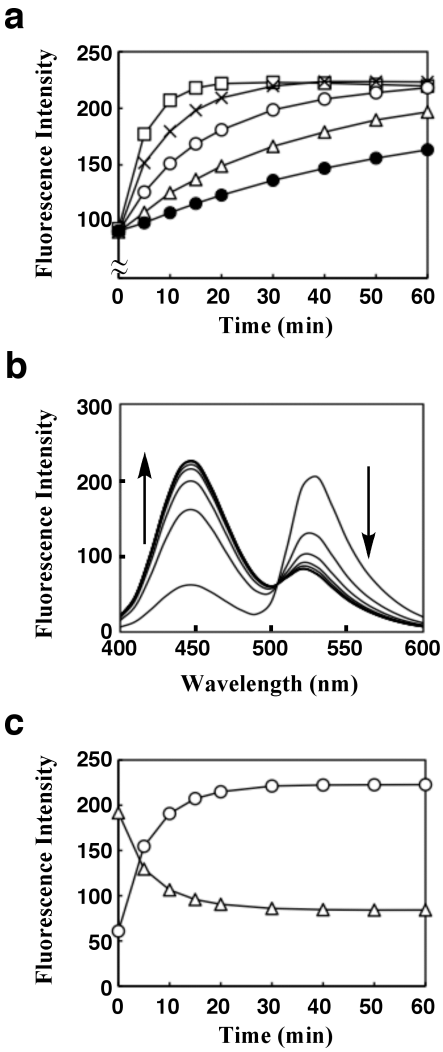
Wavelength (nm)

Fig. 4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20
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40
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b
Fluorescence Intensity
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200
100
0
400
450
500
550
600
Wavelength (nm)
c
Fluorescence Intensity
250
200
150
100
50
0
0
10
20
30
40
50
60
Time (min)

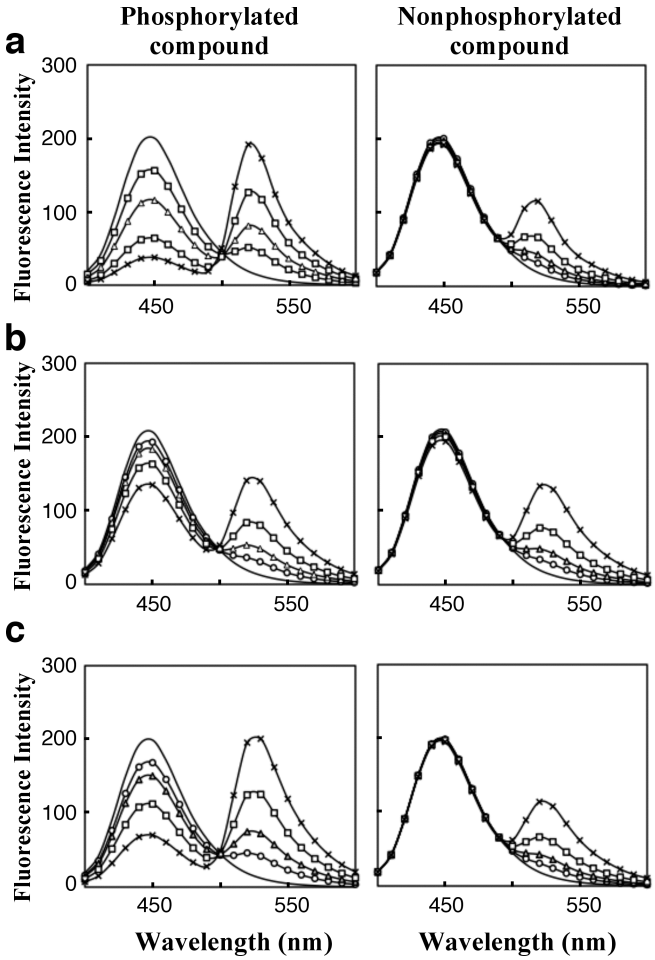
Supplementary material

The purity and MALDI-TOF MS data of all peptides used

As for 5-FAM-labeled Kemptide (5-FAM-LRRA-S-LG-OH), HPLC (eluent: 0.1% [v/v] TFA/H_2O from 90% to 20% [v/v] and 0.1% [v/v] TFA/CH_3CN from 10% to 80% [v/v], 20 min): Retention time = 11.7 min, purity 98%. MALDI-TOF MS: 5-FAM-labeled Kemptide (100 μM) dissolved in a 25% (v/v) CH_3CN solution containing 2,4,6-trihydroxyacetophenone (2.5 mg/mL) and 0.1% (v/v) TFA, *m/z* 1130.2. As for 5-FAM-labeled phosphorylated Kemptide (5-FAM-LRRA-*p*S-LG-OH), HPLC (eluent: 0.1% [v/v] TFA/H_2O from 90% to 20% [v/v] and 0.1% [v/v] TFA/CH_3CN from 10% to 80% [v/v], 20 min): Retention time = 11.5 min, purity 91%. MALDI-TOF MS: 5-FAM-labeled phosphorylated Kemptide (100 μM) dissolved in a 25% (v/v) CH_3CN solution containing 2,4,6-trihydroxyacetophenone (2.5 mg/mL) and 0.1% (v/v) TFA, *m/z* 1210.1. As for the 5-FAM-labeled nonphosphorylated PTP1B substrate (5-FAM-DADE-Y-LIPQQG-OH), HPLC (eluent: 0.1% [v/v] TFA/H_2O from 90% to 20% [v/v] and 0.1% [v/v] TFA/CH_3CN from 10% to 80% [v/v], 20 min): Retention time = 11.8 min, purity 95%. MALDI-TOF MS: the 5-FAM-labeled nonphosphorylated PTP1B substrate (40 μM) dissolved in a 50% (v/v) CH_3CN solution containing 2,4,6-trihydroxyacetophenone (2.5 mg/mL) and 0.1% (v/v) TFA, *m/z* 1605.6. As for the 5-FAM-labeled phosphorylated PTP1B substrate (5-FAM-DADE-*p*Y-LIPQQG-OH), HPLC (eluent: 0.1% [v/v] TFA/H_2O from 90% to 20% [v/v] and 0.1% [v/v] TFA/CH_3CN from 10% to 80% [v/v], 20 min): Retention time = 11.0 min, purity 97.2%. MALDI-TOF MS: the 5-FAM-labeled phosphorylated PTP1B substrate (40 μM) dissolved in a 50% (v/v) CH_3CN solution containing 2,4,6-trihydroxyacetophenone (2.5 mg/mL) and 0.1% (v/v) TFA, *m/z* 1685.3. As for the 5-FAM-labeled histone H1-derived peptide (5-FAM-GGGPA-T-PKKAKKL-OH), HPLC (eluent: 0.1% [v/v] TFA/H_2O from 100% to 50% [v/v] and 0.1% [v/v] TFA/CH_3CN from 0% to 50% [v/v], 20 min): Retention time = 12.3 min, purity 96%. MALDI-TOF MS: the 5-FAM-labeled histone H1-derived peptide (100 μM) dissolved in a 50% (v/v) CH_3CN solution containing 2,4,6-trihydroxyacetophenone (2.5

mg/mL) and 0.1% (v/v) TFA, *m/z* 1610.1. As for the 5-FAM-labeled phosphorylated histone H1-derived peptide (5-FAM-GGGPA-*p*T-PKKAKKL-OH), HPLC (eluent: 0.1% [v/v] TFA/H_2O from 100% to 50% [v/v] and 0.1% [v/v] TFA/CH_3CN from 0% to 50% [v/v], 20 min): Retention time = 12.1 min, purity 99%. MALDI-TOF MS: the 5-FAM-labeled phosphorylated histone H1-derived peptide (100 μM) dissolved in a 50% (v/v) CH_3CN solution containing 2,4,6-trihydroxyacetophenone (2.5 mg/mL) and 0.1%(v/v) TFA, *m/z* 1690.2. As for 5-FAM-labeled Abltide (5-FAM-KKGEAI-Y-AAPFA-NH_2), HPLC (eluent: 0.1% [v/v] TFA/H_2O from 90% to 20% [v/v] and 0.1% [v/v] TFA/CH_3CN from 10% to 80% [v/v], 20 min): Retention time = 11.5 min, purity 94%. MALDI-TOF MS: 5-FAM-labeled Abltide (100 μM) dissolved in a 50% (v/v) CH_3CN solution containing 2,4,6-trihydroxyacetophenone (2.5 mg/mL) and 0.1%(v/v) TFA, *m/z* 1622.1. As for 5-FAM-labeled phosphorylated Abltide (5-FAM-KKGEAI-*p*Y-AAPFA-NH_2), HPLC (eluent: 0.1% [v/v] TFA/H_2O from 90% to 20% [v/v] and 0.1% [v/v] TFA/CH_3CN from 10% to 80% [v/v], 20 min): Retention time = 10.8 min, purity 44.2%. MALDI-TOF MS: 5-FAM-labeled phosphorylated Abltide (100 μM) dissolved in a 50% (v/v) CH_3CN solution containing 2,4,6-trihydroxyacetophenone (2.5 mg/mL) and 0.1% (v/v) TFA, *m/z* 1702.1. As for 5-FAM-labeled EGF receptor 661–681 (5-FAM-KRELVEPL-T-PSGEAPNQALLR-NH_2), HPLC (eluent: 0.1% [v/v] TFA/H_2O from 100% to 50% [v/v] and 0.1% [v/v] TFA/CH_3CN from 0% to 50% [v/v], 20 min): Retention time = 15.1 min, purity 97%. MALDI-TOF MS: 5-FAM-labeled EGF receptor 661–681 (100 μM) dissolved in a 50% (v/v) CH_3CN solution containing 2,4,6-trihydroxyacetophenone (2.5 mg/mL) and 0.1% (v/v) TFA, *m/z* 2674.9. As for 5-FAM-labeled phosphorylated EGF receptor 661–681 (5-FAM-KRELVEPL-*p*T-PSGEAPNQALLR-NH_2), HPLC (eluent: 0.1% [v/v] TFA/H_2O from 100% to 50% [v/v] and 0.1% [v/v] TFA/CH_3CN from 0% to 50% [v/v], 20 min): Retention time = 14.9 min, purity 92%. MALDI-TOF MS: 5-FAM-labeled phosphorylated EGF receptor 661–681 (100 μM) dissolved in a 50% (v/v) CH_3CN solution containing 2,4,6-trihydroxyacetophenone (2.5 mg/mL) and 0.1% (v/v) TFA, *m/z* 2754.3.

Supplemental Figure S1.



Overlaid emission spectra (λ_{ex} 345 nm, 25 ˚C, pH 7.4) of the mixtures of AMCA-labeled Zn^{2+}–Phos-tag (0.80 μM) with FAM-labeled phosphorylated (left panels) and nonphosphorylated compounds (right panels): (a) 0 (solid line), 0.2 (circle), 0.4 (triangle), 0.8 (square), and 1.6 μM (cross) of 6-FAM-labeled tyrosine, (b) 0 (solid line), 0.3 (circle), 0.5 (triangle), 1.0 (square), and 2.0 μM (cross) of the histone H1-derived peptide (5-FAM-GGGPA-[*p*]T-PKKAKKL-OH), (c) left panel: 0 (solid line), 0.1 (circle), 0.2 (triangle), 0.4 (square), and 0.8 μM (cross) of phosphorylated Abltide (5-FAM-KKGEAI-*p*Y-AAPFA-NH_2), which contain 1.3 eq of nonphosphorylated counterpart; right panel: 0 (solid line), 0.2 (circle), 0.5 (triangle), 1.0 (square), or 1.9 μM (cross) of nonphosphorylated Abltide (5-FAM-KKGEAI-Y-AAPFA-NH_2).