Gel-based analysis of protein phosphorylation status by rapid fluorometric staining using TAMRA-labeled Phos-tag

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SUMMARY

Phosphorylation, one of the most common post-translational modifications of proteins, plays a critical role in many biological processes. We have previously developed several analytical methods for determining the phosphorylation status of certain proteins by using a phosphate-capturing binuclear metal complex known as Phos-tag. Here, we describe a novel method for the gel-based in vitro analysis of the phosphorylation status of a protein by a simple and rapid fluorometric staining method that uses a tetramethylrhodamine (TAMRA)-labeled Phos-tag derivative (TAMRA–Phos-tag). The entire staining protocol, which requires less than 2 h to complete, uses three buffer solutions for staining, washing, and dilution, respectively, at room temperature. The gel-based analysis of phosphoproteins in a polyacrylamide gel can be conducted by using a fluorescence imaging scanner with a 532-nm excitation laser and a 580-nm longpass emission filter. As a practical example of the use of the TAMRA–Phos-tag staining method, we examined the time course of dephosphorylation of ovalbumin by an alkaline phosphatase. In addition, inhibitor profiling of a tyrosine kinase Abl was performed by using an Abl-substrate (GST-Abl-tide) and an Abl-inhibitor (Imatinib).

Key words: phosphoprotein-selective staining, gel-based analysis, fluorescence imaging, kinase assay, phosphatase assay

INTRODUCTION

Phosphorylation of proteins is one of the most common post-translational modifications and is associated with numerous regulatory events in biological processes, such as signal transduction, cell-cycle progression, gene expression, metabolism, or apoptosis. A balance between kinase and phosphatase reactions controls the phosphorylation status of a great variety of proteins. Because genetic abnormalities of kinases and phosphatases can induce a broad range of diseases, it is important to determine how phosphorylation is regulated, not only for basic research, but also for clinical applications such as diagnosis or drug discovery. Various methods for the analysis of phosphorylated proteins in kinase and phosphatase reactions have been reported; these generally involve colorimetric, fluorometric, chromatographic, radiometric, immunochemical, electrophoretic, or mass spectrometric approaches.

We have previously reported that the binuclear metal complex 1,3-bis[bi(pyridin-2-ylmethyl)amino]propan-2-olato dizinc(II) (Phos-tag) acts as a phosphate-binding tag molecule under near-physiological conditions. As a result, a number of original analytical methods that use various Phos-tag derivatives have been developed for biological research on the phosphoproteome. In a fluorometric approach, an aminocoumarin-labeled Phos-tag molecule has been utilized in the development of a fluorescence resonance energy transfer (FRET) system for assaying the dephosphorylation of a fluorescein-labeled phosphopeptide substrate by bovine intestinal alkaline phosphatases (ALPs). This assay was based on the principle that an aminocoumarin-labeled Phos-tag derivative captures the fluorescent phosphorylated peptide in preference to its nonphosphorylated counterpart. The formation of a 1:1 complex between the Phos-tag derivative and the phosphopeptide brings the fluorescence donor near the acceptor, resulting in highly efficient FRET. We applied a similar FRET system in an examination of the reverse reaction: the phosphorylation of fluorescein-labeled peptide substrates by several kinases. Recently, a fluorescence-quenching system that uses a...
tetramethylrhodamine (TAMRA)-labeled Phos-tag derivative (TAMRA–Phos-tag) has been developed for a real-time analysis of the hydrolysis of pyrophosphate (a natural di-phosphate) by ALPs at neutral pH in aqueous solution.

The global chemical company PerkinElmer, Inc. (Waltham, MA, USA) formerly provided a fluorophore-labeled Phos-tag derivative for selective staining of phosphorylated proteins on an electrophoretic polyacrylamide gel. However, the chemical structure of the fluorescent dye and the contents of the staining buffer solutions are not open to the public, and the fluorescent Phos-tag dye is no longer commercially available. Another fluorescent dye based on a gallium(III) complex, Pro-Q Diamond (Thermo Fisher Scientific, Waltham, MA, USA), is a well-known staining reagent that is selective for phosphorylated proteins and can be used in electrophoretic research. This phosphate-affinity dye permits the direct in-gel detection of phosphate groups of phosphotyrosine (pTyr), phosphoserine (pSer), and phosphothreonine (pThr) residues. However, Pro-Q Diamond staining involves ten solution changes and the process requires more than 5 h to achieve adequate phosphoprotein selectivity. Several low-molecular-weight fluorophore-labeled aluminum(III) and titanium(IV) complexes have also been developed for the selective detection of Ser-containing proteins in an electrophoretic polyacrylamide gel; however, these assays also involve protocols of similar complexity to that of Pro-Q Diamond staining.

SDS-PAGE is a gel-based proteomic strategy for separating proteins on the basis of their molecular weight. By using an enzyme reaction mixture as an electrophoretic sample, the enzyme and its substrate proteins, which should have different molecular weights, can be separated from one another on an electrophoretic gel. In addition, SDS-PAGE experiments using Tris–HCl buffer (pH=9) or Bis-tris–HCl buffer (pH=7) cause much less chemical damage to phosphoproteins, as demonstrated in our previous study. Moreover, even if a kinase is also phosphorylated through the action of another kinase or by autophosphorylation, a phosphoprotein-selective gel staining method would permit the analysis of the phosphorylation status of both the enzyme and the substrate proteins at different migration positions in the electrophoresis gel. In the SDS-PAGE method, a target phosphorylated protein in the gel can generally be quantified by autoradiography of radioactive phosphate groups or by Western blotting with a phosphorylation-site-specific antibody. By taking advantage of the features of SDS-PAGE and of the Phos-tag technology, we previously developed a label-free kinase profiling technique based on phosphate-affinity electrophoresis (Phos-tag SDS-PAGE). The principle of Phos-tag SDS-PAGE is based on the shifts in the mobilities of phosphorylated proteins compared with those of their nonphosphorylated counterpart as a result of reversible trapping of phosphate moieties by Phos-tag molecules immobilized in the gel. In the present study, we demonstrated an alternative gel-based method for the analysis of the phosphorylation status of a protein by means of SDS-PAGE followed by simple and rapid fluorometric staining with the chemically well-characterized TAMRA–Phos-tag.

MATERIALS AND METHODS

Materials
The TAMRA–Phos-tag ligand and its zinc(II) complex were prepared by the same method as reported previously (see the phosphate-binding structure in Fig. 1). The concentration (typically 5.0 μM) of TAMRA–Phos-tag at pH 7.0 in aqueous solution was established from its molar absorption coefficient of 9.7×10^4 M⁻¹·cm⁻¹ at 557 nm and 25°C. Acrylamide, HEPES, MOPS, ammonium persulfate, DTT, glycerol, glycine, ZnCl₂, MgCl₂, 2-sulfanylethanol, Bis, SDS, TEMED, and lysozyme chloride were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). CBB G-250 and Tris were obtained from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan). Bovine milk β-casein, ovalbumin, bovine serum albumin (BSA), egg-white avidin, and bovine intestinal mucosa ALP were purchased from Sigma-Aldrich (St. Louis, MO, USA). The ALP dose is shown as a conventional enzyme-activity unit, as follows: one unit of ALP tetramethylrhodamine (TAMRA)-labeled Phos-tag derivative (TAMRA–Phos-tag) has been developed for a real-time analysis of the hydrolysis of pyrophosphate (a natural di-phosphate) by ALPs at neutral pH in aqueous solution.

![Fig. 1. Structure of TAMRA–Phos-tag, and its excitation (λmax = 558 nm) and emission (λmax = 580 nm) spectra in a 1:1 mixture of methanol and aqueous buffer (pH 7.0; 10 mM Hepes–NaOH) at 25°C.](image-url)
hydrolyzed 1 μmol of 4-nitrophenyl phosphate per minute at pH 9.8 and 37°C. β-Galactosidase was purchased from Toyobo Co., LTD. (Osaka, Japan). Glycerol 2-phosphate (1,3-dihydroxypropan-2-yl dihydrogen phosphate) and sodium orthovanadate were purchased from Calbio Chem (San Diego, CA, USA). Recombinant human Abl kinase was obtained from Carna Biosciences, Inc. (Kobe, Japan). Imatinib (a tyrosine kinase inhibitor) was supplied by Novartis (Basel, Switzerland). All chemical reagents and solvents were of the highest commercial quality and were used without further purification. All aqueous solutions were prepared by using distilled water.

Preparation of recombinant proteins derived from Escherichia coli

A fusion protein of GST with Abl kinase substrate peptide, EAIYAAPFAKKK (GST-Abltide) was prepared by the following procedure: The two oligonucleotides 5’-ggg atc ccc gga att cga agc gat tta tgc cgc gcc gtt cgc cca aa aa aa atc atc gag cgg ccg cat c-3’ and 5’-gat gcg gcc gct cga tta ttt ttt ggc gaa cgg cgc gtc aat cgc tca gtc cgg gga tcc c-3’ were annealed to produce a double-stranded DNA, and inserted between EcoR I -Xho I sites of pGEX 6p-1 vector (GE Healthcare Ltd., Little Chalfont, UK) by using an In-Fusion cloning kit (Takara-Bio Inc., Otsu, Japan). The plasmid was named pGEX-GST-Abltide. The BL21(DE3) strain of Escherichia coli was transformed with pGEX-GST-Abltide for overexpression of the N-terminal GST-fusion protein Abltide (GST-Abltide). The expressed protein was purified as a single band on SDS-PAGE by using COSMOGEL GST-Accept (Nacalai Tesque, Inc.).

Spectroscopic analyses

Fluorescence excitation and emission spectra of TAMRA–Phos-tag were recorded on a FP-8300 fluorescence spectrophotometer (JASCO Corp., Tokyo, Japan) equipped with a Peltier-thermostatted cell holder maintained at 25.0±0.1°C. The absorption and emission spectra for rhodamine B were recorded in an ethanol solution (4.0 mL) containing 20 μL of 0.1 M NaOH, and its quantum yield was assumed to be the reported value of 0.65 for excitation at 542 nm. Z

SDS-polyacrylamide gel electrophoresis

For conventional Laemmli’s SDS-PAGE, the bilayer gel that we used consisted of a stacking gel containing 4.0% w/v polyacrylamide, 125 mM Tris–HCl (pH 6.8), and 0.10% w/v SDS, together with a separating gel containing 10 or 12.5% w/v polyacrylamide, 375 mM Tris–HCl (pH 8.8), and 0.10% w/v SDS. A stock solution of 30% w/v acrylamide was prepared from a mixture of acrylamide and Bis in a 29:1 ratio. The electrophoresis running buffer was 25 mM Tris and 192 mM glycine containing 0.10% w/v SDS. Sample protein solutions were added to a half volume of 3× SDS-PAGE loading buffer consisting of 195 mM Tris–HCl (pH 6.8), 3.0% w/v SDS, 15% v/v 2-sulfanylethanol, 30% v/v glycerol, and 0.15% w/v bromophenol blue, except as noted. The PAGE apparatus used was an ATTO model AE-6500 (ATTO Co., Tokyo, Japan). Electrophoresis was performed at 30 mA/gel and room temperature until the bromophenol blue dye reached the bottom of the separating gel. After TAMRA–Phos-tag staining (see next section) followed by fluorometric detection of phosphoproteins, the gel was doubly stained with CBB G-250 to confirm the total amounts of the phosphorylated and nonphosphorylated proteins.

Basic protocol for TAMRA–Phos-tag gel staining

The basic protocol for fluorometric gel staining with TAMRA–Phos-tag was performed at ambient temperatures of between 18 and 28°C under atmospheric conditions. The optimized procedures are illustrated in Fig. 2. The TAMRA–Phos-tag staining required three methanol-containing aqueous solutions. The staining solution consisted of a 1:1 v/v mixture of methanol and a pH 7.0 aqueous solution containing 0.20 μM TAMRA–Phos-tag ligand, 0.60 μM ZnCl₂, 0.20 M NaCl, 20 mM HEPES, and 4.4 mM NaOH. The washing solution was a 1:1 v/v mixture of methanol and a pH 7.0 aqueous solution containing 10 μM ZnCl₂, 0.20 M NaCl, 20 mM HEPES, and 4.4 mM NaOH. The diluting solution was a 1:1 v/v mixture of methanol and distilled water, which was stored at 4°C before use. Because TAMRA–Phos-tag can be adsorbed on glassware, we recommend that the staining solution is stored in a polyethylene bottle. The step-by-step protocol for TAMRA–Phos-tag staining is as follows. Step 1: After electrophoresis of the target phosphoprotein on a polyacrylamide mini-gel (90×80×1 mm), the gel is placed in a plastic staining tray, completely covered with the staining solution (60 mL), and incubated at room temperature with gentle shaking for 60 min: there is no need to shield the gel from room light. Step 2: The staining solution is decanted, and
reaction was stopped by the addition of the 3× SDS-PAGE loading buffer (2.5 μL) to aliquots of the reaction mixture (5.0 μL) removed at incubation times of 0, 2, 5, 10, 30, and 60 min. The resulting sample solutions (2.0 μL each) containing 0.5 μg of protein were subjected to 10% w/v SDS-PAGE followed by TAMRA–Phos-tag staining and then CBB G-250 staining. The dose-dependent inhibition of the phosphorylation of GST-Abltide by Imatinib (a tyrosine kinase inhibitor) was determined for a total incubation time of 60 min at 30°C. The reaction mixture (5.0 μL) consisted of 20 mM MOPS–NaOH (pH 7.2), 25 mM glycerol 2-phosphate, 1.0 mM sodium orthovanadate, 1.0 mM DTT, 15 mM MgCl₂, 1.0 mM ATP, 3.0 μg of GST-Abltide, and 1.0 μg of Abl. The kinase reaction was stopped by the addition of the 2× SDS-PAGE loading buffer (10 μL) to aliquots of the reaction mixture (20 μL) removed after incubation times of 0, 0.5, 1, 3, 6, 12, and 24 h. The resulting sample solutions (3.0 μL) containing 1.0 μg of protein were subjected to 10% w/v SDS-PAGE followed by TAMRA–Phos-tag staining and then CBB G-250 staining.

Dephosphorylation of ovalbumin by ALP

The time-course study of the dephosphorylation of dual-site-phosphorylated ovalbumin by bovine intestinal mucosa ALP was carried out for a total incubation time of 24 h at 37°C. The reaction mixture (200 μL) contained 50 mM Tris–HCl (pH 9.0), 1.0 mM MgCl₂, 1.0 units of ALP, and 100 μg of ovalbumin. The dephosphorylation reaction was stopped by the addition of the 3× SDS-PAGE loading buffer (10 μL) to aliquots of the reaction mixture (20 μL) removed after incubation times of 0, 0.5, 1, 3, 6, 12, and 24 h. The resulting sample solutions (3.0 μL) containing 1.0 μg of protein were subjected to 10% w/v SDS-PAGE followed by TAMRA–Phos-tag staining and then CBB G-250 staining.

Phosphorylation of GST-Abltide by Abl

The time-course study of the phosphorylation of the Tyr residue in the Abltide sequence of GST-Abltide (a fusion protein) by Abl (a recombinant tyrosine kinase) was carried out for a total incubation time of 60 min at 30°C. The reaction mixture (50 μL) consisted of 20 mM MOPS–NaOH (pH 7.2), 25 mM glycerol 2-phosphate, 1.0 mM sodium orthovanadate, 1.0 mM DTT, 15 mM MgCl₂, 1.0 mM ATP, 20 μg of GST-Abltide, and 1.0 μg of Abl. The kinase reaction was stopped by the addition of the 3× SDS-PAGE loading buffer (2.5 μL) to aliquots of the reaction mixture (5.0 μL) removed at incubation times of 0, 2, 5, 10, 30, and 60 min. The resulting sample solutions (2.0 μL each) containing 0.5 μg of protein were subjected to 10% w/v SDS-PAGE followed by TAMRA–Phos-tag staining and then CBB G-250 staining. The dose-dependent inhibition of the phosphorylation of GST-Abltide by Imatinib (a tyrosine kinase inhibitor) was determined for a total incubation time of 60 min at 30°C. The reaction mixture (5.0 μL) consisted of 20 mM MOPS–NaOH (pH 7.2), 25 mM glycerol 2-phosphate, 1.0 mM sodium orthovanadate, 1.0 mM DTT, 15 mM MgCl₂, 1.0 mM ATP, 3.0 μg of GST-Abltide, 24 ng of Abl in the absence and presence of various concentrations of Imatinib (0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.1, 6.3, 12.5, 25, and 50 μM). The kinase reaction was stopped by the addition of the 2× SDS-PAGE loading buffer (5.0 μL). The resulting solutions (1.5 μL each) containing 0.5 μg of protein were subjected to 10% w/v SDS-PAGE followed by TAMRA–Phos-tag staining and then CBB G-250 staining.

RESULTS AND DISCUSSION

Characterization of TAMRA–Phos-tag in the staining solution

We have previously reported that Phos-tag derivatives show strong affinities toward pTyr functional groups, phenyl phosphate (dissociation constant $K_d=25$ nM)7), and pSer-, pThr-, or pTyr-containing phosphopeptides ($K_d<0.1$ μM) at neutral pH in aqueous solution13). On the basis of the strong affinity of Phos-tag toward phosphate, we conceived the idea of using a dinuclear zinc(II) complex, the 5-TAMRA-labeled Phos-tag derivative (TAMRA–Phos-tag), as a novel fluorescent dye that would bind selectively to phosphorylated proteins in an electrophoresis gel. The fluorescent Phos-tag ligand was prepared by the same synthesis method as that described previously15). A stock solution of TAMRA–
Phos-tag was prepared by mixing the ligand (0.20 μM) and three equivalents of zinc(II) chloride (0.60 μM) in a buffer solution containing 20 mM HEPES–NaOH (pH 7.0) and 0.20 M NaCl at room temperature. The aqueous solution of TAMRA–Phos-tag was bright magenta in color over a wide pH range from 5 to 11. The absorption maximum for the stock solution of TAMRA–Phos-tag occurs at 557 nm (ε=9.7×10⁴ mol⁻¹ cm⁻¹) at 25°C. The staining solution was prepared as a 1:1 (v/v) mixture of methanol and the stock solution of TAMRA–Phos-tag. The solubility of TAMRA–Phos-tag in a 1:1 mixture of methanol and H₂O was determined to be more than 1 mM at room temperature. Fig. 1 shows the excitation (λex=558 nm) and emission (λem=580 nm) spectra of TAMRA–Phos-tag in the staining solution at 25°C; these are almost identical to those in aqueous solution. TAMRA–Phos-tag in the stock solution was very stable, as shown by the lack of change in its visible absorption and fluorescence spectra on standing for six months at 4°C under atmospheric conditions. The quantum yield of TAMRA–Phos-tag in the staining solution was determined to be 0.32 at 25°C through a comparison with that of the fluorescence standard rhodamine B.

Optimization of the protocol for TAMRA–Phos-tag staining

To determine the optimal conditions for phosphate-selective gel staining by TAMRA–Phos-tag, we adopted SDS-PAGE with the pSer-containing protein β-casein on a polyacrylamide gel (90×80×1 mm). Fig. 2 shows the optimized basic protocol for mini-gel staining with TAMRA–Phos-tag. The three operational steps in the protocol are described in the Experimental section. In the first step, we employed a 1:1 mixture (60 mL) of methanol and a neutral-pH solution containing 0.20 μM TAMRA–Phos-tag, 0.20 M NaCl, and 20 mM HEPES–NaOH (pH 7.0). It is well known that the organic solvent methanol can efficiently fix SDS-denatured proteins in the gel and can simultaneously remove excess lipophilic SDS molecules from the gel. Consequently, no pretreatment, such as protein fixation using an acidic solution, is necessary, thereby reducing the total operational time and the number of solvent changes. Previously, we found that an ionic salt, NaCl or CH₃COONa, significantly enhances the phosphate-selectivity of Phos-tag molecule. Here, we adopted −0.10 M NaCl in the staining and washing solutions. The HEPES buffer system was enough to maintain the pH of the electrophoresis mini-gel at about 7, which is an appropriate pH for the preferential phosphate-binding of TAMRA–Phos-tag. The permeation of TAMRA–Phos-tag (~0.1 μM) into the gel was accomplished within 1 h under gentle shaking in the staining solution at room temperature; this was confirmed by plateauing of the fluorescence of the gel. In the second step, a 1:1 mixture (60 mL) of methanol and a neutral-pH solution containing 0.20 M NaCl, 10 μM ZnCl₂, and 20 mM HEPES–NaOH (pH 7.0) was used to wash the gel. The addition of ZnCl₂ to the washing solution prevents dissociation of zinc(II) ion from TAMRA–Phos-tag ligand, which would otherwise have resulted in dissociation of the phosphorylated protein. We recommend that the appropriate washing time for the best contrast is 5 to 30 min, depending on the amounts and types of phosphorylated proteins. Note that because only one washing step is used, as much as possible of the residual staining solution needs to be removed by absorption with a paper towel. In the third step, the washing solution used was diluted two-fold with 1:1 mixture of methanol and distilled water cooled to 4°C. This operation markedly reduces the background fluorescent signal from residues of TAMRA–Phos-tag dye on the surface of the gel. For the basic protocol, the total operational time for the TAMRA–Phos-tag staining and the visualization of phosphorylated proteins is significantly short, at less than 2 h.

Fluorometric detection of pSer-containing phosphoproteins

The selectivity and sensitivity of TAMRA–Phos-tag to the pSer-containing phosphoproteins ovalbumin (two pSer residues; 42 kDa) and β-casein (five pSer residues; 25 kDa) were evaluated by SDS-PAGE using two-fold serially diluted samples containing four nonphosphorylated proteins: β-galactosidase, BSA, avidin, and lysozyme. Each six-protein mixture in the sample solution containing SDS and 2-sulfanylethanol was denatured at 90°C for 5 min before loading. Fig. 3a shows a typical result for an experiment performed in accordance with the basic protocol using the mixed samples containing each protein in the concentration range 1000 to 4 ng/lane. It is clear that both phosphorylated proteins were selectively stained by TAMRA–Phos-tag.
whereas the fluorescence signals for the nonphosphorylated proteins were almost identical to the background values. The total amount of each protein (phosphorylated and nonphosphorylated forms) in given electrophoresis band was confirmed by subsequent staining with the blue-colored dye CBB G-250 (see Fig. 3b). There was no need for a prewashing procedure before the CBB G-250 staining of the TAMRA–Phos-tag stained gel. The detection limits for TAMRA–Phos-tag staining were almost the same as those for CBB G-250 staining: between 8 and 16 ng/lane. The linear dynamic ranges for TAMRA–Phos-tag staining of ovalbumin and β-casein were determined by using the densitographic software CS Analyzer (ATTO Co., Tokyo, Japan). The linearity coefficients of the fluorescence responses against the amounts of proteins per lane in the range from 1000 to 8 ng/lane were evaluated to be 0.995 for ovalbumin and 0.990 for β-casein. In this range of protein concentrations, the fluorescence intensities can therefore be used for quantitative determination of pSer-containing proteins. Unfortunately, lower-molecular-weight and fifth-site phosphorylated β-casein (which contains more phosphate groups per unit mass) showed almost the same fluorescence intensities as those of larger-molecular-weight and dual-site phosphorylated ovalbumin (which has fewer phosphate groups per unit mass). In other words, the fluorescence response per phosphate group of β-casein is obviously weaker than that of ovalbumin. The weak response of β-casein might be due to intermolecular fluorescence quenching between the TAMRA–Phos-tag molecules or to steric hindrance toward the binding of TAMRA–Phos-tag molecules to the tandem-type pSer residues of β-casein (pSer-Leu-pSer-pSer-pSer-pSer). However, the linear fluorescence response of each phosphorylated protein suggests that TAMRA–Phos-tag staining can be used in gel-based in vitro analyses of phosphatase and kinase reactions involving certain enzyme–substrate protein pairs, as shown below.

Gel-based analyses of phosphatase and kinase reactions

As a first analysis of a phosphorylated protein by TAMRA–Phos-tag staining, we examined the time-dependent dephosphorylation of ovalbumin in the presence of bovine intestinal mucosa ALP during an incubation period of 24 h at 37°C and pH 9.0. After the enzyme reaction had been stopped by addition of the SDS-PAGE loading buffer, each reaction mixture was left to stand at room temperature before loading. The upper view in Fig. 4a shows typical electrophoresis results for incubation times of 0, 0.5, 1, 3, 6, 12, and 24 h. The fluorescence intensity of ovalbumin decreased with time, and the intensity at 24 h reached less than 5% of that at 0 h. Subsequent CBB G-250 staining of the TAMRA–Phos-tag-stained bands (Fig. 4a, lower view) showed that the total signals of the electrophoresis bands were almost constant, indicating that no other reaction, such as degradation of ovalbumin in the phosphatase reaction, occurs. We believe that a similar gel-based assay using the TAMRA–Phos-tag staining method might be used to analyze the activity and inhibition of phosphatase isoforms, even in complex biological samples.

Similarly, we examined the time course of the kinase reaction of Abl (a tyrosine kinase) with a fusion protein substrate, GST-linked dodecapeptide (Abltide: Glu-Ala-Ile-Tyr-Ala-Ala-Pro-Phe-Ala-Lys-Lys-Lys). The tyrosine residue in the Abltide sequence was selectively phosphorylated by Abl at 30°C and pH 7.2. The upper view in Fig. 4b shows a typical fluorescence image of TAMRA–Phos-tag stained GST-Abtlide (0.5 μg/lane) for incubation times of 0, 2, 5, 10, 30, and 60 min. The fluorescence signal from GST-
Abltide after an incubation time of 0 min was almost the same as the background value. The fluorescence of the kinase reaction product increased in a time-dependent manner, and the intensity at 60 min reached a plateau value. The TAMRA–Phos-tag stained gel was subsequently stained with CBB G-250 (Fig. 4b, lower view), with the result that almost the same signal intensity of blue-color was observed for all the TAMRA–Phos-tag stained bands. This showed that the total amount of nonphosphorylated and phosphorylated GST-Abltide remained nearly constant throughout the kinase reaction, and that both proteins have the same migration distance in the gel. Thus, a combination of the SDS-PAGE and TAMRA–Phos-tag staining method can be used to profile the activity of the tyrosine kinase Abl without the use of delicate reagents such as an anti-phosphorylated Abltide-antibody or 32P-labeled ATP.

We have previously analyzed the inhibition profile of an Abl-specific competitive inhibitor, Imatinib, in the Abl kinase reaction by means of the alternative gel-based method Phos-tag PAGE22, 26). In the present study, we applied the TAMRA–Phos-tag staining method to a similar inhibition profiling using Abl, GST-Abltide, and Imatinib. The experimental conditions in the absence and presence of Imatinib (0.1–50 μM) were the same as those described above, except that the constant incubation time was 60 min. The upper view in Fig. 4c shows a typical electrophoresis image of TAMRA–Phos-tag stained GST-Abltide (0.5 μg/lane). Clearly, the fluorescence intensity decreased with increasing concentration of Imatinib. The fluorescence intensity for 50 μM Imatinib was less than 10% of that in the absence of the inhibitor. The inhibitor concentration required to halve the fluorescence intensity (IC₅₀) was between 1.6 and 3.1 μM under the experimental conditions. The dose-dependent inhibition by Imatinib is in good agreement with the reported result obtained by the Phos-tag SDS-PAGE method22). Also, the total amounts (0.5 μg/lane) of nonphosphorylated and phosphorylated GST-Abltide were nearly constant, as confirmed by CBB staining (Fig. 4c, lower view). Because Imatinib is a competitive kinase inhibitor, differences in the reported IC₅₀ values such as 1.6 μM22, 0.44 μM25, and 0.13 μM28 may reflect differences in experimental conditions such as Abl concentrations, ATP concentrations, and incubation times. From these results, we are convinced that TAMRA–Phos-tag staining can be used as in a simple gel-based method for screening Abl-inhibitor candidates in an in vitro kinase reaction.

CONCLUSIONS

In this article, we have introduced the novel fluorescent gel-staining dye TAMRA–Phos-tag. This Phos-tag derivative selectively captures phosphorylated proteins in an SDS-PAGE gel, permitting a gel-based analysis of the phosphorylation status of proteins in kinase and phosphatase reactions. As practical examples, the dephosphorylation of ovalbumin by an ALP and the phosphorylation of GST-Abltide by a tyrosine kinase, Abl, were examined. The TAMRA–Phos-tag staining method has the following advantages: (i) the gel staining involves a simple procedure using three solutions for the staining, washing, and dilution steps, respectively; (ii) the pH of the solution before mixing with the same volume of methanol is around 7, which results in much less damage to phosphoproteins; (iii) the total time for the staining method is less than 2 h; and (iv) a quantitative analysis of phosphoproteins can be conducted by using a visible-fluorescence imaging scanner through excitation at 532 nm with the use of a 580-nm longpass emission filter at room temperature. Thus, the TAMRA–Phos-tag staining method using a general SDS-PAGE apparatus should be useful for performing simple and comprehensive phosphorylation and dephosphorylation assays to analyze the activity of enzymes and to identify activators or inhibitors.

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