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A chromism-based assay (CHROBA) technique for in situ detection of protein kinase activity

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Abstract—A unique chromism-based assay technique (CHROBA) using photochromic spiropyran-containing peptides has been firstly established for detection of protein kinase A-catalyzed phosphorylation. The alternative method has advantages that avoid isolation and/or immobilization of kinase substrates to remove excess reagents including nonreactive isotope-labeled ATP or fluorescently-labeled anti-phosphoamino acid antibodies from the reaction mixture. Such a novel protocol based on thermocoloration of the spiropyran moiety in the peptide can offer not only an efficient screening method of potent kinase substrates but also a versatile analytical tool for monitoring other post-translational modification activities.

In the post-genomic era, identification and characterization of all proteins encoded are increasingly exciting challenges for researchers, and great efforts have been devoted in development of a high-throughput detection of a vast number of proteins.[1,2] Proteins, however, exhibit highly diverse functions and structures altered by post-translational modifications such as phosphorylation, glycosylation, acetylation, methylation, and so on, causing complicated manipulation protocols. Especially, phosphorylation catalyzed by protein kinases that play important roles in signal transduction in biological systems has been vigorously studied by several approaches such as radioassay,[3] immunoassay,[4] surface plasmon resonance,[4] fluorescence measurements using artificial receptors for phosphorylated peptides[5] or Pro-Q Diamond phosphosensor,[6] and chemiluminescent microtiter plate assay.[7] In most cases of such methods, however, isolation of kinase substrates from the reaction mixture or immobilization of them onto solid surfaces are essential to remove resulting nonreactive isotope-labeled ATP or anti-phosphoamino acid antibodies tethering a probe. As an alternative approach for in situ phosphorylation detection, fluorescence sensing systems using substrate peptides tethering a fluorophore have been demonstrated on the basis of the increase in fluorescence intensity by phosphorylation.[8,9] Such techniques, however, sometimes limit the positions of amino acid residues to be phosphorylated. Another approach is a fluorescence anisotropy assay that is independent upon background fluorescence signals, in which fluorescent kinase substrates were complexed with poly(L-arginine) (PLR, Mw = 75,000–150,000) or poly(L-lysine) (PLK, Mw = 80,000–100,000) to induce fluorescence polarization activity characteristic to phosphorylated substrates.[10] However, the sensitivity is expected to be highly influenced by the size of complexes formed and the method would not be suitable for a colorimetric assay.

Meanwhile, spiropyran derivatives are one of the most popular photochromic compounds and have been mainly studied in the field of molecular memories and switches.[11] In general, a spiropyran molecule is in the equilibrium between fluorescent merocyanine (MC)-form (colored in pink) and nonfluorescent colorless spiropyran (SP)-form with characteristic equilibrium constants depending upon the dielectric constant of solvents and can be controlled by UV or visible light-irradiation (Fig. 1).[12] With such an environmentally-sensitive properties in hand, we attempted to develop a novel phosphorylation detecting system using a spiropyran-containing kinase substrate. The analysis is based on changes in SP-to-MC isomerization (thermocoloration) rates by the phosphorylation, and it can avoid

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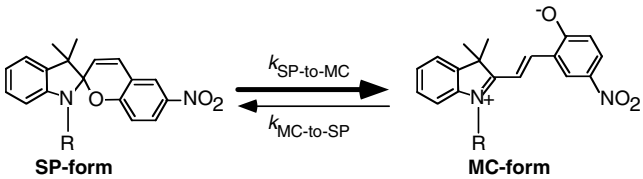

Polarity of solvent	$k_{SP\text{-}to\text{-}MC}$	$k_{MC\text{-}to\text{-}SP}$
Non-polar	UV light	in Dark or Visible light
Polar	in Dark	Visible light

Figure 1. Spiropyran derivatives show a characteristic photochromism in polar and nonpolar solvents. In nonpolar solvents, the SP-form is exclusively a dominant species and the chemical structures can be controlled by UV light and in dark/visible light converting to the MC- and SP-forms, respectively. In polar solvents such as water, equilibrium constants, K_{eq} ($=k_{SP\text{-}to\text{-}MC}/k_{MC\text{-}to\text{-}SP}$) tend to elevate with stabilization of the MC-form by solvation, in which the MC-form can be given in dark and the SP-form by visible light.

immobilization of kinase substrates onto solid surfaces, reduce background signals derived from reagents using thermocoloration shifts, and thus can provide reproducible and accurate results. Here, we describe the first example of chromism-based assay (CHROBA) for the in situ detection of protein kinase A (PKA)-catalyzed phosphorylation toward a colorimetric assay protocol.

In this study, Kemptide sequence, LRRASLG,[13] was employed as a PKA-specific substrate, which was linked to the spiropyran-tethering lysine via a glycine spacer (Fig. 2). *S*-Protected cysteine was attached to the N-terminus of the sequence for immobilization onto surfaces in future. A phosphorylated peptide was also designed as an authentic sample for the completion of phosphorylation. The peptides were synthesized by solid-phase synthesis using Fmoc chemistry and a spiropyran moiety was also attached at the lysine side chain on the resin. The peptide-bound resin was treated with TFA and the obtained crude peptides were purified by HPLC, followed by lyophilization, affording pure compounds as a yellow powder. Molecular ion peaks for both compounds were observed by MALDI-TOFMS. When the yellow powder colored by a co-existing protonated MC-form was dissolved in a neutral aqueous solution, the solution colored in yellow turned to pink immediately, corresponding to the mixture of SP- and zwitterionic MC-forms.[14] The synthetic procedures and physicochemical properties of spiropyran-containing peptides will be detailed elsewhere.[15]

In order to monitor a relaxation process from SP-form to an equilibrium state in **PKA-SP**, the solution of a mixture of SP- and MC-forms was irradiated with 510 nm-light to afford a solution containing only SP-form, and subsequent incubation in the dark started the relaxation. The relaxation process was monitored by the increase in fluorescence intensity at 600 nm where the MC-form emits a characteristic fluorescence by excitation at 510 nm (Fig. 3). The time course showed that the spiropyran moiety reached a thermally equilibrium state over 300 min and gave a mostly linear correlation

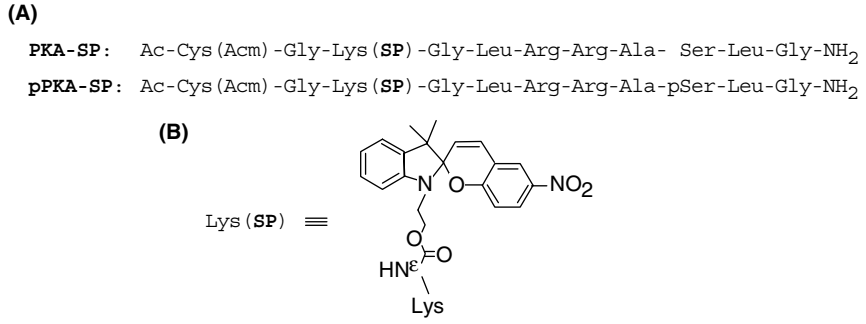

Figure 2. (A) Amino acid sequences of spiropyran-containing peptides and (B) structure of the Lys residue attached with a spiropyran dye (**SP**). pSer = phosphoserine.

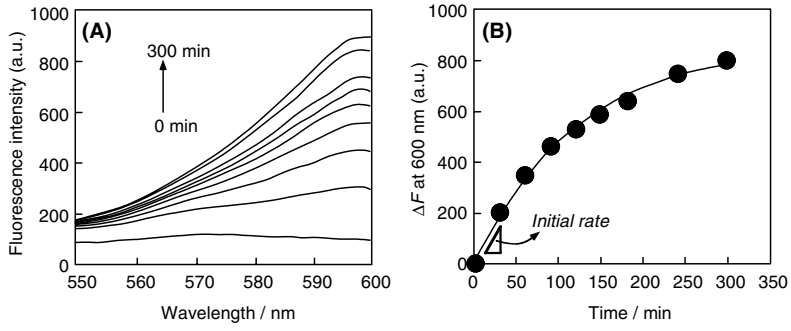

Figure 3. (A) Fluorescence spectra of **PKA-SP** showing the SP-to-MC isomerization at the incubation time points of 0, 30, 60, 90, 120, 150, 180, 240, and 300 min in the dark after the MC-to-SP photoisomerization by irradiation with a 510 nm-light and (B) the increase in fluorescence intensity at 600 nm along with the SP-to-MC isomerization. [**PKA-SP**] = 2.0 μM in 20 mM Tris–HCl buffer (pH 7.4) at 25 °C, λ_{ex} = 510 nm, λ_{em} = 550–600 nm.

between the thermocoloration and elapsed time within an initial 30 min. Therefore, we attempted to carry out the phosphorylation assay of the peptide based on the increase in fluorescence intensity for 30 min after incubation started in the dark as a discrimination factor.

Figure 4 shows relative SP-to-MC isomerization rates (V/V_0) of **PKA-SP** and **pPKA-SP** in the absence/presence of additives such as sodium chloride, PLK hydrobromic acid salts (Mw = 70,000–150,000), PLR hydrochloric acid salts (Mw = 70,000–150,000), and poly(L-aspartate) sodium salts (PLD, Mw = 40,000). First, a sample solution containing a mixture of SP- and MC-forms was irradiated with 510 nm-light to convert the peptide to the colorless SP-form. Subsequent incubation of the solution in the dark leaded to a thermally equilibrium state with characteristic initial rates depending upon environments. The V/V_0 value of **PKA-SP** in the presence of sodium chloride was increased by 10% more than that of **PKA-SP** in the absence of additives, which is the standard V_0 value. The addition of PLK or PLR to the reaction mixture containing **PKA-SP** also caused the increases in the V/V_0 values by 20%, due to higher ionic strength environments stabilizing the zwitter ionic MC-form with ions such as sodium chloride, PLK hydrobromic acid salts, or PLR hydrochloric acid salts. Surprisingly, the addition of PLD to the reaction mixture reduced the V/V_0 value by 40% relative to the standard V_0 value. These results strongly support that PLD binds to cationic **PKA-SP** more favorably than PLK or PLR, lowering the mobility of the spiropyran moiety in the peptide-polyanion complex formed and/or destabilizing the MC-form (changing the SP/MC ratio in the equilibrium state) in such electrostatic environments. On the other hand, the SP-to-MC isomerization rate of **pPKA-SP** in the absence of additives showed a slightly lowered value compared with the standard V_0 value, probably due to a mutual repulsion between a negatively charged phosphoserine residue and the MC-form within the peptide.

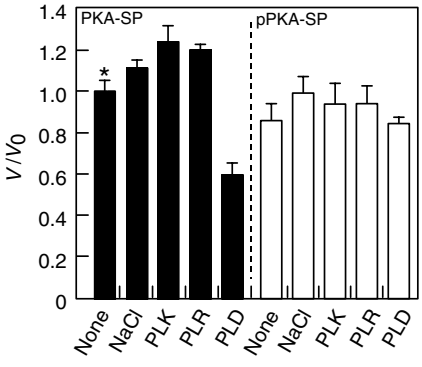

Figure 4. The SP-to-MC isomerization rates of **PKA-SP** and **pPKA-SP** depending upon various additives. Samples of a peptide (2.0 μM) and an additive were irradiated with a 510 nm-light in 20 mM Tris–HCl buffer (pH 7.4) to convert to the SP-form and subsequent incubation at 25 °C for 30 min in the dark afforded the initial rate of the SP-to-MC isomerization. The *y*-axis shows relative rates normalized with the standard V_0 value marked with an asterisk. Additives: [NaCl] = 143 mM; [PLK], [PLR], or [PLD] = 9.5 μM.

The addition of sodium chloride and polymers did not significantly affect the V/V_0 values in the SP-to-MC isomerization of **pPKA-SP** and the values varied from 0.85 to 1, resulting from a combination of higher ionic strength environments made by external additions of sodium chloride, PLK hydrobromic acid salts, or PLR hydrochloric acid salts (for positive effects) and a mutual repulsion of negatively charged phosphate group and PLD (for negative effects). The findings clearly suggest that comparison of the ratio in the SP-to-MC isomerization rate with PLD and that with PLK can discriminate phosphorylated peptides from nonphosphorylated ones without any isolation of peptides from excess reagents, encouraging us to use such a powerful 'CHROBA' technique in analyses of a kinase activity.

Next, we attempted to in situ monitor the PKA-catalyzed phosphorylation of **PKA-SP** without removal of excess reagents. Phosphorylation was performed in the presence of PKA, cAMP, and ATP at 25 °C for 30 min followed by the MC-to-SP photoisomerization then incubation in the dark to initiate the SP-to-MC isomerization. The assay protocol is detailed in the references and notes.[16] Figure 5A shows the V/V_0 values in the SP-to-MC isomerization under various conditions. All data were normalized with the value of **PKA-SP** without PKA and external polymers (condition 1). In nonphosphorylation condition lacking PKA (condition 2), the addition of PLK to the reaction mixture resulted in the increased rate and PLD decelerated the SP-to-MC isomerization, making a wide gap in the V/V_0 values between PLK and PLD [$V_{PLD}/V_{PLK} = 0.57$, condition 2 in Fig. 5B]. Meanwhile, in the phosphorylation condition (condition 3), the V/V_0 value with PLK was close to that with PLD, raising the V_{PLD}/V_{PLK} value from 0.57 to 0.88 comparable to the value obtained in

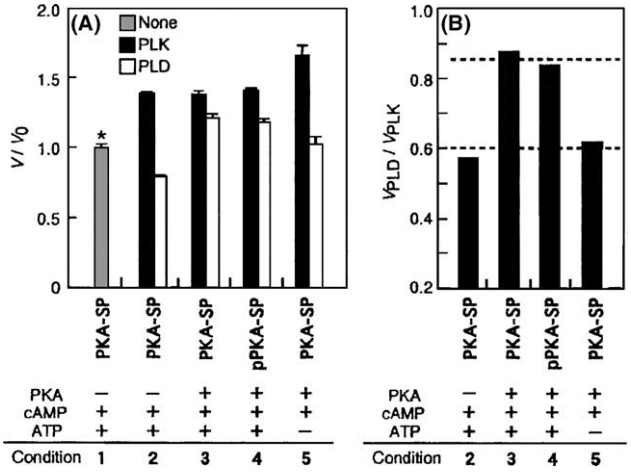

Figure 5. Phosphorylation catalyzed by PKA was assayed with 'CHROBA' technique. (A) The SP-to-MC isomerization of **PKA-SP** and **pPKA-SP** was carried out under various conditions. The *y*-axis shows relative signals in the isomerization monitoring peptide phosphorylation by PKA normalized with the standard V_0 value marked with an asterisk. (B) Representation of the ratios of V_{PLD}/V_{PLK} obtained in Figure 5A. Assay protocol is detailed in the references and notes.[16]

the case of **pPKA-SP** (V_{PLD}/V_{PLK} = 0.84, condition 4), suggesting that the PKA-catalyzed phosphorylation could be monitored successfully based on the 'CHROBA' technique. The assay condition with PKA and without ATP in the presence of external polymers (condition 5), in which phosphorylation does not occur, afforded the totally increased V/V_0 values in the isomerization compared with those obtained in the condition 2. Although it is not clear the accelerated isomerization rates observed in the condition 5, the lack of ATP in the reaction mixture might stabilize the MC-form more favorably by reducing mutual repulsion with negatively charged groups in ATP. The V_{PLD}/V_{PLK} value of the condition 5, however, showed 0.62 that is coincident to that given in the nonphosphorylation condition 2. These results strongly support that the present 'CHROBA' technique would be applicable for detecting protein kinase phosphorylation activities without laborious isolation steps.

Although the V_{PLD}/V_{PLK} values in the SP-to-MC isomerization rates were quite effective to monitor phosphorylation by PKA as described above, only V_{PLD} values also provided significant changes good enough to discriminate phosphorylation activities in the present case. Thus, in order to simplify the assay protocol, we examined the concentration dependence of PKA and the detection limit in the phosphorylation based on the 'CHROBA' technique coupled with the addition of external PLD (Fig. 6). In the phosphorylation conditions, the relationship between detectable SP-to-MC isomerization rates and PKA concentrations varying in three orders of magnitude (0.1–10 μg/mL) was observed. The product formation predicted from the coloration at each time point was also confirmed by MALDI-TOFMS with a reasonable correlation to results obtained by the 'CHROBA' protocol,[17] implying that the lower limit for detection according to the present protocol is settled around 0.1–1.0 μg/mL of PKA. Prolongation of the incubation time for phosphorylation and/or the SP-to-MC isomerization enable to improve sensitivity in the assay.

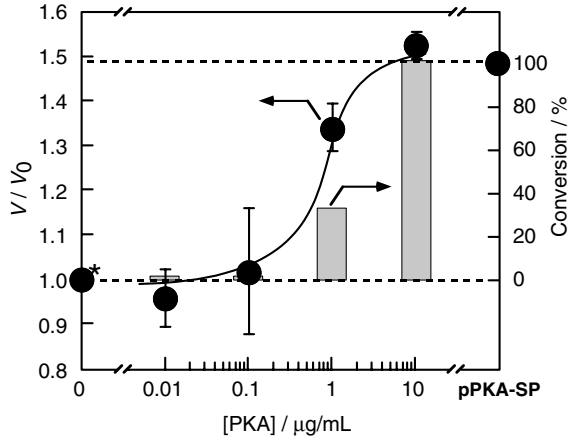

Figure 6. PKA concentration dependence in phosphorylation obtained with 'CHROBA' technique in the presence of PLD (closed circles, left-axis) and confirmed by MALDI-TOFMS (bars, right-axis). The left *y*-axis shows relative initial rates normalized with the standard V_0 value marked with an asterisk. Experimental procedure is detailed in the references and notes.[16,17]

In summary, we firstly established the 'CHROBA' technique using photochromic spiropyran-containing peptides and successfully demonstrated the usability for the in situ detection of PKA-catalyzed phosphorylation based on the thermocoloration from the SP-to-MC-forms. Such a novel 'CHROBA' technique involves the addition of ionic polymers to a reaction mixture followed by measurement of the SP-to-MC isomerization rate to acquire a fluorescence signal. Isolation and/or immobilization of kinase substrates are not required to remove nonreactive isotope-labeled ATP or fluorescently-labeled anti-phosphoamino acid antibodies from the reaction mixture. The present method can offer not only an efficient method for screening potent kinase substrates but also a versatile analytical tool for monitoring other post-translational modification activities.

Acknowledgements

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16. Kinase assay protocol: Following a modification to the literature,[7] samples of **PKA-SP** (40 μM), PKA (10 μg/mL), cAMP (1 mM), and ATP (1 mM) were incubated in 100 mM HEPES (pH 7.0), 5 mM $MgCl_2$ (100 μL) at 25 °C for 30 min in the dark. Aliquot (50 μL) was removed from the reaction mixture, transferred into an assaying buffer (950 μL) containing an additive ([PLK] or [PLD] = 10 μM in 20 mM Tris–HCl buffer, pH 7.4) that is placed at the cell holder in fluorescence photopolarimeter, and irradiated with a 510 nm-light to convert from the MC- to SP-forms. Subsequent incubation in the dark for 30 min followed by acquiring fluorescence spectrum (λ_{ex} = 510 nm, λ_{em} = 550–600 nm) afforded the initial rate of the SP-to-MC isomerization.
17. PKA-catalyzed phosphorylation was judged by MALDI-TOFMS after isolation of the phosphorylated/nonphosphorylated peptides by HPLC performed on a Hitachi L7000 system equipped with a Wakosil 5C18 (4.6 × 150 mm) with a linear gradient of 30–60% acetonitrile/0.1% TFA at a flow rate of 1.0 mL/min^{-1} for 30 min detected at 220 nm (both peptides were co-eluted at t_R = 15.5 min).