A dot-blot-staining method for detecting phosphoproteins with a Phos-tag Aqua fluorescent dye

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SUMMARY

We describe a method for detecting phosphoproteins by dot-blot staining with a Phos-tag Aqua fluorescent dye. By using the method, three types of in vitro protein kinase assays for visualizing Tyr-, His-, and Asp-phosphoproteins, respectively, were performed. The staining procedure, which requires less than 2.5 h to complete, is conducted under conditions of neutral pH that are particularly advantageous for detecting labile His- and Asp-phosphoproteins. This method promises rapid and easy detection of phosphoproteins, and should be useful in high-throughput profiling of in vitro kinase activities or in vitro kinase inhibition.

Key words: Phos-tag Aqua, dot-blot staining, tyrosine phosphorylation, histidine phosphorylation, aspartic acid phosphorylation

INTRODUCTION

Phosphorylation, one of the most common post-translational modifications of proteins, plays a critical role in regulatory events of many biological processes, such as signal transduction, cell-cycle progression, gene expression, metabolism, or apoptosis. Because the coordinated modification is controlled by protein kinases and phosphatases, abnormal activities of these enzymes can trigger a broad range of human diseases. Consequently, it is important to monitor how phosphorylation is regulated, not only for basic research, but also for clinical applications such as diagnosis or drug discovery. We have recently developed a gel-based strategy in which Phos-tag fluorescent gel-staining reagents are used to determine the phosphorylation status of proteins and to evaluate protein kinase activities. The gel-based method is reliable and useful for quantitative monitoring of protein phosphorylation, but it is not appropriate for high-throughput assays. In this report, we describe a novel non-gel-based dot-blot-staining method that can be applied in high-throughput profiling of in vitro kinase activities or in vitro kinase inhibition.

MATERIALS AND METHODS

Materials

Phos-tag Aqua, Phos-tag acrylamide AAL107, and PVDF membrane was purchased from Fujifilm Wako (Osaka, Japan). Bovine serum albumin, carbonic anhydrase, β-galactosidase, α-casein, β-casein, ovalbumin, and pepsin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Abelson tyrosine kinase (ABL) were obtained from Merck Millipore (Darmstadt, Germany). Imatinib was supplied by Novartis (Basel, Switzerland).

Dot-blot staining with Phos-tag Aqua

Proteins were dot-blotted on a PVDF membrane according to the method previously described. The protein-dot-blotted membrane was completely dried and then rehydrated with an equilibrating solution consisting of 5 mM Tris–HCl (pH 7.5), 50 mM NaCl, and 0.05% v/v Tween 20 in 50% v/v methanol for 60 min. The well-rehydrated membrane was probed with the Staining Solution of Phos-tag Aqua, as provided by the manufacturer, in a plastic bag for 60 min under light-shielded conditions. The membrane was then washed with the equilibrating solution for 1 min, and the fluorescent signals were visualized by using an FLA-7000 fluorescence-imaging scanner (Cytiva, Sheffield, UK).
**Kinase assays**

The ABL kinase reaction was conducted in 10 μL of a solution containing 20 mM MOPS–NaOH (pH 7.2), 25 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 15 mM MgCl₂, 0.1 mM ATP, 0.2 μg ABL, and 2 μg of the ABL peptide substrate (EAIYAAPFAKKK) tagged with glutathione S-transferase (GST-Abtide) at 30°C for 30 min. The GST-Abtide was prepared as described previously. The reaction mixture (2 μL) was immediately dot-blotted onto a PVDF membrane, and then the membrane was stained with the Phos-tag Aqua fluorescent dye, as described above.

The autophosphorylation reaction of EnvZ, prepared as described previously, was conducted in 10 μL of a solution containing 50 mM Tris–HCl (pH 8.0), 25 mM KCl, 5.0 mM MgCl₂, 10 mM dithiothreitol, 0 or 10 mM ATP, and 2 μg EnvZ at 25°C for 30 min. The autophosphorylation reaction of ArcA, prepared as described previously, was conducted in 10 μL of a solution containing 50 mM Tris–HCl (pH 8.0), 25 mM KCl, 5.0 mM MgCl₂, 10 mM dithiothreitol, 0 or 100 mM acetyl phosphate, and 2 μg ArcA at 25°C for 30 min. The reaction mixture (2 μL) was immediately dot-blotted onto a PVDF membrane, and then the membrane was stained with the Phos-tag Aqua fluorescent dye.

**ABL kinase inhibition assay**

The ABL kinase reaction was conducted in 7 μL of a solution containing MOPS–NaOH (pH 7.2), 25 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 15 mM MgCl₂, 0 or 1 mM ATP, 14 ng ABL, 3 μg GST-Abtide, and imatinib (0–100 μM) at 30°C for 60 min. The assays were performed in triplicate. The reaction mixture (2 μL) was immediately dot-blotted onto a PVDF membrane, and then the membrane was stained with the Phos-tag Aqua fluorescent dye. The assays were performed in triplicate.

**RESULTS AND DISCUSSION**

**Dot-blot-staining of serine-phosphorylated purified proteins**

In this study, we used Phos-tag Aqua (λ<sub>Ex</sub>=551 nm; λ<sub>Em</sub>=564 nm), one of a series of fluorophore-labeled Phos-tag-derived dyes newly developed by the NARD Institute (Amagasaki, Japan) and marketed by Fujifilm Wako (Osaka, Japan) as gel-staining reagents for detecting phosphorylated proteins in SDS-PAGE. To confirm that Phos-tag Aqua fluorescent dye permits the selective detection of phosphorylated proteins on a PVDF membrane, we first performed dot-blot staining of nonphosphorylated proteins (bovine serum albumin, carbonic anhydrase, and β-galactosidase), standard phosphoproteins (α-casein, β-casein, ovalbumin, and pepsin) containing a phosphorylated serine residue, and the corresponding proteins dephosphorylated by treatment with alkaline phosphatase (AP-treated). The typical fluorescent image shown in the left-hand panel of Fig. 1 confirms that the dot-blotted Ser-phosphoproteins (α-casein, β-casein, ovalbumin, and pepsin) were specifically detected on the PVDF membrane. The detection limits for Phos-tag Aqua staining were 62.5 ng for α-casein, β-casein, and ovalbumin and 15.6 ng for pepsin. Although a weak signal was detected for spots in which 500–2000 ng of AP-treated pepsin were dot-blotted, there was no signal in the same range for AP-treated α-casein, β-casein, or ovalbumin. After scanning, the same blot was stained with CBB for reference (right-hand panel of Fig. 1). Note that it is difficult to detect the acidic protein pepsin by CBB staining.
Dot-blot-staining of tyrosine-phosphorylated protein and inhibition profiling of tyrosine kinase ABL

Next, we performed dot-blot staining of a Tyr-phosphoprotein produced by an in vitro tyrosine kinase reaction. The signal for phosphorylated GST-Abtlde was specifically detected in the sample containing ATP [ATP(+)] (Fig. 2A). The same blot was stained with CBB to show that both the ATP(−) and ATP(+) samples contained equal amounts of the substrate protein. The same kinase reaction mixture was also analyzed by Phos-tag SDS-PAGE7) followed by CBB gel staining, and an upshifted band corresponding to phosphorylated GST-Abtlde was observed in the ATP(+) lane, indicating that the kinase reaction proceeded successfully.

Next, we applied this dot-blot-staining method to an in vitro inhibition profiling by using ABL and GST-Abtlde together with imatinib, an ABL-specific inhibitor. As shown in a typical dot-blot image (Fig. 2B), the fluorescent intensity decreased markedly with increasing concentration of imatinib. The inhibitor concentration required to halve the intensity (IC50) was determined to be 14 μM. The dose-dependent inhibitory effect was almost similar to that reported previously8).

Fig. 2  Dot-blot-staining analysis for in vitro kinase assays.
(A) Typical images of dot-blotted GST-Abtlde after the kinase reaction in the absence (−) or presence (+) of ATP by using Phos-tag Aqua staining and CBB staining. The same reaction mixture (5 μL) was subjected to Phos-tag SDS-PAGE (10% w/v polyacrylamide, 20 μM Zn2+–Phos-tag) followed by CBB gel staining. (B) Inhibition profile of the ABL-specific inhibitor imatinib in the ABL kinase reaction by using Phos-tag Aqua staining. An inhibition curve is shown under the gel image. The plotted points and the error bars indicate the average and standard deviation, respectively, from three independent experiments. Standard deviations were within almost 20%. (C) Typical images of dot-blotted EnvZ after the autophosphorylation reaction in the absence (−) or presence (+) of ATP by using Phos-tag Aqua staining and CBB staining (left panel). Typical images of dot-blotted ArcA after the autophosphorylation reaction in the absence (−) or presence (+) of acetyl phosphate by using Phos-tag Aqua staining and CBB staining (right panel). The same reaction mixtures (5 μL) were subjected to Phos-tag SDS-PAGE (10% w/v polyacrylamide, 20 μM Zn2+–Phos-tag) followed by CBB gel staining.
Dot-blot-staining of histidine-, or aspartic acid-phosphorylated protein and inhibition profiling of tyrosine kinase ABL

Finally, we performed dot-blot staining of His- and Asp-phosphoproteins produced by in vitro autophosphorylation reactions of a histidine kinase (HK) and a response regulator (RR), respectively. Two-component signal-transduction systems consisting of a sensor HK and an RR are ubiquitous among bacteria, and they are associated with the virulence of pathogens. The signaling systems are potential targets for new antibiotics and antivirulence agents. It is therefore important to visualize His- and Asp-phosphoproteins and to determine HK activities in bacterial systems. The sensor kinase has an HK domain containing an invariant His residue that is autophosphorylated in an ATP-dependent manner. The RR has a receiver domain that receives a phosphoryl group from its cognate HK. The receiver domain contains a conserved Asp residue that can be directly autophosphorylated by treatment with acetyl phosphate as a phosphoryl donor. In this study, we used recombinant EnvZ and ArcA proteins derived from Escherichia coli as our HK and RR, respectively, to examine the His- and Asp-phosphoproteins generated by their respective autophosphorylation reactions.

As shown in Fig. 2C, the signals of autophosphorylated EnvZ and ArcA were specifically detected in the ATP(+) and acetyl phosphate(+) samples, respectively. These results demonstrate that this dot-blot-staining method can be also used to analyze His- and Asp-phosphoproteins in bacterial two-component signal-transduction systems.

CONCLUSIONS

We have introduced the novel fluorescent dot-blot-staining dye Phos-tag Aqua. This Phos-tag derivative selectively captures phosphorylated proteins dot-botted on a PVDF membrane, permitting the analysis of the phosphorylation status of proteins in protein kinase and phosphatase reactions. As initial examples, the Ser-phosphoproteins of caseins, ovalbumin, and pepsin and the corresponding dephosphorylated proteins produced by treatment with AP were examined, and the selectivity of Phos-tag Aqua toward phosphorylated proteins was confirmed. In addition, Tyr-, His-, and Asp-phosphoproteins produced by kinase reactions were visualized. We also demonstrated that this Phos-tag Aqua staining method can be applied to in vitro inhibition profiling of ABL, a human tyrosine kinase, by using its specific inhibitor, imatinib.

The dot-blot-staining method has the following advantages. (i) The blot staining involves a simple procedure and requires only two solutions: an equilibrating solution and a staining solution. The equilibrating solution is used in both the equilibrating step and the final washing step. The staining solution, as provided by the manufacturer, is used in the staining step. (ii) The pH of the solution before mixing with an equal volume of methanol is neutral; this results in much less damage to phosphoproteins, particularly to His- and Asp-phosphoproteins in bacterial two-component signal-transduction systems. (iii) The total time required for the staining procedure is less than 2.5 h. (iv) Quantitative analyses of phosphoproteins can be conducted by using a fluorescence-imaging scanner through excitation at 551 nm with the use of a 564-nm long-pass emission filter. (vii) Finally, the method is likely to be applicable in experiments conducted in the 96 or 384 format with multitasking equipment such as dot blotters or automated liquid-handling workstations, thereby permitting high-throughput profiling of in vitro kinase activities or in vitro kinase inhibition. Consequently, the dot-blot-staining method with Phos-tag Aqua fluorescent dye should be useful in performing simple and comprehensive assays of phosphorylation and dephosphorylation to analyze the activities of enzymes and to identify their activators or inhibitors.

CREDIT AUTHORSHIP CONTRIBUTION

Emiko Kinoshita-Kikuta: Conceptualization, Methodology, Resources, Investigation, Writing: original draft, Funding acquisition. Keisuke Akayama: Investigation. Eiji Kinoshita: Project administration, Investigation, Writing: original draft, Writing: review & editing, Funding acquisition. Tohru Koike: Writing: original draft, Writing: review and editing, Supervision, Funding acquisition.

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ABBREVIATIONS

ABL, Abelson tyrosine kinase; AP, alkaline phosphatase; GST, glutathione S-transferase; HK, histidine kinase; RR, response regulator; SDS-PAGE

No potential conflicts of interest were disclosed.

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