Histochemical Imaging of Alkaline Phosphatase Using a Novel Fluorescent Substrate

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Received June 19, 2014; accepted July 25, 2014; advance publication released online August 9, 2014

Highlight Paper selected by Editor-in-Chief

Histochemical visualization of phosphatase is exclusively required for Western immunoblotting and antigen-positive cell staining using an alkaline phosphatase (AP)-labeled secondary antibody. This detection has been performed by several reagents including 5-bromo-4-chloro-3-indolyl-phosphate (X-Phos), nitro blue tetrazolium (NBT), 3-(2’-spiroadamantane)-4-methoxy-4-(3’-phosphoryl oxy)phenyl-1,2-dioxetane and 2-(5’-chloro-2’-phosphoryloxyphenyl)-6-chloro-4-[3H]-quinazolinone (ELF® 97 Phosphate). We previously reported that 2-(benzothiazol-2-yl)-4-bromophenol bonded with N-acetylneuraminic acid (BTP3-Neu5Ac), enabled fluorescent histochemical visualization of sialidase activity. 2-(Benzothiazol-2-yl)-4-bromophenol (BTP3), which is formed from BTP3-Neu5Ac by sialidase reaction, is a crystalline, insoluble and stable fluorescent compound, deposited at the site of enzyme activity. We developed a BTP3 phosphate ester (BTP3-Phos) for the purpose of fluorescent histochemical visualization of phosphatase activity. BTP3-Phos emitted fluorescence in a manner dependent on the concentration of the AP-labeled antibody. BTP3-Phos also enabled fluorescent histochemical visualization of AP-blotted dots in a manner dependent on the concentration of the AP-labeled antibody. The detection sensitivity of BTP3-Phos was estimated to be greater than that of the conventional method using X-Phos and NBT. Influenza A virus-infected cells were fixed and reacted with anti-influenza A virus antibodies and incubated continuously with an AP-labeled secondary antibody. BTP3-Phos stained the infected cells with distinct green fluorescence. These results indicate that BTP3-Phos can enable fluorescent immunohistochemical staining analysis using an AP-labeled antibody. BTP3-Phos would be beneficial for histochemical staining of AP activity, and may be applicable for multi-color staining or a cell sorter.

Key words phosphatase; fluorescent imaging; histochemical staining; benzothiazolylphenol; fluorescent substrate

Among the wide variety of substrates for detection of alkaline phosphatase (AP) activity, several substrates including p-nitrophenol phosphate, 4-methylumbelliferyl phosphate, 3,6-fluorescein diphasate, and 6,8-difluoro-4-methylumbelliferyl phosphate have been devised as conventional substrates.1–5) However, these substrates are not used for histochemical staining, because of their solubility in water after phosphatase reaction. 5-Bromo-4-chloro-3-indolyl-phosphate (X-Phos) has been mainly used for histochemical detection of phosphatase activity.3) Color production of X-Phos is sensitively improved by addition of nitro blue tetrazolium (NBT).6,7) Additionally, several reagents including 3-(2’-spiroadamantane)-4-methoxy-4-(3’-phosphoryloxy)phenyl-1,2-dioxetane,5,9) 2-(5’-chloro-2’-phosphoryloxyphenyl)-6-chloro-4-[3H]-quinazolinone (ELF® 97 Phosphate),10,11) and 3-hydroxy-N’-2-biphenyl-2-naphthalenecarboxamide phosphateester12) have been developed for immunohistochemical staining such as Western immunoblotting and antigen-positive cell staining with an AP-labeled antibody. Moreover, 2-(2’-hydroxyphenyl)benzothiazole phosphate ester (HBT-Phos), 2-(2’-hydroxyphenyl)-4-phenylthiazole phosphate ester, and 2-benzothiazoleacetontitrile, α-[4-(diethylamino)-2-(phosphonoxy)phenyl]methylene have been reported as fluorescent substrates of phosphatase.13–15) However, the usefulness of these substrates for histochemical staining has not been examined.

We have recently developed a new sialidase substrate that enables fluorescent histochemical visualization of sialidase activity.16) The new substrate is 2-(benzothiazol-2-yl)-4-bromophenol bonded with N-acetylneuraminic acid (BTP3-Neu5Ac). 2-(Benzothiazol-2-yl)-4-bromophenol (BTP3), which is formed by sialidase reaction of BTP3-Neu5Ac, is a crystalline, insoluble, acid-resistant, and fluorescently stable compound (Ex/Em=372/526 nm).17) Its sufficiently large Stokes’ shift has an advantage in preventing the influence of excitation light, self-absorption, and noise fluorescence derived from cells and tissues. Although BTP3-Neu5Ac itself is not fluorescent, BTP3 locally deposits at the site of sialidase activity after removal of Neu5Ac by sialidase. Therefore, BTP3-Neu5Ac can be used for fluorescent histochemical visualization of sialidase activity.18,19) We developed a new substrate using BTP3 bonded with phosphate (BTP3-Phos) for the purpose of fluorescent histochemical visualization of phosphatase activity (Fig. 1).

First, we examined the reactivity of BTP3-Phos for an AP activity of AP-labeled antibody. Then, we investigated the usefulness of BTP3-Phos for histochemical detection and compared the detection sensitivity of BTP3-Phos to the AP-labeled antibody with a conventional method using X-Phos and NBT. Finally, we confirmed the usefulness of BTP3-Phos for fluorescent immunohistochemical detection of influenza A virus antigen-positive cells using the AP-labeled secondary antibody.

MATERIALS AND METHODS

Cells and Reagents Madin–Darby canine kidney

Received June 19, 2014; accepted July 25, 2014; advance publication released online August 9, 2014

The authors declare no conflict of interest.

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(MDCK) cells were maintained in Eagle’s minimum essential medium (MEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS). BTP3-Phos was synthesized by Dr. Tadamune Otsubo and Dr. Kiyoshi Ikeda at Hiroshima International University, Japan (Fig. 1, see supplementary information). AP-labeled anti-mouse immunoglobulin G (IgG) (H+L) was purchased from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, U.S.A. Western Blue Stabilized substrate for alkaline phosphatase was purchased from Promega Corp., Madison, WI, U.S.A.

**Fluorescent Visualization of AP Activity Using BTP3-Phos** To determine the appropriate concentration of BTP3-Phos, we prepared an AP solution of goat AP-labeled anti-mouse IgG (H+L) (60 ng/mL) in AP reaction buffer (10 mM Tris–HCl pH 9.5, 5 mM MgCl2, 100 mM NaCl). Five microliters of five-serially diluted BTP3-Phos solution from 60 ng/mL to 1.54 × 10^{-6} ng/mL was added to the AP solution (Final concentrations of BTP3-Phos during AP reaction were from 500 nM.). After AP reaction at 37°C for 10 min, fluorescent images were obtained during UV irradiation for 2 s by using Lumivision Pro HR (AISIN SEIKI Co., Ltd., Aichi, Japan) with DR655 filter and green enhancer filter.

To confirm that the fluorescence was dependent on AP concentration, 5 µL of 800 nM BTP3-Phos was added to 100 µL of five-serially-diluted AP solution (from 60 ng/mL for final concentrations of goat AP-labeled anti-mouse IgG and 40 nM for final concentration of BTP3-Phos). After AP reaction at 37°C for 10 min, fluorescent images were obtained during UV irradiation for 2 s by using Lumivision Pro HR.

**Histochemical Visualization of AP Activity Blotted on a Membrane Using BTP3-Phos and X-Phos** AP-labeled goat anti-mouse IgG (H+L) was five-serially diluted with Tris-buffered saline (TBS; TaKaRa Bio Inc., Shiga, Japan) from 60 ng/mL to 1.54 × 10^{-6} ng/mL. Fifty microliters of each dilution was dot-blotted on a polyvinylidene difluoride (PVDF) membrane (from 3 ng/dot to 7.68 × 10^{-6} ng/dot of the AP-labeled antibody). The PVDF membrane was soaked in methanol at room temperature for 5 min and washed with TBS immediately before dot-blotting of the AP-labeled antibody. The AP-blotted membrane was reacted with 3 mL of 40 nM BTP3-Phos in AP reaction buffer at room temperature for 10 min. After drying the membrane, a fluorescent image was obtained during UV irradiation for 1 s by using Lumivision Pro HR.

To compare detection sensitivity of AP activity with a conventional method using X-Phos and NBT, the AP-blotted membrane was developed with Western Blue Stabilized substrate for alkaline phosphatase including X-Phos and NBT at room temperature for 10 min. An image was obtained with a GT-S620 scanner (Seiko Epson Corp., Nagano, Japan).

**Fluorescent Immunohistochemical Visualization of Influenza A Virus-Infected Cells** MDCK cells (0.5×10^5 cells/well) were seeded on a 24-well plate and cultured at 37°C under 5% CO2 overnight. After washing the cells with 250 µL/well of sterilized phosphate buffered saline (PBS; pH 7.2, 131 mM NaCl, 14 mM Na2HPO4, 1.5 mM KH2PO4, and 2.7 mM KCl), the cells were inoculated with 100 µL/well of human influenza A virus strain A/Memphis/1/1971 (H3N2) at 20 hemagglutination units in serum-free medium (SFM; Invitrogen Corp., Carlsbad, CA, U.S.A.) at 37°C for 30 min. After washing the cells with 250 µL/well of sterilized PBS, the infected cells were cultured in 500 µL/well of MEM supplemented with 5% FBS at 37°C under 5% CO2 for 8 h. The infected cells were fixed with 500 µL/well of methanol for 5 s and washed with 500 µL/well of PBS. The cells were reacted with 250 µL/well of mouse anti-influenza A virus A/Memphis/1/1971 (H3)-A/Bellamy/42 (N1) hemagglutinin (HA) 2E10 and nucleoprotein (NP) 4E6 monoclonal antibodies (IgG). 20-23) followed by 250 µL/well of AP-labeled goat anti-mouse IgG (H+L) at room temperature for 40 min each. After washing the cells with 250 µL/well of TBS, the cells were reacted with 250 µL/well of 40 nM BTP3-Phos in TBS or Western Blue Stabilized substrate for alkaline phosphatase at room temperature for 10 min. A fluorescent image and an optical image were observed by using an OLYMPUS XL 71 fluorescence microscope (Olympus Co., Ltd., Tokyo, Japan) equipped with a fluorescent filter (U-MWU2, DM400, BP336–385, BA420).

**RESULTS**

**BTP3-Phos Fluorescently Detected AP Activity** We examined the reactivity of BTP3-Phos against AP activity of an AP-labeled antibody. AP-labeled goat anti-mouse IgG antibody (60 ng/mL) was fluorescently detected in a concentration-dependent manner of BTP3-Phos by reaction with BTP3-Phos at room temperature for 10 min (Fig. 2A). BTP3-Phos at 100 nM showed strong fluorescent intensity, while 20 nM BTP3-Phos showed weak intensity. Therefore, we used 40 nM BTP3-Phos in the next experiments. AP activity was also detected by reaction with BTP3-Phos (40 nM) at room temperature for 10 min in a manner dependent on the concentration of the AP-labeled antibody (Fig. 2B). We confirmed the usefulness of BTP3-Phos for fluorescent detection of an AP-labeled anti-
rescence of BTP3 can be additionally enhanced by extending with 40 nM BTP3-Phos at room temperature for 10 min. BTP3-a PVDF membrane. The AP-blotted membrane was reacted for BTP3-Phos and 5 4 dilution of the antibody (4.80 ng/dot) during AP reaction). BTP-Phos only (no AP) was used as a negative control. AP-labeled goat anti-mouse IgG antibody (from 60 ng/mL as final concentrations during AP reaction) was reacted with five serial dilutions of BTP3-Phos (from 500 nM as final concentrations as a final concentration during AP reaction). Therefore, BTP3-Phos would be very beneficial as another entity.

Fluorescent Histochemical Visualization of AP Activity

We have demonstrated that insolubility of fluorescent BTP3 enables histochemical visualization of the site of enzyme activity.16–19) AP-labeled antibody was dot-blotted on a PVDF membrane. The AP-blotted membrane was reacted with 40 nM BTP3-Phos at room temperature for 10 min. BTP3-Phos fluorescently and histochemically visualized dots of AP activity in a manner dependent on the concentration of the AP-labeled antibody (Figs. 3A, C). To compare the sensitivity of BTP3-Phos with a conventional method using X-Phos, the AP-dotted membrane was also reacted with an AP-labeled antibody. Western Blue Stabilized substrate for alkaline phosphatase including X-Phos and NBT at room temperature for 10 min, as a positive control. BTP3-Phos enabled fluorescent immunohistochemical visualization of the antigen-positive cells (Fig. 4). There was no distinct fluorescence derived from BTP3-Phos reaction in non-infected cells. For the infected cells, the fluorescent image was almost completely merged with the optical image of the cells, indicating that the fluorescence was specifically developed from incubation of the antigen-positive cells.

DISCUSSION

X-Phos has been used as a conventional substrate for histochemical staining of phosphatase. This substrate is frequently used for immunohistochemical detection with an AP-labeled antibody such as Western immunoblotting and antigen-positive cell staining. By addition of NBT, sensitivity of the method using X-Phos has been improved. In the present study, BTP3-Phos appeared to be higher sensitivity and more definitive staining for histochemical detection than did the conventional method using X-Phos and NBT. Fluorescent histochemical visualization of BTP3-Phos will enable multi-color fluorescent staining. UV excitation (372 nm at the maximum excitation wave length) and green emission (526 nm at the maximum emission wave length) of BTP3 can use usually commercial fluorescent filters and lasers for excitation. Therefore, BTP3-Phos would be very beneficial as another fluorescent substrate for immunohistochemical detection using an AP-labeled antibody.

AP is highly expressed in embryonic stem (ES) cells24) and induced pluripotent stem (iPS) cells derived from humans and bovine.25) Histochemical staining of AP activity has been used for detection of ES cells and iPS cells by the conventional method using X-Phos. It is expected that BTP3-Phos will enable fluorescent histochemical visualization of ES cells and iPS cells. These BTP3-stained cells can apply to multi-color fluorescent histochemical staining together with several specific antibodies and cell isolation using flowcytometric cell sorter. BTP3-Phos might be a useful, easy, and rapid tool for histochemical detection of ES cells and iPS cells.
This study is the first study showing the histochemical staining property of BTP3-Phos. Our BTP3-Phos is a bromine atom-bonded derivative of HBT. Bromine addition to the 4-position of 2-(benzothiazol-2-yl)-phenol increases the insolubility more than does HBT in order to be more suitable for histochemical staining and induces a shift from 518 nm to

Fig. 3. Histochemical Visualization of AP Activity Blotted on a Membrane Using BTP3-Phos or X-Phos

Five serial dilutions of AP-labeled goat anti-mouse IgG antibody (from 3 ng/dot to 7.68×10^{-6} ng/dot) were dot-blotted on a PVDF membrane. The AP-blotted membrane was reacted with 40 nM BTP3-Phos in AP reaction buffer (A) or Western Blue Stabilized substrate for alkaline phosphatase (X-Phos) including X-Phos and NBT (B), at room temperature for 10 min. Relative fluorescent intensities were measured from (A) using Image J 1.46r software (National Institutes of Health, U.S.A.) (C). Mean fluorescent data (grey column) were expressed as a relative fluorescent intensity of that in 3 ng of AP-labeled antibody (filled column).

Fig. 4. Fluorescent Immunohistochemical Visualization of Influenza A Virus-Infected Cells

MDCK cells were inoculated with human influenza A virus strain A/Memphis/1/1971 (H3N2) and cultured at 37°C for 8 h. The infected cells were fixed with methanol and reacted with mouse anti-viral HA (2E10) and NP (4E6) monoclonal antibodies, followed by AP-labeled goat anti-mouse IgG antibody. Then, the infected cells were incubated with BTP3-Phos at room temperature for 10 min. The infected cells were also developed with Western Blue Stabilized substrate for alkaline phosphatase (X-Phos) including X-Phos and NBT as a positive control. A bar indicates 100 µm.
526 nm at the maximum emission wave length, close to the maximum emission (530 nm) of fluorescein isothiocyanate (FITC), which is most frequently used in fluorescent staining.  

Acid phosphatase has been used as a historical biomarker of prostate cancer, although serum prostate-specific antigen is now the major diagnostic marker.  

BTP3-Phos might enable fluorescent histochemical imaging of prostate cancer. Furthermore, since BTP3 is an acid-resistant fluorescent compound, it is suitable for an enzyme reaction under an acid condition such as acid phosphatase reaction. AP is highly expressed in osteosarcoma, which has been histochemically stained by the conventional method using X-Phos and NBT.  

BTP3-Phos might also be applicable to fluorescent histochemical staining of osteosarcoma.

BTP3-Phos is beneficial tool as one of the sensitive developing reagents for histochemical detection of an AP-labeled antibody. BTP3-Phos can locally stain AP-labeled antibody by 10-min AP reaction after incubation simultaneously with AP-labeled antibody and fluorescent antibodies. Additionally, for detection of fluorescence, UV excitation of BTP3 differentiates many representative fluorescent compounds showing excitation of blue, green and red lasers, such as FITC. It is expected that BTP3-Phos will enable multi-color fluorescent staining in combination with some fluorescent antibodies or enable AP-positive or AP-labeled cell isolation using a flow-cytometric cell sortet. Taken together, the results indicate that BTP3-Phos would be more beneficial than the conventional method for histochemical detection of AP. Further study may demonstrate the usefulness of BTP3-Phos for fluorescently sensitive histochemical imaging of phosphatase-positive cells and tissues such as pluripotent cells and cancer.

Acknowledgments This work was in part supported by a Grant from Nakatani Foundation for Advancement of Measuring Technologies in Biomedical Engineering, the Kurata Memorial Hitachi Science and Technology Foundation, the Futaba Electronics Memorial Foundation, Tokai Foundation for Technology, Takahashi Industrial and Economic Research Foundation, and MEXT/JSPS KAKENHI Grant Number (Scientific Research C, 23590549; challenging Exploratory Research, 26670064).

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