Available online at www.sciencedirect.com
ScienceDirect
J. Biochem. Biophys. Methods 70 (2008) 1014–1019
JOURNAL OF biochemical and biophysical methods
www.elsevier.com/locate/jbbm

A new detection method for arginine-specific ADP-ribosylation of protein — A combinational use of anti-ADP-ribosylarginine antibody and ADP-ribosylarginine hydrolase

Harumi Osago*, Masaharu Terashima, Nobumasa Hara, Kazuo Yamada, Mikako Tsuchiya

Department of Biochemistry, Faculty of Medicine, Shimane University, Izumo 693-8501, Japan

Received 16 March 2007; accepted 19 November 2007

Abstract

Arginine-specific ADP-ribosylation is one of the posttranslational modifications of proteins by transferring one ADP-ribose moiety of NAD to arginine residues of target proteins. This modification, catalyzed by ADP-ribosyltransferase (Art), is reversed by ADP-ribosylarginine hydrolase (AAH).

In this study, we describe a new method combining an anti-ADP-ribosylarginine antibody (αADP-R-Arg Ab) and AAH for detection of the target protein of ADP-ribosylation. We have raised αADP-R-Arg Ab with ADP-ribosylated histone and examined the reactivity of the antibody with proteins treated by Art and/or AAH, as well as *in situ* ADP-ribosylation system with mouse T cells. Our results indicate that the detection of ADP-ribosylated protein with αADP-R-Arg Ab and AAH is a useful tool to explore the target proteins of ADP-ribosylation. We applied the method to search endogenously ADP-ribosylated protein in the rat, and detected possible target proteins in the skeletal muscle, which has high Art activity.

Keywords: ADP-ribosylation; ADP-ribosylarginine hydrolase; Target protein

1. Introduction

Arginine-specific ADP-ribosylation, originally identified as the mechanism of action of certain bacterial toxins [1], is catalyzed by mono-ADP-ribosyltransferases (Art) (EC 2.4.2.31) that transfer an ADP-ribose residue from NAD to arginine residues of the acceptor protein. After the discovery of bacterial Arts, similar enzymatic activities were detected in vertebrates and cloned from rabbit skeletal muscle [2], mouse lymphocytes [3] and chicken heterophils [4] and lung [5]. These Arts were classified into seven members (Art1–Art7), based on their similarities in amino acid sequence and conservation of gene structures [6,7].

The extent of protein modification by ADP-ribosylation depends on the activity of Art and ADP-ribosylhydrolase (AAH) (EC 3.2.2.19), which hydrolyses arginine-ADP-ribosylated protein to ADP-ribose and arginine-protein. The presence of Art and AAH activities in eucaryotic cells suggests that reversible protein mono-ADP-ribosylation acts as a regulatory mechanism for the protein substrates of these reactions [8].

This modification has a well-known regulatory function of target proteins in the procaryotic world while the biological function in eucaryotic cells remains to be elucidated, partly because target proteins of the modification have not yet been established. In eucaryotes, Arts have been reported to modify a number of proteins, including the purinergic receptor P2X7 [9], actin [10], desmin [11] and integrins [12]: however, to date, there has been no direct evidence of their *in vivo* modification

Abbreviations: AAH, ADP-ribosylarginine hydrolase; Ab, antibody; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt; ADP-R, ADP-ribose; αADP-R-Arg, anti-ADP-ribosylarginine; Art, ADP-ribosyltransferase; BSA, bovine serum albumin; CLMS, confocal laser scanning microscopy; Con A, concanavalin A; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FITC, fluoresceinisothiocyanate; GST, glutathione *S*-transferase; Hanks, Hanks' balanced salt solution; HRP, horseradish peroxidase; NAD, nicotinamide adenine dinucleotide; TCA, trichloroacetic acid; Tris, tris (hydroxymethyl)-aminomethane; Tween 20, polyoxyethylene sorbitan monolaurate.

* Corresponding author. Tel./fax: +81 853 20 2120.
E-mail address: biochem1@med.shimane-u.ac.jp (H. Osago).

0165-022X/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jprot.2007.11.008

[13]. Recently, ADP-ribosylated human neutrophil-derived peptide (HNP-1, an antimicrobial peptide secreted by immune cells) has been identified in the bronchoalveolar lavage fluid from individuals who smoke cigarettes [14]. This is the first demonstration of endogenous ADP-ribosylation in humans. Molecular identification of the cellular target protein, however, remains to be elucidated.

To detect the endogenously ADP-ribosylated protein, the antibodies previously produced by Meyer and Hilz [15] and by Eide et al. [16] predict low specificity, because the antigens are not natural steric relative to the protein. Schwab et al. developed a polyclonal antiserum against ADP-ribosyl-polyarginine, and identified ADP-ribosylated proteins *in vivo* in combination with hydroxylamine (NH_2OH) to which ADP-ribosylarginine bond is labile [17]. Here, we describe a new specific method using an anti-ADP-ribosylarginine antibody (αADP-R-Arg Ab) and AAH. We raised αADP-R-Arg Ab with ADP-ribosylated histone and examined the reactivity of the antibody with proteins treated by Art and/or AAH, as well as *in situ* ADP-ribosylation system with mouse T cells. Our results indicate that the combination of αADP-R-Arg Ab with AAH is a useful tool to detect the target proteins of ADP-ribosylation. Using this method, we detected possible target proteins in rat skeletal muscle.

2. Materials and methods

2.1. Materials

The reagents included the following: Freund's complete adjuvant, histone, protamine, ADP-R, ConA, RPMI 1640 and Hanks (Sigma), Tween 20, Tris, NaCl, NH_2OH, and paraformaldehyde (nacalai tesque), ECL Western blotting detection reagents (Amersham Biosciences), CNBr-activated Sepharose (Pharmacia), NAD (ORIENTAL YEAST CO.), biotinylated NAD (TREVIGEN), FITC-conjugated avidin (Caltag Laboratories), HRP-conjugated αrabbit IgG Ab (MBL), Alexa fluor 594-conjugated αrabbit-IgG Ab (Molecular Probes) FITC-conjugated αCD4 Ab (Pharmingen), [*adenylate*-^{32}P]NAD (NEN) and His-Bind metal chelation resin (Novagen).

2.2. ADP-ribosylation system

2.2.1. Preparation of proteins

Art7 (0.3 ng proteins/μl) was purified from chicken spleen according to the methods described by Terashima et al. [5]. Recombinant rat AAH was prepared as described [18], except that GST-tag was changed to histidine-tag to purify with His-Bind metal chelation resin.

Mouse brain was cut and homogenized with 5 volumes of 0.25 M sucrose containing 20 mM Tris–Cl (pH 7.5), 0.1 mM EDTA and 1 mM PMSF. The suspension was centrifuged at 8000 ×*g* for 10 min, and the supernatant was further centrifuged at 105,000 ×*g* for 1 h. The supernatant was used as rat brain cytosol fraction.

Each fresh rat tissue was cut and homogenized with 5 volumes of 0.25 M sucrose containing 20 mM Tris–Cl (pH 7.5), 0.1 mM EDTA and 1 mM PMSF. The suspension was centrifuged at 600 ×*g* for 10 min, and the supernatant was further centrifuged at 105,000 ×*g* for 1 h. The pellet was suspended in the original volume of the medium containing 1 mM ADP-R.

2.2.2. Sensitivity of ADP-ribosylation

Modified proteins were treated with AAH (2 – 4 μg), 10 mM $MgCl_2$ and 50 mM DTT for 1 h at 37 °C or 1 M NH_2OH (pH 7.0) in 1% SDS for 3 h at 37 °C.

2.3. Antibody preparation

2.3.1. Preparation of antigen

NAD (0.1 mM) and histone (1 mg) were incubated for 10 h at 25 °C with Art7 (50 μl) in 50 mM Tris–Cl (pH 8.0) in a total volume of 5 ml. Following incubation, histone was precipitated with 10% (weight/volume) TCA, and rinsed and suspended with PBS. The preparation contained 25 nmol ADP-R-residues/0.2 mg histone based on the small-scale assay with [*adenylate*-^{32}P] NAD (220 Bq/nmol).

2.3.2. Immunization of rabbits

The PBS suspension of ADP-ribosylated histone was emulsified in the same volume of Freund's complete adjuvant. Rabbits (about 2.5 kg) were injected with a total of 0.2 mg of antigen at multiple sites, and boosted and bled at 2-week intervals.

2.4. Antibody purification

2.4.1. Preparation of ADP-ribosyl-protamine Sepharose

Protamine (2 mg) incubated with NAD (3 mM), Art7 (10 μl) in 50 mM Tris–Cl (pH 8.0) in a total volume of 1 ml for 10 h at 25 °C was mixed with 0.5 ml of coupling buffer (0.1 M $NaHC0_3$ (pH 8.3), 0.5 M NaCl) and 0.13 ml of CNBr-activated Sepharose 4B for 10 h at 4 °C. Subsequently, the gel was transferred to a column and washed successively with coupling buffer and wash buffer (0.5 M NaCl, 0.1 M CH_3COONa (pH 4.0)), alternately 3 times. The matrix contained 80 nmol ADP-R-residues/2 mg protamine based on the small-scale assay with [*adenylate*-^{32}P] NAD (220 Bq/nmol).

2.4.2. Affinity purification of antibody

The serum was fractionated with 40% saturated ammonium sulfate. The precipitate was dissolved in PBS and dialyzed against PBS. The dialysate was applied to the ADP-ribosyl-protamine Sepharose blocked with 0.5% BSA in TPBS (PBS containing 0.05% Tween 20) for 1 h at 25 °C and the Sepharose was washed with TPBS. The antibody was eluted by 0.2 M glycin (pH 2.2) containing 0.05% Tween 20.

2.5. Evaluation of antibody specificity

2.5.1. Assessment of the antibody titer by ELISA

The titer of the antibody was examined by ELISA. Histone (10 μg) or casein (12.5 μg) was reacted with or without NAD (200 μM) in Art7 (1 μl), 50 mM Tris–Cl (pH 8.0) in a total volume of 50 μl on the ELISA plates for 10 h at 25 °C. Each