

Identification in human erythrocytes of mono(ADP-ribosyl) protein hydrolase that cleaves a mono(ADP-ribosyl) G_i linkage

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A novel enzymatic activity, the hydrolysis of linkages between mono(ADP-ribose) and cysteine residues in G_i prepared by eukaryotic ADP-ribosyltransferase C [(1988) *J. Biol. Chem.* 263, 5485–5489] was found in the cytosol of human erythrocytes. The mono(ADP-ribosyl) G_i hydrolase, tentatively named ADP-ribosyl protein hydrolase C was partially purified by sequential chromatographies on DEAE-cellulose and Blue Sepharose. This enzyme catalyzes the release of ADP-ribose from mono(ADP-ribosyl) G_i . Its activity was enhanced by Ca^{2+} and inhibited by ADP-ribose. The presence of this enzyme in eukaryotic cells suggests that endogenous mono(ADP-ribosyl)ation of G_i is a reversible post-translational modification.

Endogenous mono(ADP-ribosyl)ation; Mono(ADP-ribosyl) protein hydrolase; Mono(ADP-ribosyl)transferase; GTP-binding protein; Signal transduction

1. INTRODUCTION

Mono(ADP-ribosyl)ation of proteins is catalyzed by mono(ADP-ribosyl)transferases, which are present in both prokaryotic and eukaryotic cells [1,2]. Some bacterial toxins are known to exert their toxic effects on animal cells through the mono(ADP-ribosyl)ation of GTP-binding proteins (G proteins) that serve as transducers in a variety of transmembrane signalling systems [1,3–5]. Cholera toxin, for example, ADP-ribosylates as arginine residue of a stimulatory G protein (G_s) of the adenylate cyclase system, leading to an increase in cellular cAMP [3,4]. Pertussis toxin catalyzes the ADP-ribosylation of a cysteine residue of an inhibitory G protein (G_i) of adenylate cyclase [5].

Enzymes similar to these bacterial toxins have been identified and purified from several eukaryotic cells [2,6–11]. Recently we found that eukaryotic cells contain a cysteine-specific mono(ADP-ribosyl)transferase, named ADP-ribosyltransferase C [10,11], which catalyzes mono(ADP-ribosyl)ation of a cysteine residue of the α subunit of G_i [11,12]. Mono(ADP-ribosyl)ation of G_i by this transferase C attenuates inhibition of adenylate cyclase by epinephrine [12]. However, the mechanism of the reversal of mono(ADP-ribosyl)ation of G_i has not been elucidated. We investigated whether there was an enzyme that cleaves the mono(ADP-ribose)-cysteine linkages in G_i . Here we report a novel enzymatic activi-

ty in extracts of human erythrocytes for hydrolysis of mono(ADP-ribose) attached to G_i via a cysteine residue. This enzyme is tentatively named ADP-ribosyl protein hydrolase C.

2. MATERIALS AND METHODS

2.1. Materials

[32 P]NAD (10–50 Ci/mmol) was purchased from DuPont-New England Nuclear. ADP-ribose and snake venom phosphodiesterase were from Sigma and Boehringer Mannheim, respectively. Polyethyleneimine (PEI)-cellulose thin-layer plates were from Merck. Blue Sepharose and DEAE-cellulose (DE-52) were from Pharmacia LKB Biotechnology Inc. and Whatman, respectively.

2.2. Preparation of mono(ADP-ribosyl) G_i

Inside-out membrane vesicles from human erythrocytes were prepared as described previously [11]. The inside-out membranes were mono(ADP-ribosyl)ated in 50 mM potassium phosphate buffer (pH 7.0) containing 0.3 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ATP, 0.1 mM GDP, and 0.6 mM [32 P]NAD in the presence of 0.1 μ g/ml ADP-ribosyltransferase C, which was purified from human erythrocytes [11]. Mono(ADP-ribosyl)ation was carried out at 30°C.

2.3. Assay of ADP-ribosyl protein hydrolase C

Enzyme activity was determined by measuring the radioactivity of 5% trichloroacetic acid-soluble material (assay 1), or the disappearance of the mono(ADP-ribose) moiety from proteins (assay 2). The reaction mixture contained 50 mM potassium phosphate (pH 7.6), 3 mM 2-mercaptoethanol, 0.1 mM PMSF, 30 μ M $CaCl_2$, 100 μ g/ml [32 P]mono(ADP-ribosyl) G_i -membranes (8000 cpm), and an appropriate amount of enzyme in a total volume of 100 μ l. The reaction was carried out at 37°C.

2.4. Purification of ADP-ribosyl protein hydrolase C

Freshly prepared human erythrocytes were disrupted by dilution in hypotonic buffer (1 mM potassium phosphate, pH 7.6, 3 mM

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2-mercaptoethanol, 0.3 mM PMSF) and then centrifuged at $105000 \times g$ for 1 h [10,11]. The supernatant cytosol fraction obtained was mixed with DE-52 for 1 h and then washed with buffer H (30 mM potassium phosphate, pH 7.6, 3 mM 2-mercaptoethanol, 0.3 mM PMSF, and 30% ethylene glycol). The washed resin was packed into a column, and proteins were eluted with buffer H containing 0.5 M NaCl. The fractions containing ADP-ribosyl protein hydrolase C were pooled and dialyzed against buffer H. The dialyzed material was applied to a Blue Sepharose column preequilibrated with the same buffer. The column was washed with the same buffer, and the enzyme was eluted with a linear gradient of 0–0.6 M NaCl in buffer H. This purification procedure resulted in about 180-fold purification of the hydrolase C from the cytosol fraction. Unless otherwise indicated, experiments were performed with the Blue Sepharose-purified enzyme.

2.5. Chromatographic analysis

Samples were spotted onto PEI-cellulose plates and developed with Solvent B (1 M LiCl and 1 N acetic acid (9:1, v/v)) [11,13]. Samples were analyzed by strong anion exchange (Hitachi CHI gel no.3013-N) HPLC. Material was eluted with a linear gradient from Solvent C (1.5% acetonitrile, 15 mM ammonium chloride, 2.5 mM potassium phosphate monobasic and 2.5 mM potassium phosphate dibasic) to Solvent D (6% acetonitrile, 300 mM ammonium chloride, 50 mM potassium phosphate monobasic and 50 mM potassium phosphate dibasic).

3. RESULTS AND DISCUSSION

The de-mono(ADP-ribosyl)ation of G_i by partially purified ADP-ribose protein cleavage enzyme was investigated using [32 P]mono(ADP-ribosyl) G_i in inside-out membrane vesicles of human erythrocytes, which was prepared by purified ADP-ribosyltransferase C [11]. The time course of removal of mono(ADP-ribose) from G_i by the enzyme was linear with time for up to 10 min, when about 30% of the substrate was cleaved (fig.1). The rate of release was proportional to the amount of enzyme. Heat-denatured enzyme cleaved little mono(ADP-ribosyl) G_i (fig.1). De-mono(ADP-ribosyl)ated membranes served as a substrate for the ADP-ribosyltransferase C (not shown). These results suggest that the cleavage enzyme is a mono(ADP-ribosyl) protein hydrolase that catalyzes the hydrolysis of linkages between mono(ADP-ribose) and cysteine residues in G_i .

To determine whether cleavage of mono(ADP-ribosyl) G_i by the ADP-ribosyl protein hydrolase C resulted in the release of ADP-ribose, we analyzed the reaction products by thin-layer chromatography (fig.2). The radiolabeled product exhibited similar mobility to that of ADP-ribose. The reaction product was confirmed to be ADP-ribose by its separation by HPLC (fig.3A). Digestion of the product with snake venom phosphodiesterase resulted in liberation of 5'-AMP (fig.3B). These results provide evidence for the existence of a novel mono(ADP-ribosyl) protein hydrolase that catalyzes the release of mono(ADP-ribose) from G_i . The enzyme preparation was not contaminated by phosphodiesterase or phosphatase activity because no 5'-AMP or adenosine was detected as a reaction product (figs 2,3).

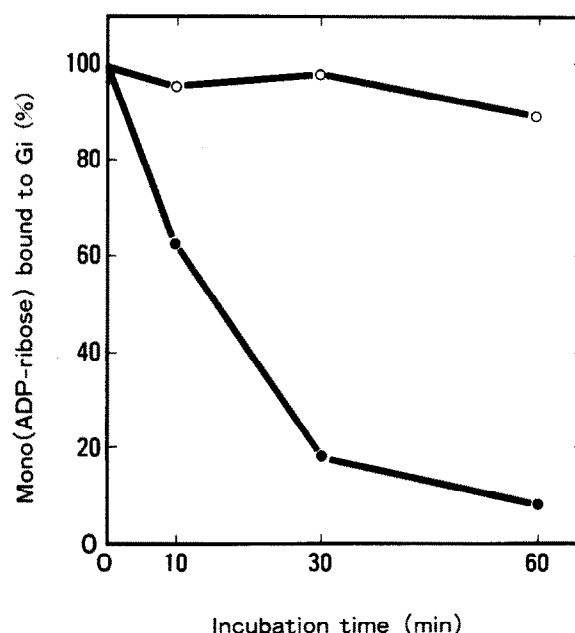


Fig.1. Time course of hydrolysis of mono(ADP-ribosyl) G_i in inside-out membranes of human erythrocytes by ADP-ribosyl protein hydrolase C. Hydrolysis of mono(ADP-ribose)- G_i linkages was measured by assay 2 (●). The enzyme was denatured by heating in boiling water for 3 min (○).

The cleavage of mono(ADP-ribose)- G_i linkage by the partially purified ADP-ribosyl protein hydrolase C was enhanced by a micromolar order of Ca^{2+} (fig.4). Activation was maximal with 30 μ M $CaCl_2$. Mg^{2+} at 1–3 mM slightly stimulated the hydrolase C activity. As shown in table 1, the hydrolase C activity was inhibited by ADP-ribose. But ADP, AMP and NAD^+ had essentially no effect on the hydrolase C reaction,

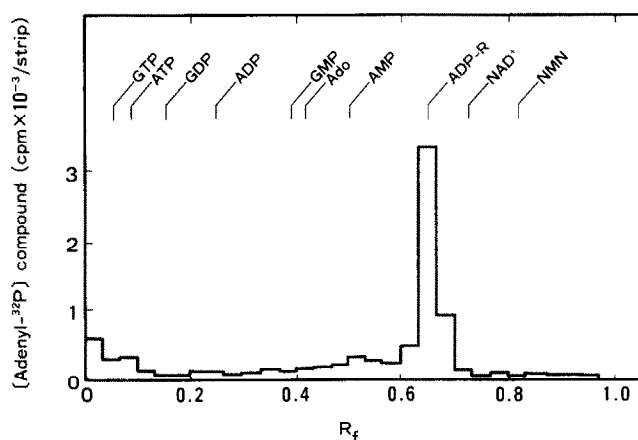


Fig.2. Thin-layer chromatography of the reaction products. The reaction was carried out at 37°C for 30 min in the standard assay mixture. The reaction was terminated by chilling. The supernatant obtained by centrifugation was applied to PEI-cellulose. Chromatography was performed in Solvent B. The thin-layer plates were cut into strips (0.5 cm) and radioactivity was determined in a γ -counter. Ado, adenosine; ADP-R, ADP-ribose.

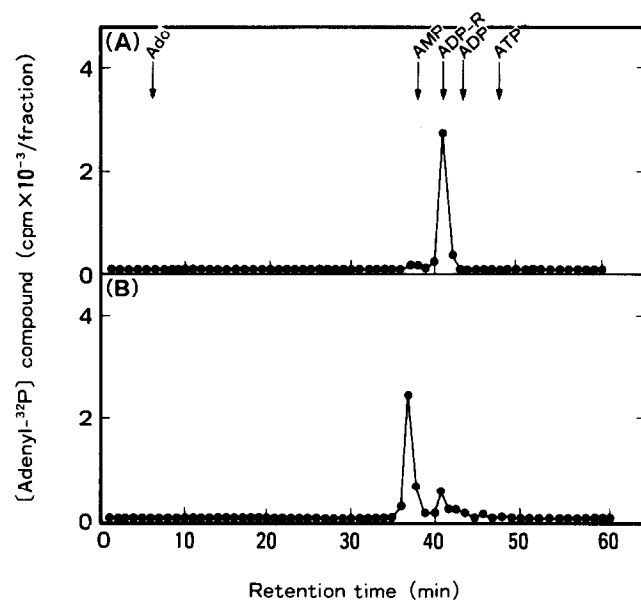


Fig.3. Identification of ADP-ribose as the product of ADP-ribosyl protein hydrolase C by HPLC. The reaction product separated by thin-layer chromatography (fig.2) was extracted and incubated without (A) or with (B) 30 µg/ml snake venom phosphodiesterase at 37°C for 1 h. The digestion product was applied to HPLC and radioactivity was determined as described in section 2.

even at 1 mM. Moreover cysteine and cysteine methylester did not significantly affect cleavage of the mono(ADP-ribose)-G_i linkages. Thus, the primary recognition site for the hydrolase C may be the mono(ADP-ribose) moiety rather than a cysteine residue.

This is the first report that eukaryotic cells contain a mono(ADP-ribosyl) protein hydrolase that can catalyze the hydrolysis of mono(ADP-ribose)-G_i linkages in G_i. The presence of this enzyme suggests that endogenous mono(ADP-ribosyl)ation of G_i is a reversible post-translational modification. The enzyme appears to

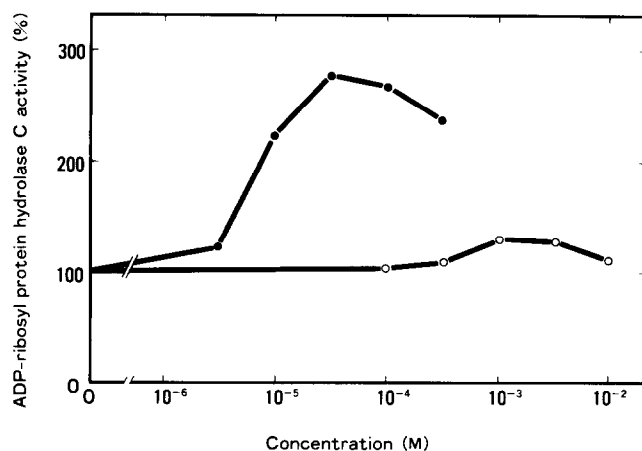


Fig.4. Effects of CaCl₂ and MgCl₂ on ADP-ribosyl protein hydrolase C. The hydrolase C activity was assayed under standard conditions for 10 min except that the concentrations of CaCl₂ (●) and MgCl₂ (○) were varied as indicated.

Table 1

Effects of compounds on ADP-ribosyl protein hydrolase C

Compound	Concentration (mM)	Inhibition (%)
ADP-ribose	0.1	67
	1.0	94
ADP	0.1	4
	1.0	14
AMP	0.1	2
	1.0	8
NAD ⁺	0.1	2
	1.0	7
Cysteine	0.1	3
	1.0	7
Cysteine methyl ester	0.1	4
	1.0	10

ADP-ribosyl protein hydrolase C activity was assayed under the standard conditions (assay 1) except for the additions of the indicated concentrations of various compounds. Incubation was carried out at 37°C for 10 min. Values are means for two experiments

catalyze the hydrolysis of mono(ADP-ribosyl) G_i to ADP-ribose and G_i by a reaction that is stimulated by Ca²⁺. There are several possible explanations for this stimulation: Ca²⁺ may act directly on ADP-ribosyl protein hydrolase C or on membranes to stimulate the cleavage of mono(ADP-ribosyl) G_i.

Recently, G_i and another G protein, G_o, which is substrate for pertussis toxin, were found to act as transducers between receptors and effectors in adenylate cyclase, phospholipase C and A₂ and ionic channel systems [14]. Thus, our observations that G_i in membranes is reversibly modified with mono(ADP-ribose) by ADP-ribosyltransferase C and ADP-ribosyl protein hydrolase C support our hypothesis [10–12] that endogenous mono(ADP-ribosyl)ation of G_i or G_o regulates not only the adenylate cyclase system but also these other signal transduction systems. Further studies on the metabolism of endogenous mono(ADP-ribose) may increase our understanding of the regulation of signal transduction.

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