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# ADP-ribosylation of cell membrane proteins by staphylococcal α-toxin and leukocidin in rabbit erythrocytes and polymorphonuclear leukocytes

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Staphylococcal α-toxin resulted in ADP-ribosylation of the 37 and 41 kDa proteins of a membrane preparation from rabbit erythrocytes. In the presence of 100 μM GTP, the toxin ADP-ribosylated proteins of 54 and 59 kDa and potentiated ADP-ribosylation of the 37 and 41 kDa forms. GTP had no effect on ADP-ribosylation of membrane proteins in the absence of α-toxin. Incubation of a membrane preparation of rabbit polymorphonuclear leukocytes with the S and F components of staphylococcal leukocidin resulted in ADP-ribosylation of the 37 and 41 kDa proteins, respectively. Furthermore, the 37, 41, 54 and 59 kDa proteins were ADP-ribosylated by leukocidin in the presence of GTP. The ADP-ribosylation of these proteins was observed to be dependent on the incubation time and toxin dose and was abolished by prior boiling. Addition of agmatine did not attenuate ADP-ribosylation of these proteins. These results demonstrate that staphylococcal α-toxin and leukocidin possess ADP-ribosyltransferase activities which are potentiated by GTP and suggest that ADP-ribosylation reactions are responsible for development of the cytolytic activities of these staphylococcal toxins.

ADP-ribosylation; Toxin, α-; Leukocidin; (Polymorphonuclear leukocyte, Erythrocyte)

## 1. INTRODUCTION

Staphylococcal α-toxin is produced as a water-soluble 34 kDa polypeptide chain by most strains of *Staphylococcus aureus* and is one of the strongest bacterial hemolytic toxins known. In previous papers, we reported that after binding of α-toxin to the receptor of the erythrocyte membrane, the toxin firstly induces the perturbation of the erythrocyte membrane through stimulation of membrane-associated phospholipase $A_2$ and C activities [1,2].

Staphylococcal leukocidin consists of two protein components designated S (31 kDa) and F (32 kDa) [3,4]. Neither component shows leukocytolytic activity when tested alone, however, in combination they act synergistically to induce leukocytolysis [4]. In previous studies, we reported that component S specifically bound to ganglioside $G_{M1}$ of the rabbit polymorphonuclear leukocyte (leukocyte) membrane [5,6] and subsequently activated phospholipase $A_2$, resulting in the stimulation of arachidonic acid metabolism [7]. In our recent experiments, leukocidin also stimulated the phospholipase C activity of phosphatidylinositol metabolism in rabbit leukocytes [8].

Several bacterial toxins modify the functions in eukaryotic cells by ADP-ribosylation of cellular target proteins. One of the authors of the current article and Honjo et al. [9] discovered that diphtheria toxin possesses ADP-ribosyltransferase activity catalyzing ADP-ribose transfer from NAD to elongation factor-2, thereby inhibiting protein synthesis. Cholera and pertussis toxins also ADP-ribosylate and modify GTP-binding proteins involved in the transmembrane signal transduction system [10].

Here, we show that the staphylococcal cytolytic

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toxins, $\alpha$-toxin and leukocidin, possess ADP-ribosyltransferase activities that catalyze the transfer of the ADP-ribose moiety from NAD predominantly to target-cell membrane proteins.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

GTP, NAD, dithiothreitol (DTT) and agmatine were purchased from Sigma, GTP$\gamma$S from Boehringer Mannheim and [*adenylate*-$^{32}$P]NAD (250 Ci/mmol) from ICN. Other chemicals used were of reagent grade.

### 2.2. *Toxins*

Staphylococcal $\alpha$-toxin was purified from culture media of *S. aureus* Wood 46 strain according to Kato [11]. Staphylococcal leukocidin was purified from *S. aureus* V8 strain as described by Noda et al. [4].

### 2.3. *ADP-ribosylation*

Assay samples (total volume 300 $\mu$l) contained 1 $\mu$M [$^{32}$P]-NAD (2 $\mu$Ci), 10 mM thymidine, 1 mM EDTA, 5 mM $MgCl_2$, 2 mM DTT, 100 mM Tris-HCl (pH 7.5) and other additions as indicated. Rabbit erythrocyte membranes (25 $\mu$g protein/assay) were incubated with $\alpha$-toxin (5 $\mu$g). Rabbit leukocyte membranes (50 $\mu$g protein/assay) were incubated with $\alpha$-toxin (5 $\mu$g) or leukocidin (5 $\mu$g S and F components). After incubation at 37°C for 40 min, 2 ml cold 7.5% trichloroacetic acid and 10 $\mu$g bovine serum albumin were added, and samples kept on ice for 30 min. Precipitated proteins were pelleted by centrifugation and dissolved in 1% SDS/5% mercaptoethanol (60°C, 10 min). Samples were subjected to electrophoresis in 12% polyacrylamide gels by the method of Laemmli [12]. Gels were exposed to Kodak X-Omat AR film.

### 2.4. *ADP-ribosylagmatine assay*

The formation of ADP-ribosylagmatine was determined as described by Moss et al. [13,14]. Assay samples contained 50 mM potassium phosphate (pH 7.5), 5 mM $MgCl_2$, 100 $\mu$M GTP, 100 $\mu$M [*adenine*-U-$^{14}$C]NAD ($\sim 6 \times 10^4$ cpm), 10 mM agmatine, ovalbumin (0.1 mg/ml), and other additions as indicated (total volume 300 $\mu$l). Reactions were initiated with toxins. After 60 min at 30°C, two 50-$\mu$l samples were transferred to columns of AG1-X2 which were washed four times with 1.25 ml water. Eluates containing [*adenine*-U-$^{14}$C]ADP-ribosylagmatine were collected for radioassays.

## 3. RESULTS AND DISCUSSION

As shown in fig.1, when a membrane preparation (25 $\mu$g protein) from rabbit erythrocytes was incubated without (fig.1, lane 1) or with staphylococcal $\alpha$-toxin (5 $\mu$g) in the presence of [$^{32}$P]NAD and 5 mM $MgCl_2$ at 37°C for 40 min, the 37 and 41 kDa proteins were ADP-ribosylated by $\alpha$-toxin (fig.1, lane 2). The intensity of radioactivity incorporated into the 37 kDa protein was stronger vs

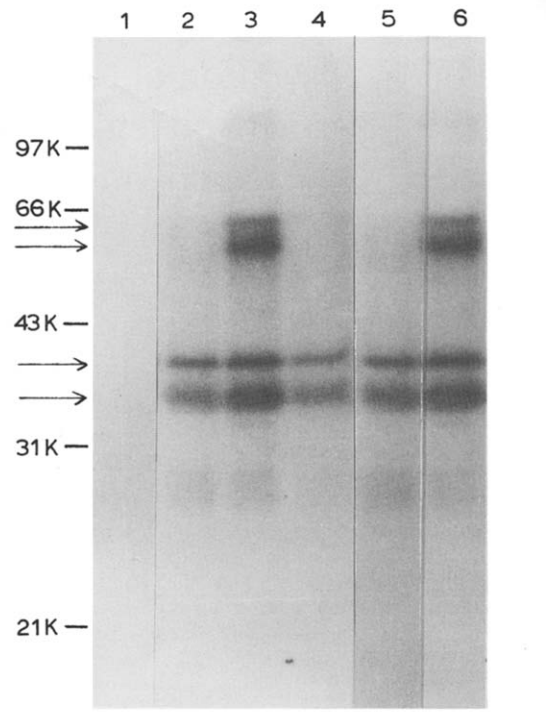


Fig.1. ADP-ribosylation of rabbit erythrocyte membrane proteins by staphylococcal $\alpha$-toxin. A rabbit erythrocyte membrane preparation (25 $\mu$g protein) and 1 $\mu$M [$^{32}$P]NAD (2 $\mu$Ci) were incubated without (lane 1) or with 5 $\mu$g staphylococcal $\alpha$-toxin (lanes 2–6) in the presence of 100 $\mu$M GTP (lanes 3,6), 100 $\mu$M GTP$\gamma$S (lane 4), or 10 mM agmatine (lanes 5,6) for 40 min at 37°C as described in section 2. The autoradiogram of the SDS-polyacrylamide gel (12%) of the labeled proteins is shown. Arrows denote positions of ADP-ribosylation substrates of 37, 41, 54 and 59 kDa.

that of the 41 kDa protein. Addition of 100 $\mu$M GTP potentiated ADP-ribosylation of the 37 and 41 kDa proteins and ADP-ribosylated proteins of 54 and 59 kDa which were not ADP-ribosylated in the absence of GTP (fig.1, lane 3). The intensity of ADP-ribosylation for the 37 kDa protein was similar to that of the 54 kDa protein, which was stronger than those of the 41 and 59 kDa proteins. Although ADP-ribosylation of the 37 and 41 kDa proteins was caused by $\alpha$-toxin, the 54 and 59 kDa proteins were not ADP-ribosylated in the presence of 100 $\mu$M GTP$\gamma$S, a nonhydrolyzable analog of GTP, and 5 mM $MgCl_2$ (fig.1, lane 4).

GTP$\gamma$S-dependent inhibition of bacterial toxin-catalyzed ADP-ribosylation is a characteristic property of the GTP-binding proteins, $G_s$, $G_i$ and $G_b$ (botulinum toxin substrates) [15,17]. Therefore,

it is strongly suggested that the 54 and 59 kDa forms are GTP-binding proteins themselves or components involved in the GTP-dependent regulatory system. ADP-ribosylation of membrane proteins caused by α-toxin was not affected by the addition of other nucleotides such as GDP, ATP, ADP, CTP, CDP, UTP and UDP (1 μM mM). The addition of 10 mM agmatine, an arginine analog, lacking the carboxyl group, had no effect on ADP-ribosylation of membrane proteins induced by α-toxin in the absence (fig.1, lane 5) or presence of 100 μM GTP (fig.1, lane 6). When agmatine was incubated with α-toxin in the presence of [*adenine*-U-$^{14}$C]NAD at 30°C for 60 min, no [*adenine*-U-$^{14}$C]ADP-ribosylagmatine was detectably formed (table 1). These results suggest that α-toxin differs in catalytic properties from cholera toxin and is not an NAD:arginine ADP-ribosyltransferase [14,18]. α-Toxin ADP-ribosylated specific membrane proteins of molecular masses 37, 41, 54 and 59 kDa in a dose- and time-dependent manner (not shown). Prior boiling of α-toxin completely abolished both hemolytic and ADP-ribosyltransferase activities.

As shown in fig.2, when a membrane preparation (50 μg protein) from rabbit polymorphonuclear leukocytes was incubated without (fig.2, lane 1) or with staphylococcal leukocidin (5 μg of S and F components) in the presence of [$^{32}$P]NAD and 5 mM $MgCl_2$ at 37°C for 40 min, the 37 and 41 kDa membrane proteins were ADP-ribosylated by leukocidin (fig.2, lane 2). In the presence of 100 μM GTP, leukocidin ADP-ribosylated the 37, 41, 54 and 59 kDa forms (fig.2, lane 3). ADP-ribosylation of the 54 and 59 kDa species did not occur in the presence of 100 μM GTPγS (fig.2, lane 4). The S and F components ADP-ribosylated the 37 and 41 kDa membrane proteins, respectively (fig.2, lanes 5,8). However, ADP-ribosylation of the 54 and 59 kDa forms was not caused by these components when tested alone in the presence of GTP or GTPγS (fig.2, lanes 6,7,9,10). ADP-ribosylation of the 37, 41, 54 and 59 kDa membrane proteins by leukocidin was found to take place in a dose- and time-dependent manner (not shown). Agmatine had no effect on ADP-ribosylation of these proteins by leukocidin. The formation of [*adenine*-U-$^{14}$C]ADP-ribosyl-

Table 1

NAD:agmatine ADP-ribosyltransferase activity of bacterial toxins

| Additions | | Formation of [*adenine*-U-$^{14}$C]-ADP-ribosylagmatine (pmol/assay) | |
|---|---|---|---|
| | | − DTT | + 20 mM DTT |
| None | | < 10 | < 10 |
| Cholera toxin A subunit (1 μg) | | 40 | 1320 |
| α-Toxin | (1 μg) | < 10 | < 10 |
| | (3 μg) | < 10 | < 10 |
| | (10 μg) | < 10 | < 10 |
| Leukocidin | | | |
| S component | (1 μg) | < 10 | < 10 |
| | (3 μg) | < 10 | 10 |
| | (10 μg) | < 10 | 10 |
| F component | (1 μg) | < 10 | < 10 |
| | (3 μg) | < 10 | < 10 |
| | (10 μg) | < 10 | < 10 |
| S + S components | (1 μg + 1 μg) | < 10 | 10 |
| | (3 μg + 3 μg) | < 10 | 20 |
| | (10 μg + 10 μg) | < 10 | 20 |

NAD:agmatine ADP-ribosyltransferase activity was assayed as described in section 2

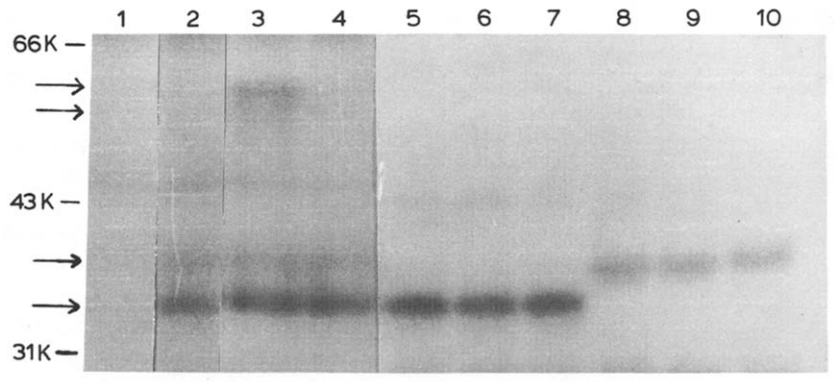


Fig.2. ADP-ribosylation of rabbit leukocyte membrane proteins by staphylococcal leukocidin. A rabbit leukocyte membrane preparation (50 μg protein) and 1 μM [$^{32}$P]NAD (2 μCi) were incubated without (lane 1) or with 5 μg S and F components of leukocidin (lanes 2–4), 5 μg S component (lanes 5–7), or 5 μg F component (lanes 8–10) in the presence of 100 μM GTP (lanes 3,6,9), or 100 μM GTPγS (lanes 4,7,10) for 40 min at 37°C as described in the text. The autoradiogram of the SDS-polyacrylamide gel (12%) of the labeled proteins is shown. Arrows denote the positions of ADP-ribosylation substrates of 37, 41, 54 and 59 kDa.

agmatine was not observed when agmatine was incubated with leukocidin in the presence of [*adenine*-U-$^{14}$C]NAD at 30°C for 60 min (table 1). Prior boiling of leukocidin abolished ADP-ribosyltransferase activity. It is clear that staphylococcal α-toxin and leukocidin possess ADP-ribosyltransferase activity but they may differ in catalytic properties from cholera toxin, and NAD:arginine ADP-ribosyltransferase. ADP-ribosylation of the 37, 41, 54 and 59 kDa membrane proteins of cells such as ESK cells that are insensitive to α-toxin and leukocidin, was not observed. Therefore, it is suggested that ADP-ribosylation of the membrane proteins of 37, 41, 54 and 59 kDa, resulting from α-toxin or leukocidin, may play an important role in cytolysis by these staphylococcal cytolytic toxins. Further detailed study on the role of ADP-ribosylation of these membrane proteins by staphylococcal toxins is now underway.

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