# CTP-dependent endogenous ADP-ribosylation of a 38 kDa protein in HL-60 cell membranes

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Incubation of membranes of human promyelocytic leukemia HL-60 cells with [$^{32}$P]NAD led to ADP-ribosylation of several proteins including a 38 kDa protein by endogenous ADP-ribosyltransferases. The ADP-ribosylation of the 38 kDa protein was distinctly different from others on the basis of pH dependency and heat stability at 50°C, suggesting that there are at least two endogenous ADP-ribosyltransferases. It was enhanced by CTP, but not affected by ATP, GTP and UTP, whereas it was inhibited by GTPγS. [α-$^{32}$P]CTP bound to the 38 kDa protein immobilized on a nitrocellulose sheet, indicating that the 38 kDa protein which bound CTP is strongly ADP-ribosylated by an endogenous ADP-ribosyltransferase.

ADP-ribosylation; CTP; GTPγS; (HL-60 cell)

## 1. INTRODUCTION

A variety of microbial toxins, i.e. diphtheria [1,2], cholera, pertussis toxins [3] and staphylococcal α-toxin and leukocidin [4] are known to have mono-ADP-ribosyltransferases, which catalyze transfer of an ADP-ribose moiety of NAD to the target cellular GTP-binding proteins. Recently, the existence of endogenous ADP-ribosyltransferases in eukaryotic cells has been published and characterized [5–8]. To elucidate the endogenous substrates of the ADP-ribosyltransferases and the roles of cellular ADP-ribosyltransferases, several works have been published [9–14]. We have studied endogenous ADP-ribosylation of HL-60 cell membranes and found that there were two types of ADP-ribosyltransferase. Interestingly, one of the transferase activities which ADP-ribosylated a 38 kDa protein was stimulated by CTP, and the 38 kDa protein bound [$^{32}$P]CTP. We also studied several characteristics of the ADP-ribosylation. This report is the first publication reporting that endogenous ADP-ribosylation of HL-60 membrane protein is controlled by CTP and GTPγS.

## 2. MATERIALS AND METHODS

### 2.1. *Chemicals*

[α-$^{32}$P]NAD (spec. act. 800 Ci/mmol) and [α-$^{32}$P]CTP (spec. act. 3000 Ci/mmol) were purchased from New England Nuclear. ATP, GTP, CTP, UTP, CDP and NAD were from Sigma. Cholera toxin subunit A was purchased from List Biochemical Laboratories and pertussis toxin was from Funakoshi.

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### 2.2. *Membrane preparation*

HL-60 cells were grown in RPMI 1640 containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% $CO_2$. The cells were washed with phosphate-buffered saline, suspended in homogenization buffer containing 20 mM Tris (pH 7.5), 0.25 M sucrose, 2 mM EDTA, 0.5 mM EGTA and 0.2 mM phenylmethylsulfonyl fluoride, and kept for 10 min at 4°C. The cells were sonicated for 30 s and centrifuged at 2000 × *g* for 10 min. The supernatant was then centrifuged at 105000 × *g* for 1 h and the pellet was suspended in the homogenization buffer without 0.25 M sucrose and stored at −20°C as membranes.

### 2.3. *Assay of ADP-ribosylation*

Membranes (80 μg protein) were incubated for 60 min at 37°C in a volume of 200 μl containing 100 mM Tris buffer (pH 7.5), 10 mM thymidine, 1 mM EDTA, 5 mM $MgCl_2$, 1 μM NAD, 2 mM dithiothreitol and 5 μCi [α-$^{32}$P]NAD. Reactions were terminated by the addition of 800 μl of 10% trichloroacetic acid, kept for 30 min at 4°C and centrifuged for 10 min at 7000 × *g*. The pellets were dissolved in 1% SDS/5% mercaptoethanol, boiled for 10 min at 60°C and resolved in SDS-polyacrylamide gel (10%) electrophoresis by the method of Laemmli [15]. Gels were autoradiographed with a Kodak X-Omat film with an intensifying screen at −80°C for 20 h.

### 2.4. *[α-$^{32}$P]CTP binding*

The experiment performed referred to [α-$^{32}$P]GTP-binding experiment to G proteins on nitrocellulose sheets [16,17]. 100 μg of HL-60 cell membranes was applied to a 15% SDS-polyacrylamide gel and transferred to a nitrocellulose sheet. The sheet was incubated in 100 ml of 50 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$, 0.1% BSA for 18 h at 4°C and then incubated with 1 nM [α-$^{32}$P]CTP (2 μCi/ml) in 20 ml of the same buffer including 1 μM CTP or 1 mM CTP for 18 h at 4°C. The sheet was washed ten times with the buffer, dried and autoradiographed with a Kodak X-Omat film with an intensifying screen at −80°C for 3 days.

## 3. RESULTS AND DISCUSSION

We have studied endogenous ADP-ribosylation in HL-60 cell membranes. 80 μg of the membrane proteins was incubated with 5 μCi [$^{32}$P]NAD at 37°C for the indicated time, and membrane proteins were

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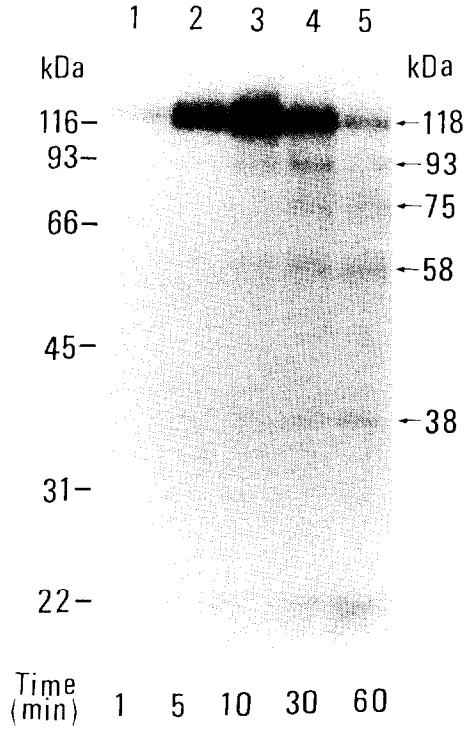


Fig. 1. Endogenous ADP-ribosylation of HL-60 cell membranes. Membrane preparations of HL-60 cells (80 $\mu$g) were incubated with 5 $\mu$Ci [$\alpha$-$^{32}$P]NAD for the indicated time at 37°C. The samples were subjected to 10% SDS-polyacrylamide gel electrophoresis and autoradiogram of the gel of the labeled proteins is shown.

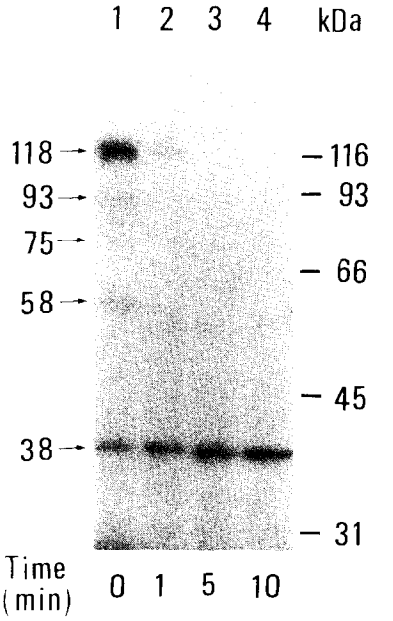


Fig. 2. Stability to 50°C. Membranes were preincubated at 50°C for the indicated time and ADP-ribosylation reaction was carried out as described in section 2. Autoradiogram of the gel is shown.

separated by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 1, [$^{32}$P]NAD led to covalent modifications of 118, 93, 75, 58 and 38 kDa proteins. Treatment of the $^{32}$P-labeled membranes with 30 U/ml snake venom phosphodiesterase at 37°C for 2 h [18] eliminated most of the radiolabel from these proteins, indicating the proteins were ADP-ribosylated. Since the reaction mixture contained 10 mM thymidine, which is a high enough concentration to suppress poly-ADP-ribosylation [19], these modifications are likely to be mono-ADP-ribosylation by endogenous ADP-ribosyltransferases. The incorporation of radiolabel from [$^{32}$P]NAD into 118 kDa protein was maximum at 10 min and gradually decreased (Fig. 1), suggesting that de-ADP-ribosylation reaction might exist. However, the incorporation into other proteins appeared later than 5 min, and radiolabel into 58 and 38 kDa proteins increased up to 1 h. The ADP-ribosylation of 118, 93, 75 and 58 kDa proteins was markedly increased in the alkaline range, but the ADP-ribosylation of the 38 kDa protein was not affected by the pH range from 6.0 to 9.0 (data not shown). To characterize these ADP-ribosylations, we examined the heat stability of membrane at 50°C. As shown in Fig. 2, preincubation of the membrane for 1 min at 50°C abolished the ADP-ribosylation of 93, 75 and 58 kDa proteins completely and most of the 118 kDa protein. However, ADP-ribosylation of the 38 kDa protein occurred even after 10 min incubation at 50°C. Further, it was found to be stable at 55°C for 5 min and decreased by 65% at 60°C for 5 min (data not shown). These data suggested that there were at least two types of ADP-ribosyltransferases in HL-60 cell membranes. One of the enzymes may ADP-ribosylate 118, 93, 75 and 58 kDa proteins and the optimal pH range is in alkaline condition. This enzyme is labile at 50°C for 1 min. The other enzyme may ADP-ribosylate 38 kDa proteins and is not affected by pH 6–9. This enzyme is stable at 55°C for 5 min.

In further experiments, we focused on the 38 kDa ADP-ribosylation and all the other ADP-ribosylation experiments were performed in physiological conditions (pH 7.5). Fig. 3A shows the effects of 500 $\mu$M of various nucleotides on the ADP-ribosylation of the 38 kDa protein. ADP-ribosylation of the 38 kDa membrane protein was significantly enhanced by CTP and partially inhibited by GTP$\gamma$S, but not affected by ATP, GTP and UTP. To confirm the effects of CTP and GTP$\gamma$S on the ADP-ribosylation of the 38 kDa protein, the ADP-ribosylation assay was performed at various concentrations of CTP or GTP$\gamma$S. As shown in Fig. 3B, the stimulatory effect of CTP on the ADP-ribosylation of the 38 kDa protein was maximum at 250 $\mu$M. 500 $\mu$M of CDP had no effect on the ADP-ribosylation (lane 7). GTP$\gamma$S inhibited the ADP-ribosylation dependent on the dose, and 1 mM GTP$\gamma$S inhibited the 38 kDa ADP-ribosylation completely (Fig. 3C).

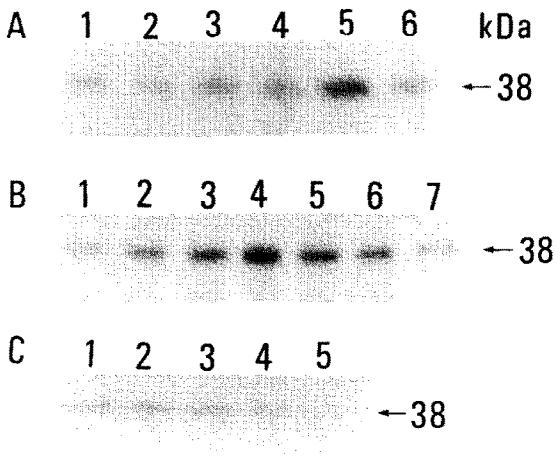


Fig. 3. The effect of nucleotides on the endogenous ADP-ribosylation of 38 kDa protein. The autoradiogram of the gel is shown. (A) ADP-ribosylation was carried out in the presence of 500 $\mu$M of various nucleotides. Control (lane 1), GTP$\gamma$S (lane 2), GTP (lane 3), ATP (lane 4), CTP (lane 5) and UTP (lane 6). (B) ADP-ribosylation was carried out in the presence of various concentrations of CTP and CDP. Control (lane 1), 50 $\mu$M CTP (lane 2), 100 $\mu$M CTP (lane 3), 250 $\mu$M CTP (lane 4), 500 $\mu$M CTP (lane 5), 1 mM CTP (lane 6) and 500 $\mu$M CDP (lane 7). (C) ADP-ribosylation was carried out in the presence of various concentrations of GTP$\gamma$S. Control (lane 1), 100 $\mu$M (lane 2), 250 $\mu$M (lane 3), 500 $\mu$M (lane 4) and 1 mM (lane 5).

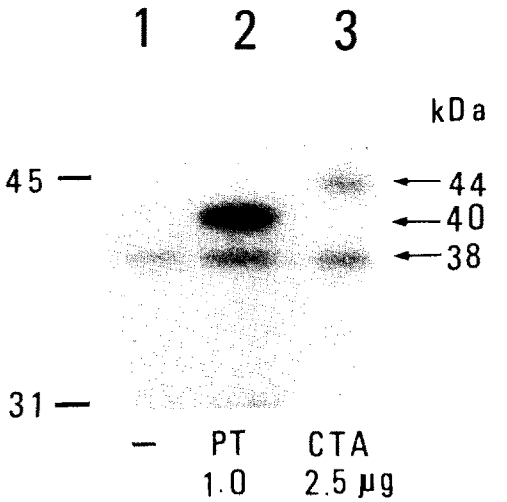


Fig. 4. ADP-ribosylation of HL-60 cell membranes by cholera and pertussis toxins. The mobility of the 38 kDa protein which was ADP-ribosylated by endogenous ADP-ribosyltransferase (lane 1) was compared to $G_s$, ADP-ribosylated by cholera toxin subunit A (lane 3), and $G_i$ which was ADP-ribosylated by pertussis toxin (lane 2).

The ADP-ribosylated 38 kDa protein was distinct from the $G_i$ (40 kDa) that was ADP-ribosylated by pertussis toxin or $G_s$ (44 kDa) that was ADP-ribosylated by cholera toxin (Fig. 4).

It was reported that binding of GTP$\gamma$S to $G_i$ decreased its ability to serve as a pertussis toxin substrate [20] and ADP-ribosylation of $G_s$ is enhanced by GTP [21]. However, there is no report of CTP-dependent ADP-ribosylation. Our hypothesis is that the 38 kDa protein might be a CTP-binding protein. To examine if the protein could bind CTP or not, HL-60 cell membrane proteins on the SDS-polyacrylamide gel were transferred to a nitrocellulose sheet and at the same time, ADP-ribosylated membranes were transferred to the same sheet to check the mobility of the 38 kDa protein. [$\alpha$-$^{32}$P]CTP binding to the proteins immobilized on the sheet was performed [16,17]. As shown in Fig. 5, the ADP-ribosylated 38 kDa band moved at the same position as one of the CTP-binding proteins, whose radioactivities were completely suppressed by 1 mM non-radioactive CTP. These data suggested that the 38 kDa protein in the CTP-binding state might be strongly ADP-ribosylated by an endogenous ADP-ribosyltransferase. Further, GTP$\gamma$S suppressed the ADP-ribosylation, suggesting that the 38 kDa protein might be a GTP-binding protein and GTP$\gamma$S suppresses the ADP-ribosylation by the similar mechanism as ADP-ribosylation by pertussis toxin.

In the present work, we showed that there were two types of ADP-ribosyltransferases. To advance our

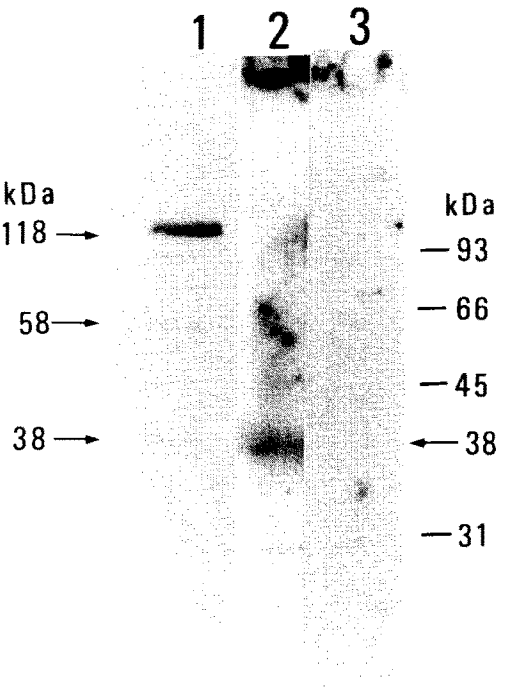


Fig. 5. [$\alpha$-$^{32}$P]CTP binding to HL-60 cell membranes immobilized on a nitrocellulose sheet transferred from SDS-polyacrylamide gel. About 100 $\mu$g of membranes were applied to two columns of 15% SDS-polyacrylamide gel and [$^{32}$P]ADP-ribosylated membranes were also applied to another column of the same gel. They were transferred to a nitrocellulose sheet and [$^{32}$P]CTP binding was performed as described in section 2. ADP-ribosylation (lane 1), and [$^{32}$P]CTP binding in the presence of 1 $\mu$M CTP (lane 2) and 1 mM CTP (lane 3). The data shown are representative of three similar experiments.

work, purification of the enzymes and substrate must be done. As the endogenous ADP-ribosyltransferases have not yet been associated with specific cellular functions, the study of physiological function of CTP-dependent endogenous ADP-ribosylation is under way.

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## REFERENCES

[1] Honjo, T., Nishizuka, Y., Hayaishi, O. and Kato, I. (1968) J. Biol. Chem. 243, 3553–3555.
[2] Ueda, K. and Hayashi, O. (1985) Annu. Rev. Biochem. 54, 73–100.
[3] Moss, J. and Vaughan, M. (1988) Adv. Enzymol. 61, 303–379.
[4] Kato, I. and Noda, M. (1989) FEBS Lett. 255, 59–62.
[5] Moss, J., Stanley, S.J. and Watkins, P.A. (1980) J. Biol. Chem. 255, 5838–5840.
[6] Yost, D.A. and Moss, J. (1983) J. Biol. Chem. 258, 4926–4929.
[7] Lee, H. and Iglewski, W.J. (1984) Proc. Natl. Acad. Sci. USA 81, 2703–2707.
[8] Tanuma, S., Kawashima, K. and Endo, H. (1988) J. Biol. Chem. 263, 5485–5489.
[9] Rosenthal, W., Binder, T. and Schultz, G. (1987) FEBS Lett. 211, 137–143.
[10] Vedia, L.M.Y., Nolan, R.D. and Lapetina, E.G. (1988) Biochem. Biophys. Res. Commun. 157, 1323–1328.
[11] Brüne, B. and Lapetina, E.G. (1989) J. Biol. Chem. 264, 8455–8458.
[12] Fendrick, J.L. and Iglewski, W.J. (1989) Proc. Natl. Acad. Sci. USA 86, 554–557.
[13] Vedia, L.M.Y., Nolan, R.D. and Lapetina, E.G. (1989) Biochem. J. 261, 841–845.
[14] Brüne, B., Vedia, L.M.Y. and Lapetina, E.G. (1990) Proc. Natl. Acad. Sci. USA 87, 3304–3308.
[15] Laemmli, U.K. (1970) Nature 227, 680–685.
[16] Kikuchi, A., Yamashita, T., Kawata, M., Yamamoto, K., Ikeda, K., Tanimoto, T. and Takai, Y. (1988) J. Biol. Chem. 263, 2897–2904.
[17] Lapetina, E.G. and Reep, B.R. (1987) Proc. Natl. Acad. Sci. USA 84, 2261–2265.
[18] Katada, T. and Ui, M. (1982) Proc. Natl. Acad. Sci. USA 79, 3129–3133.
[19] Rankin, P.W., Jacobson, E.L., Benjamin, R.C., Moss, J. and Jacobson, M.K. (1989) J. Biol. Chem. 264, 4312–4317.
[20] Bokoch, G.M., Katada, T., Northup, J.K., Ui, M. and Gilman, A.G. (1984) J. Biol. Chem. 259, 3560–3567.
[21] Gill, D.M. and Meren, R. (1983) J. Biol. Chem. 258, 11908–11914.