Identification of Glu^{173} as the critical amino acid residue for the ADP-ribosyltransferase activity of *Clostridium botulinum* C3 exoenzyme

Yuji Saito, Yasuo Nemoto**, Toshimasa Ishizaki, Naoki Watanabe, Narito Morii, Shuh Narumiya*

Department of Pharmacology, Kyoto University Faculty of Medicine, Yoshida, Sakyo-ku, Kyoto 606, Japan

Received 14 June 1995; revised version received 14 July 1995

Abstract ***Clostridium botulinum*** **C3 exoenzyme specifically ADP-ribosylates *rho-p21* in eukaryotic cells. Trp^{18} and Glu^{173} of this enzyme were substituted with other amino acids via site-directed mutagenesis. All substitutions at Glu^{173} caused a significant reduction in affinity for NAD and diminished ADP-ribosyltransferase activity. On the other hand, the activity of enzymes with the substitution at Trp^{18} remained intact. Swiss 3T3 cells treated with the enzyme with the Trp^{18} substitution showed the typical morphologic changes of the C3 exoenzyme phenotype. In contrast, no changes were found in cells incubated with the Glu^{173}-substituted enzyme. These results indicate that the Glu^{173} residue of the C3 exoenzyme plays a key role in interacting with NAD and in expression of ADP-ribosyltransferase activity, which is essential for the phenotypic change by C3 exoenzyme treatment.**

Key words: Botulinum C3 exoenzyme; ADP-ribosylation; *rho* p21; Swiss 3T3 cell

1. Introduction

Bacterial mono-ADP ribosyltransferases catalyze the transfer of an ADP-ribose moiety of NAD to specific target proteins in eukaryotic cells, thereby affecting their functions. Examples of this family of enzymes include diphtheria toxin and pertussis toxin, which specifically ADP-ribosylate and inactivate elongation factor 2 and a heterotrimeric G protein Gi, respectively [1,2]. *Clostridium botulinum* C3 exoenzyme [3] specifically ADP-ribosylates the small molecular weight GTP binding protein *rho p21* at the Asn^{41} residue located in a putative effector binding domain [4]. Treatment of cells with C3 exoenzyme inhibits stimulus-induced actin filament organization and cell adhesion [5–12], indicating that *rho p21* mediates these cellular responses as a molecular switch and that C3 exoenzyme inhibits these functions of *rho p21*. Identification of the amino acid residues of the C3 exoenzyme interacting with NAD is important in elucidating the structure of its catalytic site and in understanding a mechanism of the ADP-ribosylation. Two sites have been suggested to be involved in the NAD binding of C3 exoenzyme. In one study, we labeled the protein with a photoaffinity probe, [^{32}P]nicotinamide-2-azidoadenine dinucleotide, and isolated a peptide corresponding to Phe^{9}-Gly^{19} as the adenine ring binding domain. Based on this, we suggested that Trp^{18} may be the critical residue [13]. Another study affinity-labeled a C3-like exoenzyme from *Clostridium limosum* with [carbonyl-^{14}C]NAD by UV irradiation and suggested that the Glu^{173} residue of the C3 exoenzyme was a part of the NAD binding site of the catalytic center [14].

In this study, we have utilized site-directed mutagenesis to substitute various amino acids in place of the Trp^{18} and Glu^{173} residues and then examined the importance of these residues in NAD binding and enzymatic activity. The substitution of Glu^{173} dramatically reduced both the affinity for NAD and its ADP-ribosyltransferase activity, whereas no change was observed with the Trp^{18} substitution.

2. Materials and methods

2.1. Construction of mutant C3 exoenzyme expression vectors

A cDNA clone for the C3 exoenzyme truncated at Leu^{172} (173C3) was generated by polymerase chain reaction (PCR) using the forward primer 5′-ATAGGATCCCATAT-GGCTAGCTATGCAG-3′ and the reverse primer 5′-CAACATCTAGAGTTGTCCCGGGAAATA-3′, with pET3a C3 [3] as the template. The PCR fragment was digested with *Nde*I and *Xba*I, and then cloned into pET3a vector, resulting in a pET3a 173C3 plasmid. The Glu^{173} substitution with Gln (E173Q) was prepared by PCR using the forward primer 5′-ATTTCCCGGGA-CAACTGCAGGTGTTGC-3′ and the reverse primer 5′-GCCTCTA-GAGGATCCTATTATTTAAATATCATTGC-3′. This PCR product was digested with *Sma*I and then cloned into pET3a 173C3 to give rise to pET3a E173Q. pET3a E173D (Asp substitution) was prepared as described for pET3a E173Q, except that 5′-ATTTCCCGGGACAA-CTTGACGTCTTGC-3′ was used as a forward primer.

The Trp^{18} substitution with Gly (W18G) was generated as follows. First, PCR was performed using the forward primer 5′-GTTGAG-GAAGGCCAAAAAAGGTGGAAATGCTCAATATAAAAAATAT-G-3′ and the reverse primer 5′-TTATTGGATCCTATTATTTAAAT ATCATTGCTGTAA-3′. The purified PCR product was served as a template for the second series of PCR using the forward primer 5′-ACTGTTCATATGGCTAGCTATGCAGATACTTTCACAGAATTT AC CA ATGTTGAGGAAGCCAAAAAA-3′ and the identical reverse primer as was used in the first PCR. This was followed by digestion with *Nde*I and *Bam*HI, and then the PCR fragment was cloned into a pET3a vector, resulting in pET3a W18G. pET3a W18A (Ala substitution) was constructed as described for pET3a W18G, except that 5′-GTTGAG-GAAGCCAAAAAAGCTGGAAATGCTCAATATAAAAAATATG -3′ was used as a forward primer in the first set of PCR. All plasmids were sequenced to verify the correct substitutions.

2.2. Expression and purification of mutant C3 exoenzyme

E. coli strain BL21 (DE3) cells carrying the plasmid vectors were grown at 37°C with shaking in M9 medium containing 1 mM $MgSO_4$,

*Corresponding author. Fax: (81) (75) 753-4693.

***Present address:* Department of Biochemistry, Biomedical Research Center, Osaka University School of Medicine, Suita Osaka 565, Japan.

Abbreviations: *rho-p21*, a small GTP-binding protein of the *rho* gene product; GST-*rho p21*, recombinant *rho p21* fused to glutathione *S*-transferase; C3 exoenzyme, *Clostridium botulinum* C3 ADP-ribosyltransferase; 2-azidoNAD, nicotinamide-2-azidoadenine dinucleotide.

SSDI 0014-5793(95)00851-9

0.1 mM $CaCl_2$, 0.5% (w/v) Casamino acids, 0.4% (w/v) glucose and 0.1 mg/ml ampicillin. When the absorbance at 600 nm of the cell suspension reached 0.5, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM. Three hours later, the cells were harvested by centrifugation at 5000 × *g* for 10 min. Whole cell lysates were prepared as reported previously [3], were diluted and applied to a CM Sepharose column equilibrated with 20 mM Hepes-NaOH (pH 7.5). After washing the column with the equilibration buffer, elution was performed with a linear salt gradient between 0 and 0.5 M NaCl in the equilibration buffer.

2.3. ADP-ribosylation and cell culture

The ADP-ribosyltransferase activity of the wild type and mutant C3 exoenzymes was measured as follows. The ADP-ribosylation buffer contained 100 mM Tris-HCl (pH 8.0), 10 mM thymidine, 10 mM nicotinamide, 10 mM dithiothreitol, 5 mM $MgCl_2$, and [^{32}P]NAD (1000 dpm/pmol) (Dupont New England Nuclear). Purified wild type or mutant C3 exoenzyme was incubated with 1 μg of recombinant GST-*rho A p21* (fusion protein) or with 100 μg protein of crude cell homogenate in this buffer and quantification of the ADP-ribosylation was carried out as previously described [12].

Phenotypic changes induced by the wild type and mutant C3 exoenzymes and in situ ADP-ribosylation of *rho p21* in cultured cells were examined as follows. Swiss 3T3 cells were cultured in DMEM containing 10% fetal bovine serum until near confluent. The cells were then treated with or without 30 μg/ml of the various C3 exoenzymes for 72 h. During treatment, the morphology of the cells was examined under a phase-contrast microscope and photographed. The cells were then rinsed twice with phosphate-buffered saline (PBS) and incubated with 0.05% (w/v) trypsin in PBS. The dissociated cells were collected, resuspended in the ADP-ribosylation buffer as above and sonicated. The ADP-ribosylation reaction was then carried out as described above.

3. Results and discussion

Previous studies using two different photoaffinity probes [13,14] suggested two residues of the C3 exoenzyme, Trp^{18} and Glu^{173}, as parts of the NAD binding site. We used a site-directed mutagenesis technique and tested the relative importance of the two residues. We replaced Trp^{18} with Gly (W18G) or Ala (W18A), replaced Glu^{173} with Asp (E173D) or Gln (E173Q), and examined the effects of these substitutions on the ADP-ribosyltransferase activity of C3 exoenzyme. These mutant and wild type C3 exoenzymes were expressed in *E. coli* and purified using CM-Sepharose column chromatography. Each preparation showed a single protein band on SDS-polyacrylamide gel electrophoresis (Fig. 1A). As shown in Fig. 1B, the ADP-ribosylation of GST-*rho p21* by the wild type or the W18A or W18G mutants of the C3 enzyme proceeded linearly with time with almost the same reaction rates. ADP-ribosylation by the E173D mutant proceeded also linearly, but the rate was about one-tenth of that of the wild type enzyme. In contrast, no ADP-ribosylation was detected in the reaction using the E173Q mutant. To quantitatively compare the enzymatic activities of each mutant, the ADP-ribosylation reaction was performed with various concentrations of the enzymes in the presence of 20 μM NAD. As shown in Fig. 1C, the enzymes with the substitution at Trp^{18} showed essentially the same dose-activity relationship as the wild type, indicating that these substitutions did not affect the activity. In contrast, a conservative substitution of Glu^{173} to Asp caused a decrease in the catalytic activity (>10 fold) under the same assay conditions. Furthermore, ADP-ribosylation was not detected with up to 1000 ng of the E173Q mutant C3 exoenzyme, indicating that this mutant showed a substantial reduction (>10000 fold) in catalytic activity. These results indicate that Glu^{173} forms a critical site within the active center of the enzyme.

We next determined whether the mutant exoenzymes showed a change in their binding affinity for NAD. To test this, the ADP-ribosylation reaction was performed using various concentrations of NAD, and the affinities of the enzymes for NAD were determined. As shown in Fig. 1D, the enzymes substituted at Trp^{18} demonstrated lower K_m values than the wild type enzyme, indicating that the Trp^{18} substitution did not adversely alter the NAD binding. In contrast, the Glu^{173} substitution with Asp reduced the affinity for NAD about 88 fold, and little ADP-ribosylation activity was observed with the E173Q mutant with up to 50 μM NAD. The K_m values for NAD calculated from double-reciprocal plots of the wild type and the W18G, W18A and E173D mutants of the C3 exoenzymes were 0.125 μM, 0.098 μM, 0.096 μM and 11.1 μM, respectively. These results demonstrate that Glu^{173} plays a key role in the binding with NAD.

C3 exoenzyme has been widely used in many systems to examine the involvement of *rho p21* and its functions. Although the enzyme specifically ADP-ribosylates *rho p21* and the observed effect is then presumably due to an inhibition of the *rho p21*-mediated function, these experiments sometimes lack a proper control. Therefore, to test if the reported phenotypic changes induced by the C3 exoenzyme treatment were due to the ADP-ribosyltransferase activity of the enzyme, we examined the effects of the C3 exoenzyme mutants on cultured cells. Swiss 3T3 cells were treated with 30 μg/ml of the wild type or mutant C3 exoenzymes for 72 h. Fig. 2A shows the [^{32}P]ADP-ribosylation reaction of lysates from control and C3 exoenzyme-treated cells. [^{32}P]ADP-ribosylation of *rho p21* in cells incubated with the wild type, W18G, W18A, E173D and E173Q mutants were 19, 22, 25, 105 and 107% of the control cells, respectively. This indicates that the exoenzymes with the substitution at Trp^{18} did not display any significant change in their ADP-ribosylation activity in situ in these cells, whereas the exoenzymes with the replacement at Glu^{173} showed no enzymatic activity in situ, which was consistent with the results obtained by the in vitro experiments. Fig. 2B–E show the morphology of the Swiss 3T3 cells treated with the wild type and mutant C3 exoenzymes. As reported previously [12], cells treated with the wild type enzyme demonstrated a marked cell rounding, with beaded dendritic processes (Fig. 2C). The cells treated with the Trp^{18}-substituted C3 exoenzymes showed the same morphology as cells treated with the wild type C3 (Fig. 2D). In contrast, the cells incubated with the E173D or E173Q mutants did not show any of the phenotypic changes observed in cells treated with the wild type enzyme (Fig. 2E). Taken together, the results suggest that the ADP-ribosylation activity of the mutant enzymes are strongly correlated with the phenotypic changes in the 3T3 cells, indicating that the inhibition of *rho p21*-mediated signalling by ADP-ribosylation induces the specific morphological changes shown in Fig. 2C.

The present study has thus shown that the Glu^{173} residue plays a critical role in NAD binding and in both the catalytic and biological activities of the C3 exoenzyme. Previous studies by other groups have compared amino acid sequence of C3 exoenzyme with those of other mono ADP-ribosyltransferases and aligned this residue with Glu^{148} in diphtheria toxin, Glu^{553} in *Pseudomonas* exotoxin A, Glu^{112} in *E. coli* heat-labile enterotoxin and Glu^{240} in rabbit muscle ADP-ribosyltransferase [14,15]. Mutagenesis studies have revealed that all of these Glu residues are essential in the NAD binding and the ADP-

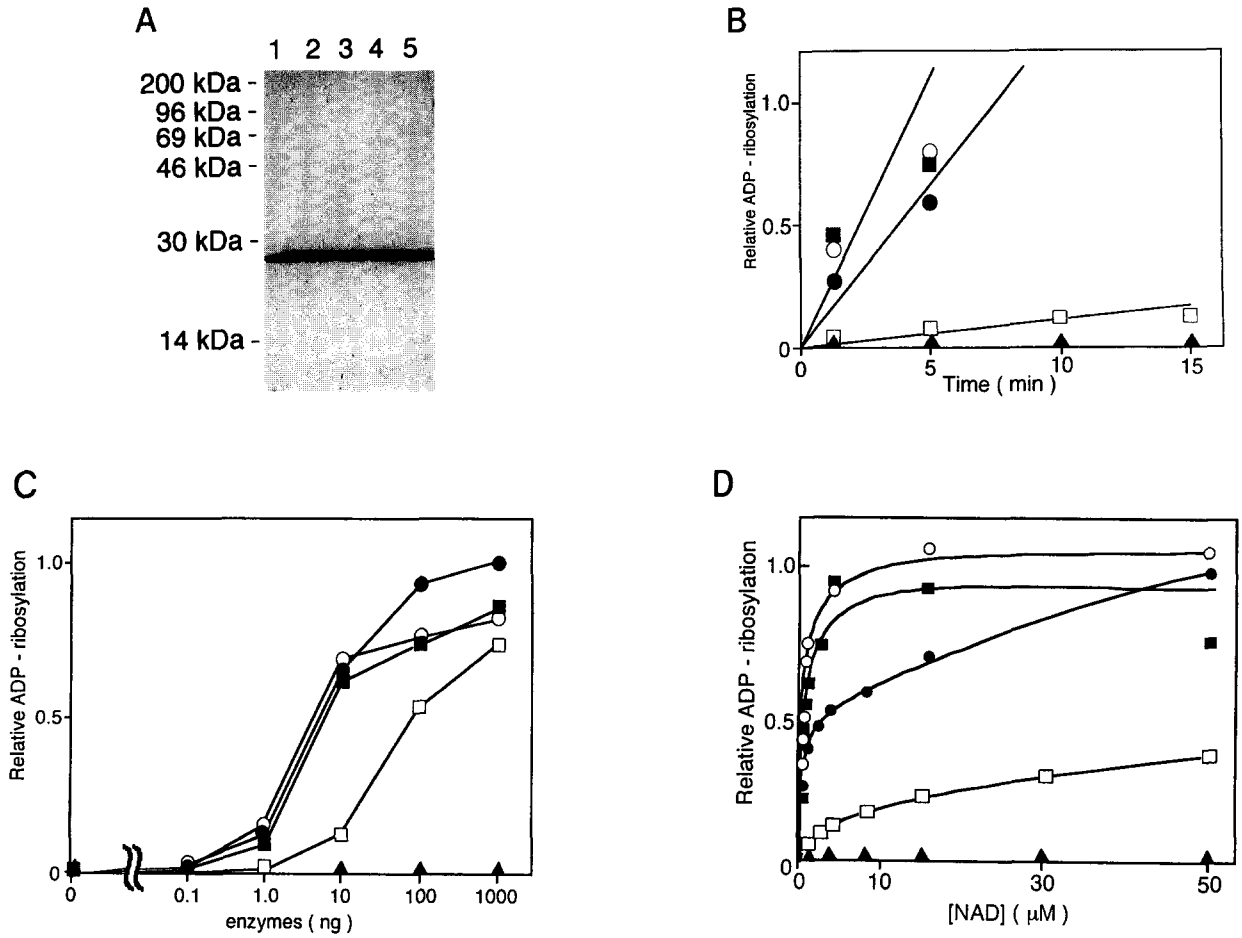

Fig. 1. (A) Purification of the wild type and mutant C3 exoenzymes. The wild type (lane 1) and the W18G (lane 2), W18A (lane 3), E173D (lane 4) and E173Q (lane 5) mutants of the C3 exoenzyme were expressed in *E. coli* and purified. Three μg of protein from each enzyme were subjected to SDS-PAGE on a 12% polyacrylamide gel, and then stained with Coomassie Brilliant blue. The positions of molecular weight markers are indicated on the left. (B) Time course of ADP-ribosylation with the wild type and mutant C3 exoenzymes. GST-rho p21 was incubated with 20 μM [^{32}P]NAD and with 3 ng of the wild type (●) or the W18G (■), W18A (○), E173D (□) or E173Q (▲) mutant in a total volume of 50 μl at 30°C for indicated times. ^{32}P incorporation was quantified by cutting out the GST-*rho p21* bands and determining their radioactivity. The relative ADP-ribosylation was expressed as a ratio of the ^{32}P incorporated into GST-*rho p21* at each point out of the maximal ^{32}P incorporation given by the wild type C3 exoenzyme in the reaction. (C) Dose–response curves for the ADP-ribosyltransferase activity of the C3 exoenzyme and its mutants. GST-*rho p21* was incubated with 20 μM [^{32}P] NAD and the indicated amounts of the wild type (●) or the W18G (■), W18A (○), E173D (□) or E173Q (▲) mutants of the C3 exoenzyme at 30°C. After 5 min, the reaction was terminated and the ADP-ribosylation of *rho p21* was analyzed. (D) Kinetic analysis of the wild type and mutant C3 exoenzymes. GST-*rho p21* was incubated with the indicated concentrations of [^{32}P]NAD and 3 ng of the wild type (●) or the W18G (■), W18A (○), E173D (□) or E173Q (▲) mutants of the C3 exoenzyme at 30°C for 5 min.

ribosyltransferase activity of the respective enzymes [15–19]. Recent X-ray crystallographic analyses of exotoxin A, heat-labile enterotoxin and diphtheria toxin have revealed a striking similarity in the structure of the enzymatic domains of these toxins, which form a prominent cleft presumably for NAD, and that the Glu residues noted above are located within this cleft [20–22]. In addition, several aromatic amino acid residues were located at or near this cleft, such as Tyr^{439}, Trp^{466} and Tyr^{470} in exotoxin A, Tyr^{6} in heat-labile enterotoxin, Tyr^{65} and Trp^{50} in diphtheria toxin. With their planar rings, these aromatic residues are presumed to provide a stacking interaction with the nicotinamide and adenine rings of NAD [19,23]. Indeed, the Tyr^{65} residue in diphtheria toxin was suggested as a binding site for the adenine ring of NAD by a photolabeling experiment using 8-azidoadenine [24]. By an experiment using a similar photoaffinity probe, 2-azidoNAD, we previously isolated a peptide corresponding to Phe^{9}-Gly^{19} near the N terminus as the adenine ring binding domain and suggested that Trp^{18} may be the critical residue [13]. However, on the contrary to our expectation, mutations of this residue did not alter the NAD binding nor the enzymatic activity of the C3 exoenzyme. These results suggested that if the above part of the protein does interact with the adenine ring, Trp^{18} does not play an essential role in NAD binding and catalysis.

C3 exoenzyme has been used to elucidate the role of *rho p21* in various cellular functions [5–12,24–29]. There have been, however, no reports using site-directed enzyme mutants to demonstrate the relationship between its ADP-ribosylation activity and the biological response. In this study, using the morphological change in Swiss 3T3 cells as an index of the response, we showed that mutant enzymes defective in enzyme activity could not induce the typical phenotype. These inactive enzymes

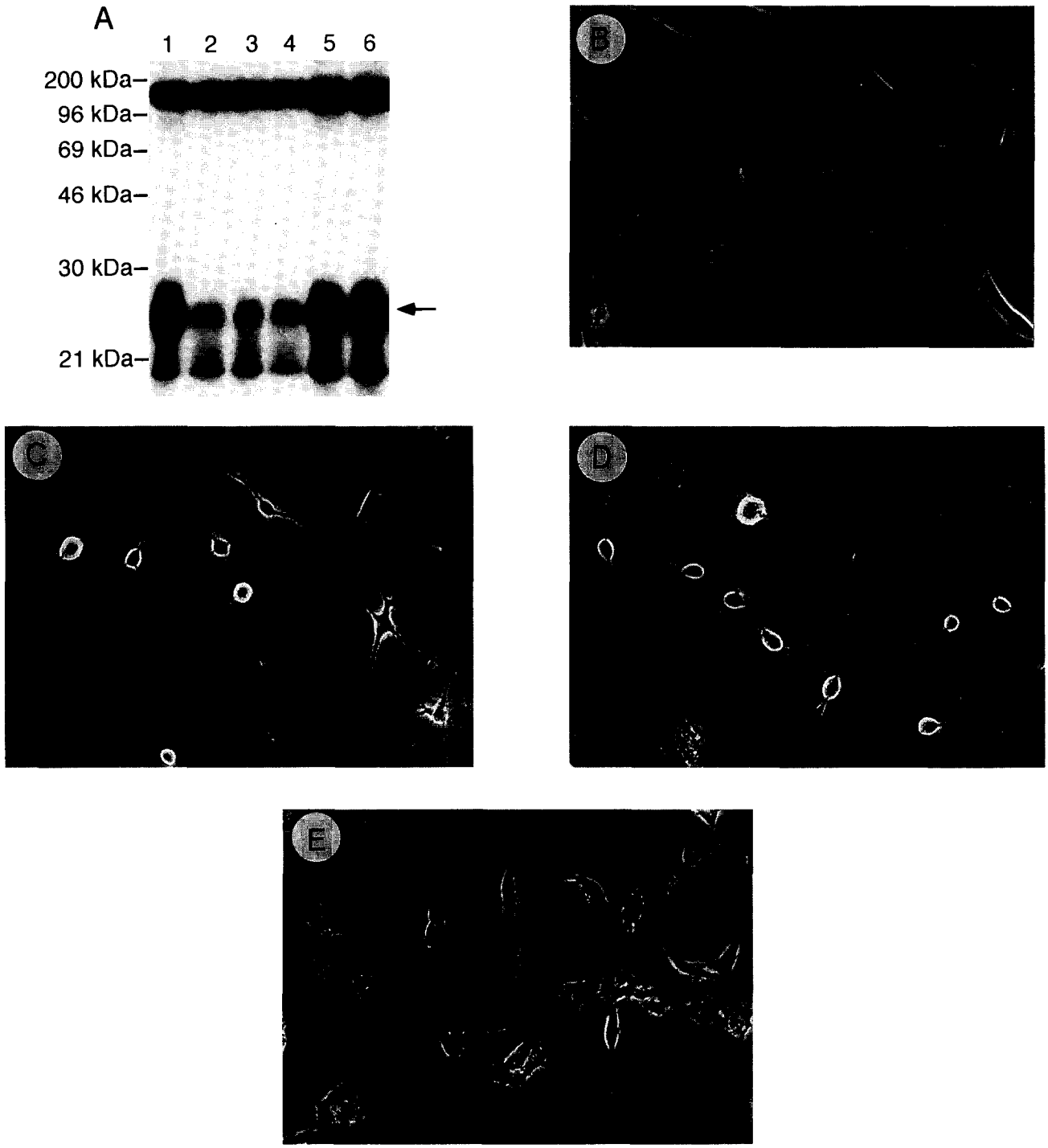

Fig. 2. Effects of the wild type and mutant C3 exoenzymes on Swiss 3T3 cells. A, autoradiogram of the [^{32}P]ADP-ribosylation of cell lysates. 3T3 cells were treated with buffer alone (lane 1), or with the wild type (lane 2), W18G (lane 3), W18A (lane 4), E173D (lane 5) or E173Q (lane 6) mutants of the C3 exoenzyme for 72 h. After treatment, cell lysates were prepared and incubated with 100 ng of the wild type C3 exoenzyme and 20 μM [^{32}P]NAD at 30°C for 2 h. The positions of the molecular weight markers are indicated on the left. The position of the ADP-ribosylated *rho-p21* is indicated by an arrow. (B–E) morphology of control cells (B), cells treated with the wild type (C), the W18G (D) and the E173Q (E) mutants of the C3 exoenzyme for 72 h. Cells treated with the W18A or the E173D mutants showed the same phenotypic change as with the W18G or E173Q mutants, respectively. Bar, 15 μm.

will be useful as controls in experiments using the C3 exoenzyme for investigating the function of *rho p21*-mediated signal transduction in many biological systems.

Acknowledgments: We thank Y. Kishimoto and K. Okuyama for their technical and secretarial assistance. This work was supported in part by Grants-in-aid for Scientific Research from the Ministry of Educa-

tion, Science and Culture of Japan (05271103, 06281231, 06275206 and 06670107), and by grants from the Human Frontier Science Program, the Senri Life Science Foundation and the Naito Memorial Foundation.

References

[1] Moss, J. and Vaughan, M. (eds) (1990) ADP-ribosylating Toxins and G-Proteins: Insights into Signal Transduction, American Society for Microbiology, Washington, DC.
[2] Weiss, A.A. and Hewlett, E.L. (1986) Annu. Rev. Microbiol. 40, 661–686.
[3] Nemoto, Y., Namba, T., Kozaki, S. and Narumiya, S. (1991) J. Biol. Chem. 266, 19312–19319.
[4] Sekine, A., Fujiwara, M. and Narumiya, S. (1989) J. Biol. Chem. 264, 8602–8605.
[5] Chardin, P., Boquet, P., Madule, P., Popoff, M.R., Rubin, E.J. and Gill, D.M. (1989) EMBO J. 8, 1087–1092.
[6] Nishiki, T., Narumiya, S., Morii, N., Yamamoto, N., Fujiwara, M., Kamata, Y., Sakaguchi, G. and Kozaki, J. (1990) Biochem. Biophys. Res. Commun. 167, 265–272.
[7] Paterson, H.F., Self, H.J., Garrett, M.D., Just, I., Aktories, K. and Hall, A. (1990) J. Cell. Biol. 111, 1001–1007.
[8] Ridley, A.J. and Hall, A. (1992) Cell 70, 389–399.
[9] Morii, N., Teruuchi, T., Tominaga, T., Kumagai, N., Kozaki, S., Ushikubi, F. and Narumiya, S. (1992) J. Biol. Chem. 267, 20921–20926.
[10] Tominaga, T., Sugie, K., Hirata, M., Morii, N., Fukata, J., Uchida, A., Imura, H. and Narumiya, S. (1993) J. Cell. Biol. 120, 1529–1537.
[11] Mabuchi, I., Hamaguchi, y., Fujimoto, H., Morii, N., Mishima, M. and Narumiya, S. (1993) Zygotes 1, 325–331.
[12] Morii, N. and Narumiya, S. (1995) Methods Enzymol. 256, 196–206.
[13] Chavan, A.J., Nemoto, Y., Narumiya, S., Kozaki, S. and Haley, B.E. (1992) J. Biol. Chem. 267, 14866–14870.
[14] Jung, M., Just, I., Damme, J., Vandekerckhove, J. and Aktories, K. (1993) J. Biol. Chem. 268, 23215–23218.
[15] Takada, T., Iida, K. and Moss, J. (1995) J. Biol. Chem. 270, 541–544.
[16] Tweten, R.K., Barbieri, J.T. and Collier, R.J. (1985) J. Biol. Chem., 260, 10392–10394.
[17] Carrol, S.F. and Collier, R.J. (1987) J. Biol. Chem. 262, 8707–8711.
[18] Tsuji, T., Inoue, T., Miyama, A., Okamoto, K., Honda, T. and Miwatani, T. (1990) J. Biol. Chem. 265, 22520–22525.
[19] Collier, R.J. (1989) in: ADP-Ribose Transfer Reactions (ed. Jacobson, M.K. and Jacobson, E.L.), pp. 458–462, Springer Verlag, New York.
[20] Allured, V.S., Collier, R.J., Carrol, S.F. and MaKay, D.B. (1986) Proc. Natl. Acad. Sci. USA 83, 1320–1324.
[21] Sixma, T.K., Pronk, S.E., Kalk, K.H., Wartna, E.S., Zanten, B.A.M., Witholt, B. and Hol, W.G.J. (1991) Nature 351, 371–377.
[22] Choe, S., Bennet, M.J., Fujii, G., Curimi, P.M.G., Kantardjieff, K.A., Collier, R.J. and Eisenbberg, D. (1992) Nature 357, 216–222.
[23] Domenighini, M., Montecucco, C., Ripka, W.C. and Rappuoli, R. (1991) Mol. Microbiol. 5, 23–31.
[24] Kumagai, N., Morii, N., Fujisawa, K., Nemoto, Y. and Narumiya, S. (1993) J. Biol. Chem. 268, 24535–24538.
[25] Rankin, S., Morii, N., Narumiya, S. and Rozengurt, E. (1994) FEBS Lett. 354, 315–319.
[26] Hirata, K., Kikuchi, A., Sasaki, T., Kuroda, S., Kaibuchi, K., Matsuura, Y., Seki, H., Saida, K. and Takai, Y. (1992) J. Biol. Chem. 267, 20921–20926.
[27] Yamamoto, M., Marui, N., Sakai, T., Morii, N., Kozaki, S., Ikai, K., Imamura, S. and Narumiya, S. (1993) Oncogene 8, 1449–1455.
[28] Kishi, K., Sasaki, T., Kuroda, S., Itoh, T. and Takai, Y. (1993) J. Cell. Biol. 120, 1187–1195.
[29] Jalink, K., Corven, E.J., Hengeveld, T., Morii, N., Narumiya, S. and Moolenaar, W.H. (1994) J. Cell. Biol. 129, 801–810.