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Bordetella bronchiseptica dermonecrotizing toxin stimulates assembly of actin stress fibers and focal adhesions by modifying the small GTP-binding protein rho

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SUMMARY

We studied the biochemical mechanism of morphological changes in cells treated with *Bordetella* dermonecrotizing toxin (DNT). DNT caused the morphological changes of serum-starved MC3T3-E1 cells from flat shapes to reflactile ones. These changes were accompanied by the assembly of actin stress fibers and focal adhesions, which is known to be regulated by the small GTP-binding protein rho. *Clostridium botulinum* C3 exoenzyme, which ADP-ribosylates and inactivates rho protein, 'rounded' the cells within 2 hours after addition to the extracellular fluid and their rounded shapes were maintained for at least 10 hours. However, when the cells were co-treated with C3 exoenzyme and DNT, they were rounded at 2 hours but recovered an apparently intact morphology after 3-8 hours of incubation. rho proteins in lysates from DNT-treated cells and untreated cells were radiolabeled by [^{32}P]ADP-ribosylation with C3 exoenzyme and analyzed by SDS-polyacrylamide gel electrophoresis. Whereas the lysate from untreated cells showed a single band of [^{32}P]ADP-ribosylated rho protein, the lysate from DNT-treated cells showed an additional two bands as well as the band identical to that of the lysate from untreated cells. Recombinant rhoA protein treated with DNT in vitro also showed a mobility shift in SDS-polyacrylamide gel electrophoresis. These results indicate that DNT causes the assembly of actin stress fibers and focal adhesions by directly modifying rho protein.

Key words: rho, *Bordetella*, toxin, focal adhesion, actin fiber

INTRODUCTION

Bordetella species such as *Bordetella pertussis*, *B. parapertussis* and *B. bronchiseptica* produce DNTs with similar biological and immunological properties (Bruckner and Evans, 1939; Evans, 1940; Wardlaw and Parton, 1983). The name DNT is derived from its ability to produce dermonecrotic lesions when injected intradermally into guinea pigs, mice, rabbits and other animals (Bruckner and Evans, 1939; Evans, 1940; Horiguchi et al., 1989, 1990; Wardlaw and Parton, 1983). DNT also has lethal, vasoconstrictive and splenoatrophic properties (Endoh et al., 1988; Horiguchi et al., 1989, 1990, 1992; Iida and Okonogi, 1971). *B. bronchiseptica* DNT is considered to be one of the virulence factors responsible for turbinate atrophy in swine atrophic rhinitis and has been suggested to damage bone tissues in the turbinates (Ackermann et al., 1991; Duncan et al., 1966; Elias et al., 1990; Hanada et al., 1979; Kimman et al., 1987). We have analyzed the action of DNT on osteogenic cells, using osteoblast-like MC3T3-E1 cells, and have demonstrated that DNT changes the morphology of the cells (Horiguchi et al., 1991), inhibits their differentiation into osteoblasts (Horiguchiet al., 1991), and stimulates protein and DNA syntheses (Horiguchi et al., 1993, 1994). The cytokinesis is probably blocked by DNT because polynucleated cells accumulate after treatment with DNT (Horiguchiet al., 1993). The morphological changes and the blocked cytokinesis in DNT-treated cells imply that DNT affects the cytoskeletal system.

In the regulation of cytoskeletal systems, the role of rho protein, a member of the family of small GTP-binding proteins, has recently received intensive attention. Microinjection of an active mutant of rho protein, rho^{Val-14} (Garrett et al., 1989), into fibroblasts changes the cell morphology to contractile shapes with finger-like processes (Paterson et al., 1990). The microinjected cells showed assembly of actin stress fibers and focal adhesions in fluorescence microscopy (Ridley and Hall, 1992). When *Clostridium botulinum* C3 exoenzyme (C3), which ADP-ribosylates and inactivates rho protein (Chardin et al., 1989; Morii et al., 1988; Narumiya et al., 1988; Ridley and Hall, 1992; Rubin et al., 1988), was introduced to cells, the cells lost their actin stress fibers and focal adhesions, and rounded up (Chardin et al., 1989; Miura et al., 1993; Ridley

and Hall, 1992; Rubin et al., 1988). These studies have revealed that rho protein is involved in the assembly of stress fibers and focal adhesions. Furthermore, it was demonstrated that rho protein mediates a variety of cell processes, such as cell adhesion (Morii et al., 1992; Nemoto et al., 1992; Tominaga et al., 1993), smooth muscle contraction (Hirata et al., 1992), G_1 to S phase progression in the cell cycle (Yamamoto et al., 1993), cytokinesis (Kishi et al., 1993; Rubin et al., 1988), cell motility (Stasia et al., 1991; Takaishi et al., 1993), and expression of the phenotype of differentiation in PC12 cells (Nishiki et al., 1990).

While investigating the effects of DNT on MC3T3-E1 cells, we found that the changes in cell morphology induced by DNT resembled those in Swiss 3T3 cells into which $rho^{Val\text{-}14}$ protein had been microinjected (Horiguchi et al., 1991; Paterson et al., 1990). DNT also affected cell functions that are considered to be regulated by rho protein, e.g. smooth muscle contraction (Endoh et al., 1988; Hirata et al., 1992), G_1 to S phase progression (DNA synthesis) (Horiguchi et al., 1993; Yamamoto et al., 1993), and cytokinesis (Horiguchi et al., 1993; Kishi et al., 1993; Rubin et al., 1988). Thus, we investigated whether rho protein is involved in the action of DNT. In this paper, we show several lines of evidence that DNT directly acts on rho protein and stimulates the rho-dependent signaling pathway that regulates the assembly of actin stress fibers and focal adhesions, leading to changes in the morphology of the cells.

MATERIALS AND METHODS

Bacterial toxins, proteins and chemicals

DNT was purified from the cell extracts of *B. bronchiseptica* S798 by the method reported previously (Horiguchi et al., 1990). C3 and component I of botulinum C2 toxin were provided by S. Kozaki, University of Osaka Prefecture, Osaka, Japan. Bovine recombinant rhoA protein was provided by Y. Nemoto, Biomedical Education and Research Center, Osaka University, Osaka, Japan. The purity of these proteins was confirmed by SDS-polyacrylamide gel electrophoresis (PAGE) with silver staining. The protein contents of test materials used in this study were determined by the methods of Lowry et al. (1951) or Bradford (1976). Guanine nucleotides and cytochalasin D were purchased from the Sigma Chemical Co., St Louis, MO. Cycloheximide was obtained from Nacalai Tesque, Kyoto, Japan.

Cell culture

MC3T3-E1 cells (Sudo et al., 1983) were cultivated in α-minimum essential medium (α-MEM, GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (Flow Laboratories, Inc., McLean, VA) at 37°C under 5% CO_2 in air. The cells were subcultured every 3 days at a dilution of 1:10 and used for experiments within 15 passages. Swiss 3T3 cells were provided by H. Inoue, Department of Tumor Virology, Research Institute for Microbial Diseases, Osaka University. C3H10T1/2, Balb/3T3, and rat embryo fibroblasts were provided by G. Kondoh, Genome Information Research Center, Osaka University, Osaka, Japan. They were maintained in Dulbecco's modified Eagle's medium.

Phase-contrast microscopy

Cells were seeded at an initial density of 2.5×10^4 cells/cm^2 into wells of 24-well plates and grown for one day. After washing the cells with Dulbecco's PBS prewarmed at 37°C, the medium was replaced with serum-free α-MEM that had been equilibrated at 37°C under 5% CO_2 in air overnight. Immediately after the replacement of the medium, C3, DNT and/or other reagents were added to the culture. This procedure made C3 fully effective on intact cells, although it was known to work inefficiently when added extracellularly. The cells were incubated for appropriate periods and examined under a phase-contrast microscope. For determination of the number of rounded cells, the cells were washed with PBS, fixed with methanol, and then stained with Giemsa solution. The rounded and unrounded cells were counted randomly to a total number of 500 cells, and the percentage of rounded cells was calculated.

Fluorescent labeling of cytoskeletal proteins

MC3T3-E1 cells were seeded at an initial density of 0.5×10^4 to 2.5×10^4 cells/cm^2 into 24-well plates wells, in each of which a 10-mm square glass coverslip was placed, and then incubated for 2 days. The cells were washed and further incubated in the serum-free medium for 24 hours. DNT was added to the culture and the cells were incubated for the indicated periods, fixed with 3% paraformaldehyde in PBS for 10 minutes, washed three times with PBS, and permeabilized with PBS containing 0.5% Triton X-100 for 5 minutes. They were incubated with the appropriately diluted mouse monoclonal anti-vinculin (clone VIN-11-5, Sigma) or anti-β-tubulin (clone TUB 2.1, Sigma) for 1 hour at room temperature. After three washes with PBS, the cells were incubated with diluted fluorescein-5-isothiocyanate-labeled anti-mouse IgG (ORGANON TEKNIKA Co., Durham, NC) and/or rhodamine-labeled phalloidin (Molecular Probes Inc., Eugene, OR) for 1 hour at room temperature. The cells were washed with PBS, mounted in 90% glycerol containing 1 mg/ml *p*-phenylenediamine, and photographed under a fluorescence microscope.

Filamentous actin (F-actin) measurement

The F-actin contents of the cells were measured by the method previously reported (Ha and Exton, 1993) with a slight modification. MC3T3-E1 cells were seeded at a density of 5.0×10^4 cells/cm^2 to 24-well plates and incubated for 24 hours. After further incubation in α-MEM without serum, the cells were incubated with DNT for the indicated periods. The cells, fixed and permeabilized as described above, were reacted with 5 units/ml of rhodamine-phalloidin for 1 hour at room temperature. After three washes with PBS, the bound rhodamine-phalloidin was extracted with 1.5 ml/well of methanol for 1 hour. The fluorescence intensity was measured with a fluorescence spectrophotometer with an excitation wavelength of 550 nm and an emission wavelength of 580 nm. The results were expressed as the ratio of the fluorescence intensity of DNT-treated cells to that of untreated cells.

ADP-ribosylation

MC3T3-E1 cells were seeded at an initial density of 2.5×10^4 cells/cm^2 into a 60-mm culture dish, incubated for 24 hours, and then treated with the test materials as described in the section on phase-contrast microscopy, above. After incubation for the indicated periods, the cells were washed with PBS and scraped with a rubber policeman in 1 ml of 10 mM sodium phosphate buffer, pH 8.5. The cells were treated by sonication and the whole homogenates were used. Ten micrograms of protein of the homogenates were incubated with 100 ng of C3 for 45 minutes at 30°C in 50 μl of reaction mixture containing 100 mM Tris-HCl buffer, pH 7.6, 10 mM thymidine, 10 mM DTT, 10 mM nicotinamide, 5 mM $MgCl_2$ and 10 μM [^{32}P]NAD (92.5 kBq, Amersham International, Amersham, UK). Five microliters of 100% (w/v) trichloroacetic acid was added to the reaction mixture. The precipitates obtained by centrifugation were washed with ice-cold ethylether, solubilized in 67.5 mM Tris-HCl, pH 6.8, containing 1% SDS, 25 mM DTT and 20% glycerol, and subjected to SDS-PAGE according to the method of Laemmli (1970). Radioactive bands were visualized by autoradiography with Fuji RX film (Fuji Film Co., Tokyo, Japan) or an imaging plate (Fuji Film) and analyzed with Fuji BAS 2000 image analyzer (Fuji Film).

In vitro treatment of recombinant rhoA protein with DNT

Recombinant rhoA protein was mixed with DNT at a molar ratio of 20:1 in 20 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 1 mM DTT and 1 mM EDTA. The mixture was incubated for 2 hours at 37°C in the presence or absence of 1 mM guanine nucleotides. The incubation was terminated by addition of 100% trichloroacetic acid to a final concentration of 10%. The precipitate obtained by centrifugation was washed with ice-cold ethylether and subjected to SDS-PAGE on a 12.5% gel. After electrophoresis, the proteins in the gel were stained with Silver Stain II Kit Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

RESULTS

Morphological changes in cells treated with DNT

Fig. 1A,B shows the morphological changes in MC3T3-E1 cells under serum-starved conditions, from flat to refractile shapes, after treatment with 5 ng/ml (approximately 33 pM) of DNT. The morphological changes in the serum-starved cells were characteristically similar to those we reported previously in cells in the logarithmic growth phase. Swiss 3T3, C3H10T1/2 and rat embryo fibroblasts showed similar changes in morphology after treatment with DNT at the same concentration. The range of DNT doses (over 0.01 ng/ml) that caused the morphological changes was almost the same as that necessary to elicit other effects of DNT, e.g. inhibition of osteoblastic differentiation (Horiguchi et al., 1991), stimulation of DNA and protein syntheses (Horiguchi et al., 1993, 1994). NIH3T3, Balb/3T3 (Fig. 1C,D), Vero, HeLa and MDCK cells did not show morphological changes after treatment with DNT to the extent of 1 μg/ml (about 7 nM).

Effects of DNT on the cytoskeleton

To elucidate the underlying mechanism of DNT-induced morphological changes, we stained actin stress fibers and microtubles with rhodamine-phalloidin and anti-β-tubulin, respectively. Serum-starved MC3T3-E1 cells showed a few thin actin fibers (Fig. 2A). Treatment of the cells with DNT for 24 hours induced the formation of dense thick actin fibers (Fig. 2B). Although the microtubles in DNT-treated cells appeared to be more densely distributed than those in untreated cells, no obvious abnormalities were found in the constitution of microtubles (Fig. 2C,D).

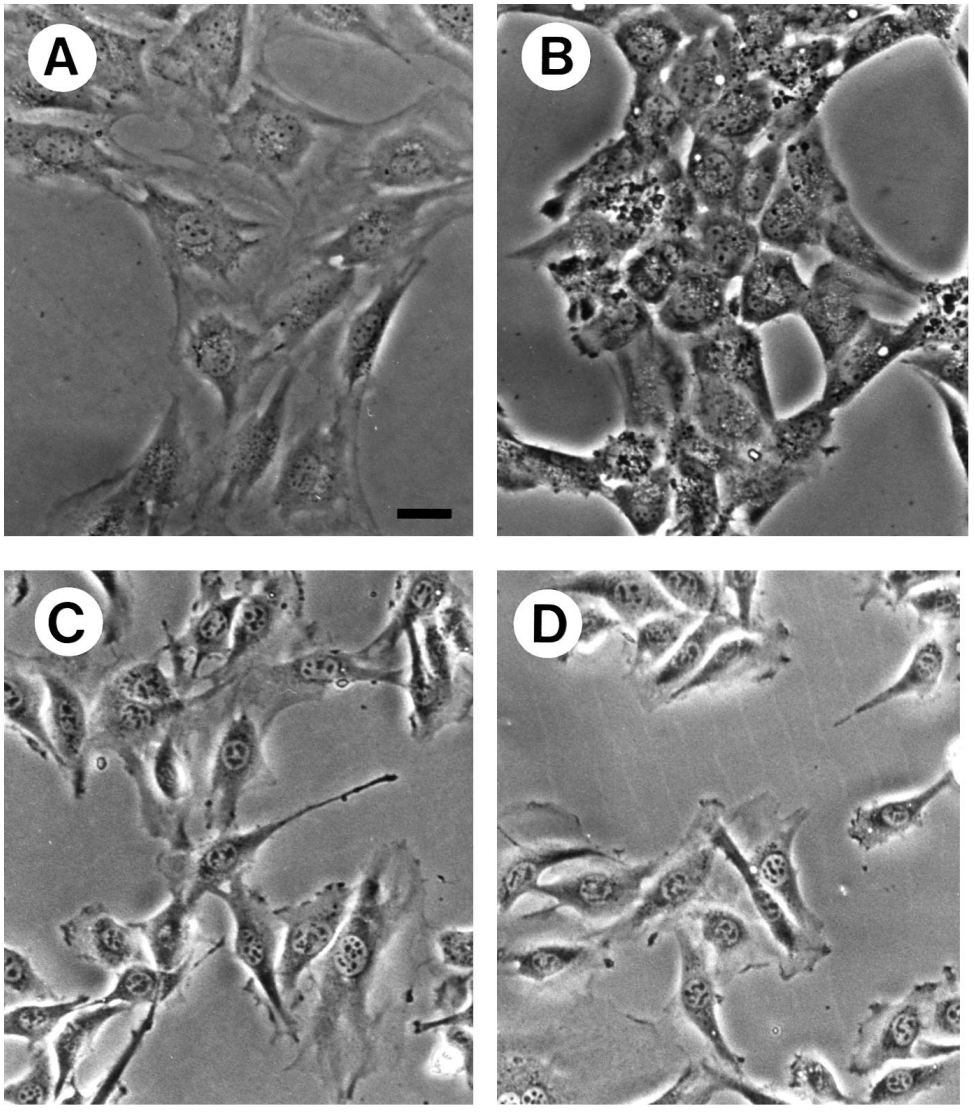

Fig. 1. Phase-contrast micrographs of MC3T3-E1 (A and B) and Balb/3T3 (C and D) cells. The cells were incubated in the absence (A and C) or presence (B and D) of DNT (5 ng/ml) for 24 hours under serum-starved conditions as described in Materials and Methods. Bar, 15 μm.

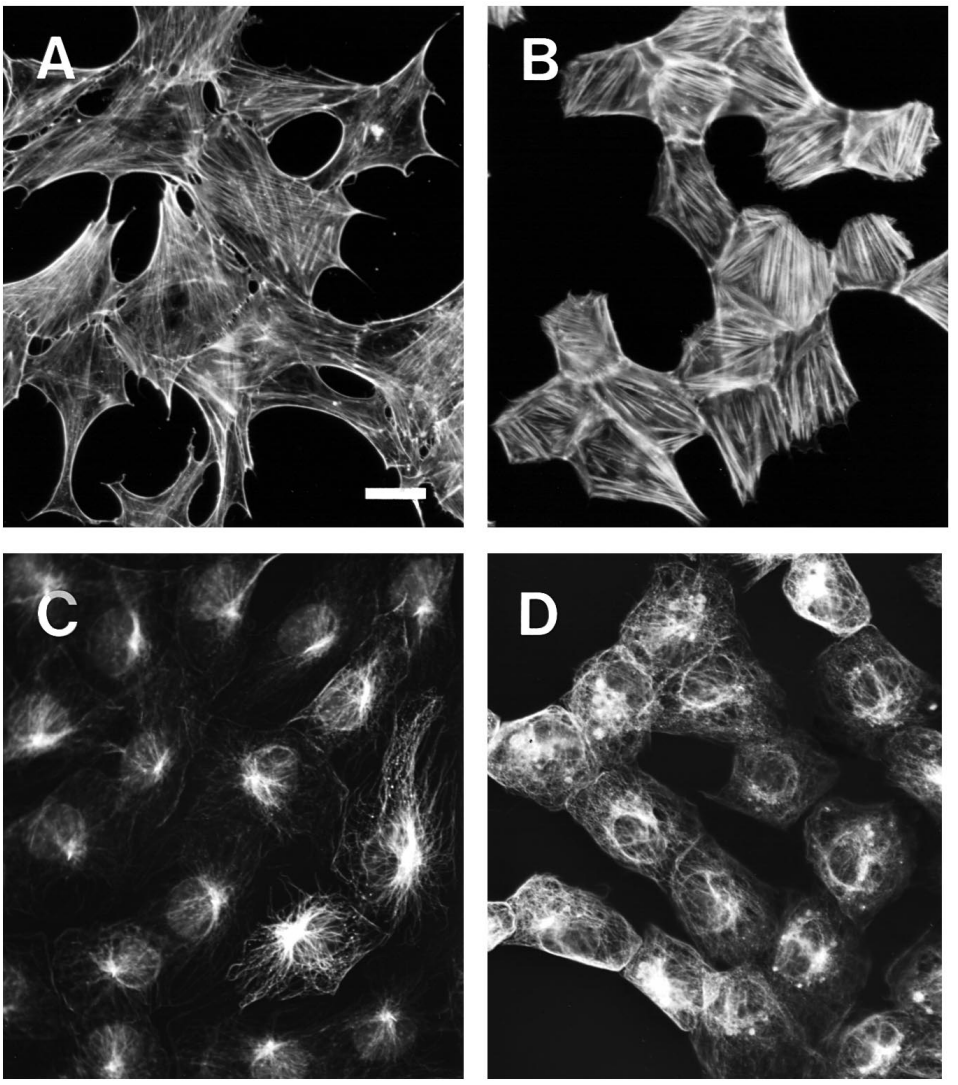

Fig. 2. Actin fibers and microtubles in MC3T3-E1 cells. The cells were incubated in the absence (A and C) or presence(B and D) of DNT at 5 ng/ml for 24 hours, fixed, and stained as described in Materials and Methods. (A and B) Actin fibers stained with rhodamine-labeled phalloidin; (C and D) microtubles stained with anti-β-tubulin and fluorescein-labeled anti-mouse IgG. Bar, 15 μm.

The cellular F-actin contents increased by treatment of the cells with DNT at 0.5 and 5 ng/ml for more than 3 hours and reached a maximum at 10 hours (Fig. 3). The cells treated with DNT at 50 pg/ml also showed an increase in F-actin contents at a similar time of incubation, but the increase was slower than that produced by treatment with higher concentrations of the toxin. Fig. 4 shows that DNT stimulates the formation of focal adhesions. Many focal adhesions were observed at the ends of newly formed actin fibers in DNT-treated cells (Fig. 4B,D), in contrast to untreated cells in which a few vinculin-containing focal adhesions were found at the ends of rare, thin stress fibers (Fig. 4A,C). The formation of actin stress fibers and focal adhesions was induced by DNT even in cells in which protein synthesis was completely blocked with 5 μM cycloheximide (data not shown), indicating that these effects of DNT do not require newly synthesized proteins.

Recovery from C3-induced cell rounding by adding DNT

C3, which ADP-ribosylates and inactivates rho protein, causes disassembly of actin stress fibers and when it is introduced into cells they become rounded. To examine whether C3 interferes with the effects of DNT, we co-treated MC3T3-E1 cells with DNT and C3. Several investigators reported that C3 added extracellularly had no obvious effects on intact cells, and therefore they delivered it into the cells by osmotic lysis of pinosomes or microinjection. We found, however, that C3 that was applied to the extracellular medium as described in Materials and Methods was fully effective. Fig. 5A,B shows that C3 (10 μg/ml) altered the cell morphology from a flat to a refractile rounded shape within 2 hours after addition. This alteration was maintained for at least 10 hours (Fig. 6). When the cells were incubated with DNT and C3 at 5 ng/ml and 10 μg/ml, respectively, they first rounded up but recovered their original flat shape after 6 hours (Fig. 5C,D,E and Fig. 6). By fluorescence microscopy, actin stress fibers were observed in cells that had recovered their flat morphology but were not seen in rounded cells (data not shown). After recovery from C3-induced rounding, the cell morphology was further changed to that characteristic of DNT-treated cells as shown in Fig. 1B. The time required for complete recovery was prolonged by decreasing the amount of DNT (Fig. 6). Such a recovery effect of DNT was not observed in MC3T3-E1 cells rounded by treatment with 1 μM cytochalasin D (Fig. 5F,G). The C3-

induced morphological changes in Balb/3T3 cells, which are resistant to DNT, were not restored by cotreatment with DNT (Fig. 5H,I).

Effects of DNT on rho protein

We examined whether DNT affects the ADP-ribosylation activity of C3. When lysate from untreated cells was incubated with C3 in the presence of [^{32}P]NAD, a single [^{32}P]ADP-ribosylated band appeared with an apparent molecular mass of 23 kDa (Fig. 7, lane 3), the position corresponding to the rho protein, as reported (Chardin et al., 1989; Morii et al., 1988; Rubin et al., 1988). The lysate of DNT-treated cells showed two bands of proteins [^{32}P]ADP-ribosylated by C3; one migrated to the position of rho protein in the lysate of control cells and the other to a lower position (lane 7). The amount of [^{32}P]ADP-ribosylated proteins in the lysates from both DNT-treated and untreated cells was decreased by treatment of the cells with C3 (lanes 5 and 6), indicating that the rho proteins had undergone ADP-ribosylation in the cells. DNT had no ability to [^{32}P]ADP-ribosylate any proteins in vitro (Fig. 7, lane 2). DNT added to the cell lysate did not apparently influence [^{32}P]ADP-ribosylation by C3 (lane 4).

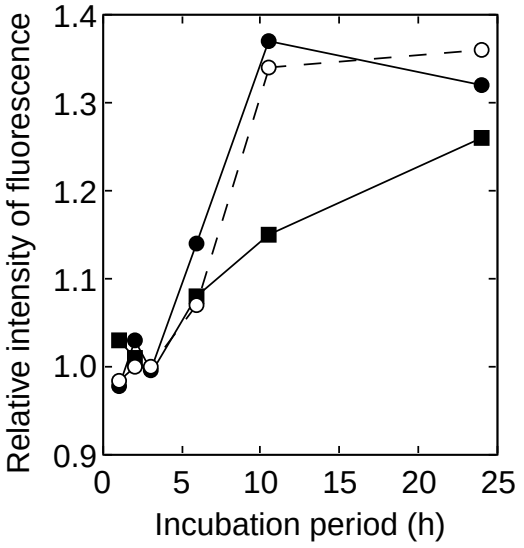

Fig. 3. Time course of changes in F-actin content. MC3T3-E1 cells were incubated with DNT at 0.05 (■), 0.5 (○), and 5 (●) ng/ml for the times indicated. F-actin contents were measured and expressed as described in Materials and Methods. The experiments were carried out independently three times and the representative data are shown.

The time course of the mobility shift of [^{32}P]ADP-ribosylated proteins in SDS-PAGE is shown in Fig. 8. The cells were incubated with DNT for the indicated periods, homogenized, and subjected to [^{32}P]ADP ribosylation by C3. The additional band appeared at a lower position than the original one after 2-3 hours of treatment of the cells with DNT. In addition, after 6-12 hours of treatment of the cells with DNT, a [^{32}P]ADP-ribosylated protein appeared at a higher position than the original one. These additional bands appeared even in cells where protein synthesis was inhibited with 5 μM cycloheximide. The total radioactivity of [^{32}P]ADP-ribosylated protein bands was constant in the cell lysates, irrespective of the period of DNT treatment (data not shown). Therefore, these additional proteins are not newly synthesized but are derived from the rho protein that is present before treatment with DNT.

To examine whether DNT directly modifies rho protein, we incubated the recombinant rhoA protein with DNT at 37°C for 2 hours and subjected the mixture to SDS-PAGE. Upon

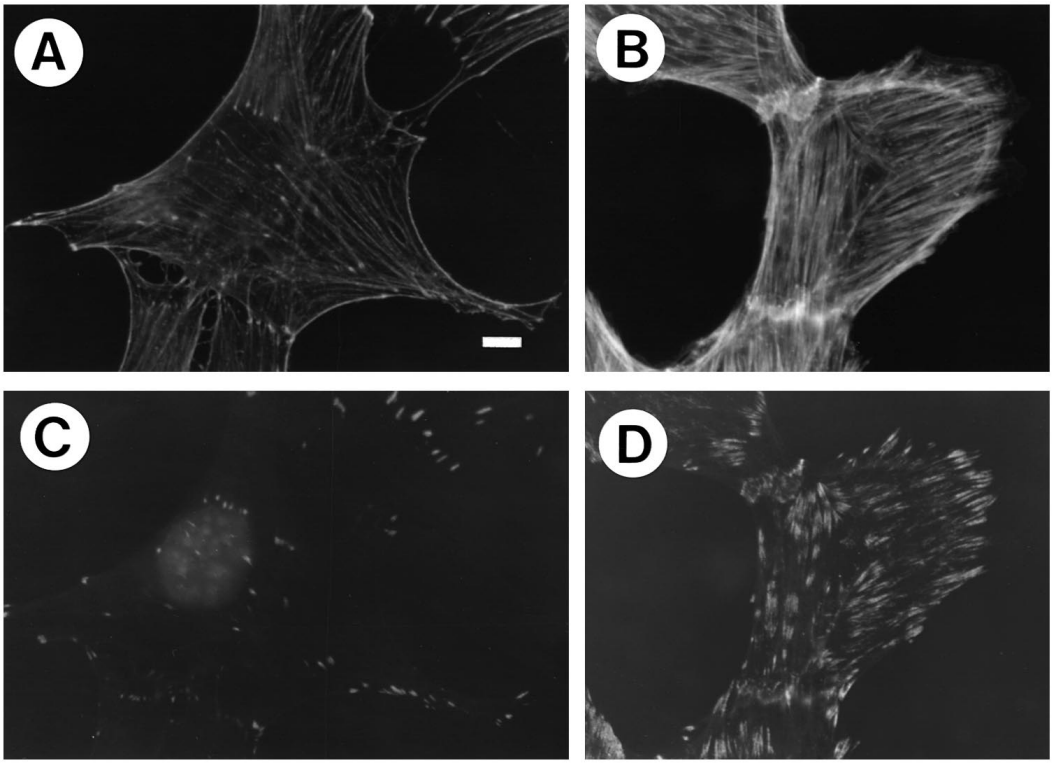

Fig. 4. Actin fiber and vinculin distribution in MC3T3-E1 cells. MC3T3-E1 cells were incubated in the absence (A and C) or presence (B and D) of DNT (5 ng/ml). Actin fiber (A and B) and vinculin (C and D) were visualized by staining with rhodamine-labeled phalloidin, and anti-vinculin and fluorescein-labeled anti-mouse IgG, respectively. Vinculin colocalizes with the ends of actin fibers. Bar, 5 μm.

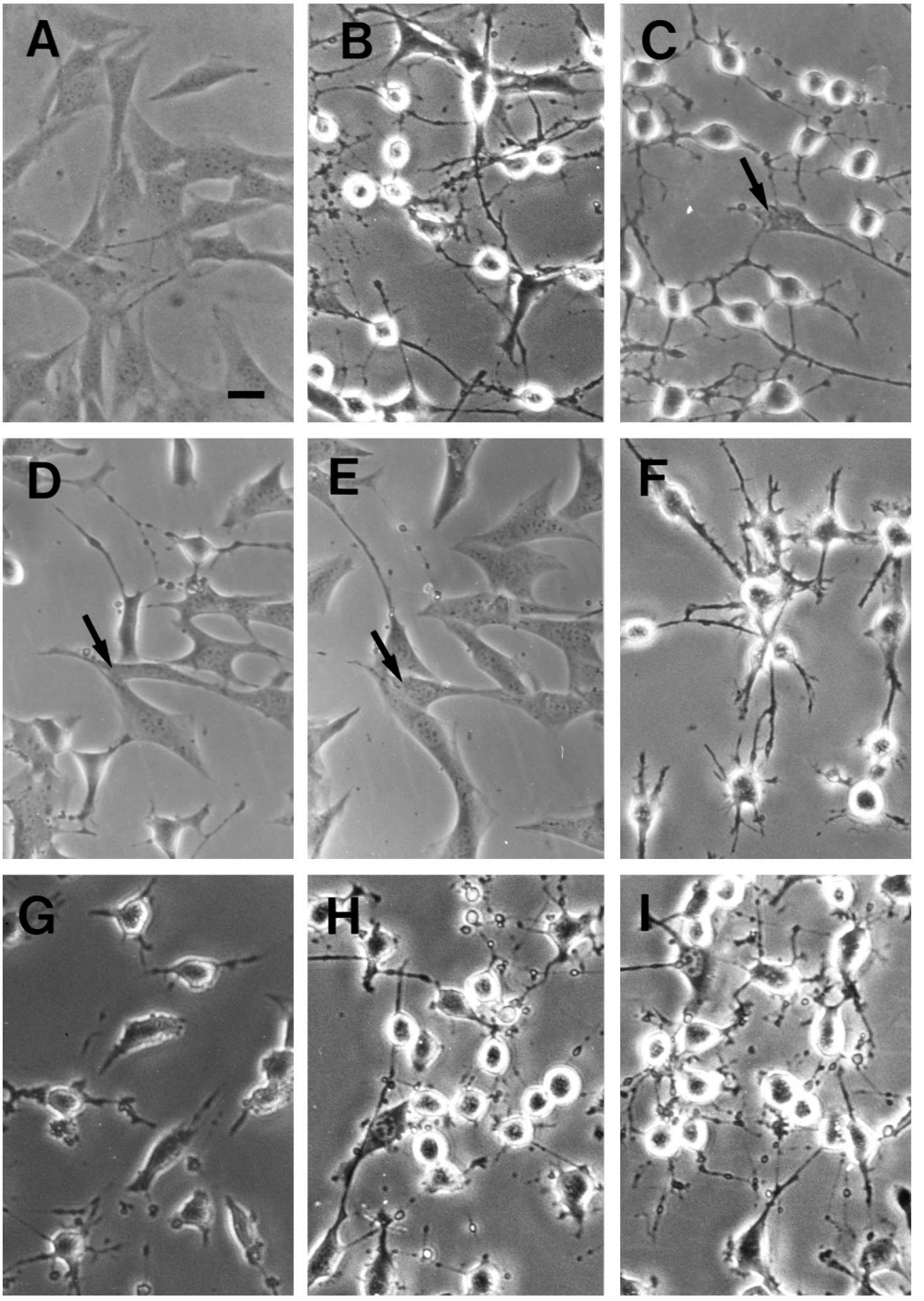

Fig. 5. Effects of DNT on the cell rounding induced by C3 and cytochalasin D. DNT (5 ng/ml) and C3 (10 μg/ml) or cytochalasin D (1 μM) were added to cell cultures immediately after replacement of α-MEM containing 10% FCS with serum-free α-MEM. (A) Untreated MC3T3-E1 cells. (B) MC3T3-E1 cells treated with C3 for 6 hours. (C,D and E) MC3T3-E1 cells treated with C3 and DNT for 2, 4 and 6 hours, respsectively. Each photograph shows an identical field of the culture. Arrows indicate identical cells. (F) MC3T3-E1 cells treated with cytochalasin D for 6 hours. (G) MC3T3-E1 cells treated with cytochalasin D and DNT for 6 hours. (H) Balb/3T3 cells treated with C3 for 6 hours. (I) Balb/3T3 cells treated with C3 and DNT. Bar, 15 μm.

treatment with DNT, the recombinant rhoA migrated to a higher position than that of untreated rhoA protein in SDS-PAGE. GTPγS, but not GMP, GDP, GDPβS or GTP, inhibits the DNT-induced mobility shift of the recombinant rhoA protein (Fig. 9). This inhibitory effect of GTPγS was observed at concentrations of 10 μM or higher.

DISCUSSION

rho protein, which consists of three members, rhoA, rhoB and rhoC (Chardin et al., 1988; Yeramian et al., 1987), belongs to a large family of low molecular mass GTP-binding proteins. The GTP-binding proteins including rho protein exist in two interconvertible forms: an inactive GDP-bound form and an active GTP-bound form (Bourne et al., 1991; Hall, 1990). In resting cells, these proteins are in the GDP-bound form. When sites where the GTP-binding proteins act are stimulated by an unknown mechanism, they are converted from the GDP-bound form to the GTP-bound form, and transfer a positive signal to downstream pathways.

In this study, we showed that DNT affects the rho protein-

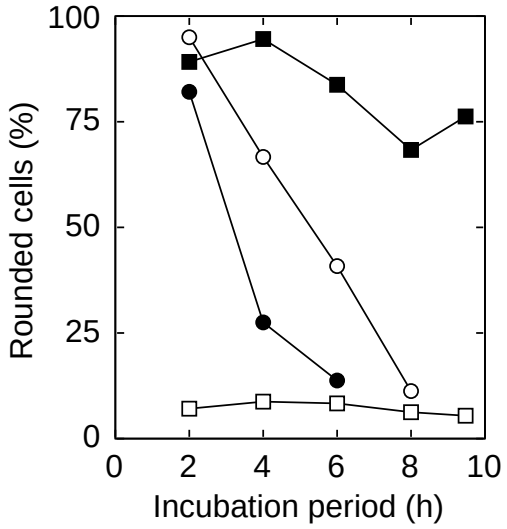

Fig. 6. Time course of recoveries of MC3T3-E1 cells from C3-induced cell rounding. MC3T3-E1 cells were incubated in the presence of 10 μg/ml of C3 (■), 10 μg/ml of C3 and 5 ng/ml of DNT (●), 10 μg/ml C3 and 0.5 ng/ml DNT (○), or in the absence of the test materials (□). The experiments were carried out independently three times and the representative data are shown.

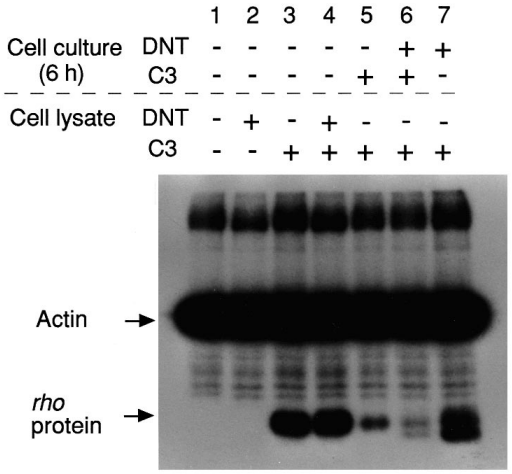

Fig. 7. ADP-ribosylation of MC3T3-E1 cell lysates. The cells were treated with 5 ng/ml DNT and/or 10 μg/ml C3 for 6 hours, and homogenized as described in Materials and Methods. The cell lysates were treated with C3, DNT, or both, in the presence of [^{32}P]NAD and subjected to SDS-PAGE. Actin was ADP-ribosylated with component I of botulinum C2 toxin as control.

dependent signal transduction pathway. DNT induces morphological changes in the cells, accompanied by intensive formation of stress fibers and focal adhesions, which is considered to involve rho protein. Many extracellular factors, such as lysophosphatidic acid, bombesin, PDGF and EGF, are also reported to stimulate the assembly of stress fibers and focal adhesions via a rho-dependent signal pathway (Ridley and Hall, 1992). These factors seem to affect the upper stream of this pathway because the responses of cells to these factors were completely inhibited by treatment of cells with C3. In contrast to these factors, DNT not only changed cells rounded

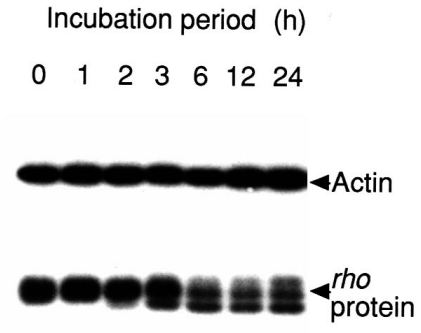

Fig. 8. Time courses of changes in mobility of ADP-ribosylated rho protein. MC3T3-E1 cells were incubated in the presence of 5 ng/ml DNT under serum-starved conditions for the indicated periods. They were homogenized and subjected to ADP-ribosylation by C3. Actin was ADP-ribosylated with component I of botulinum C2 toxin as control.

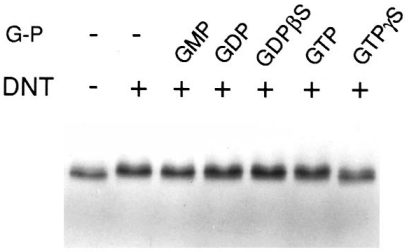

Fig. 9. Effects of DNT on mobility of recombinant rhoA protein in SDS-PAGE. Recombinant rhoA protein and DNT were mixed at a molar ratio of 20:1, incubated at 37°C for 2 hours in the presence or absence of gunanine nucleotides (G-P) at 1 mM, and subjected to SDS-PAGE.

by C3 to normal flat-shaped cells but further changed their morphology to the refractile one characteristic of DNT-treated cells. This recovery effect of DNT was not observed in Balb/3T3 cells, which are insensitive to DNT, excluding the possibility that DNT directly inactivates C3. The restoration of the cell morphology and an increase in F-actin occurred at similar times after DNT addition. This shows that the restoration of the cell morphology by DNT can probably be attributed to the reorganization of actin fibers. This is supported by the fact that the actin stress fibers were observed in the cells that had recovered from the rounded morphology but not in the cells that remained rounded. In contrast to the case of C3-treated cells, cytochalasin D-treated cells did not recover from the rounded morphology by cotreatment with DNT. Cytochalasin D acts as barbed-end capping protein and inhibits further elongation of the actin microfilaments. These results indicate that DNT acts on an intracellular target that exists on the signaling pathway from rho proteins to the F-actin elongation site and makes this pathway active, overcoming the inactivation of rho protein by C3. It is unlikely that DNT restores the cell morphology by inhibiting ADP-ribosylation by C3 or reversibly removing the ADP-ribose moiety from ADP-ribosylated rho proteins, because ADP ribosylation of rho protein by C3 was not diminished by DNT in vivo or in vitro. The [^{32}P]ADP-ribosylated rho protein in the lysate of DNT-treated cells appeared as three bands in SDS-PAGE, whereas that in

the lysate of untreated cells occurred as a single band: one appeared at the same position as the single band in the control lysate, and the other two bands were at slightly higher and lower positions. The appearance of the lower band was coincident with an increase in F-actin content and the recovery of the cells rounded by C3. We consider, therefore, that the rho protein at the lower position is probably involved in the stimulation of formation of actin stress fibers. The question remains to be answered as to why the treatment of cells with DNT yields two types of modified rho proteins that migrate as two distinct bands in SDS-PAGE. rho protein in cells was reported to be distributed to the cytosol and the membrane fractions (Nemoto et al., 1992). The rho proteins that are localized to different sites may have different sensitivities to DNT and may receive different modifications. We used purified recombinant rhoA protein and provide direct evidence of an interaction between DNT and rho protein. The recombinant rhoA protein, which is purified in the GDP-bound form, changed in its mobility in SDS-PAGE when it was incubated with DNT. GTPγS, a hydrolysis-stable analog of GTP, inhibited the mobility shift, indicating that the stable GTPγS-bound form of rhoA protein is possibly insensitive to DNT. The patterns of the mobility shift were somewhat different between the rho protein in MC3T3-E1 cells and the recombinant rhoA protein expressed in *Escherichia coli.* The rho protein in the cells was detected by [^{32}P]ADP-ribosylation by C3 whereas the recombinant rhoA protein was visualized by silver staining. These different methods for detection of rho proteins, however, do not cause the different patterns of the mobility shift, because the ADP-ribosylated recombinant rhoA protein, as well as the unribosylated one, showed a similar shift with DNT treatment (data not shown). rho protein in mammalian cells is post-translationally modified in the C-terminal region but the recombinant rhoA protein is not (Hori et al., 1991). To determine whether the different mobility shifts are attributable to the different structures between eukaryotic and procaryotic rho proteins, we are now preparing rho protein that is produced by baculoviral vectors and are modifying it with DNT.

The present results indicate that DNT induces the assembly of actin stress fibers and focal adhesions through rho-dependent signal transduction. Moreover, we show direct modification of rho protein by DNT, using purified preparations of both. We conclude that DNT directly modifies rho protein, activates downstream signal transduction, and causes the cellular events. Recently, cytotoxic necrotizing factor type 2 (CNF2) produced by virulent *E. coli* was reported to cause assembly of actin stress fibers, probably through modification of rho protein detected as a mobility shift in SDS-PAGE (Oswald et al., 1994). However, those authors only showed the mobility shift of rho protein in the lysates of *E. coli* coexpressing CNF2 and rho protein, and therefore a direct interaction between CNF2 and rho protein was not proved.

C3 and rho$^{Val\text{-}14}$ protein have been used as tools for analyses of the cell responses in which rho protein is involved. However, rho$^{Val\text{-}14}$ protein is difficult to use because of the limitation of microinjection, and C3 that inactivates rho protein has a disadvantage for analysis of the downstream pathway of rho protein. We show here that, unlike C3 and rho$^{Val\text{-}14}$, DNT added extracellularly activates rho-dependent signal transduction in many cells. DNT will provide a new useful tool for investigating cell functions that are regulated by rho protein. To clarify the nature of DNT-induced modification of rho protein, we are now conducting further work.

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