Inhibition of PMA-induced, LFA-1-dependent Lymphocyte Aggregation by ADP Ribosylation of the Small Molecular Weight GTP Binding Protein, *rho*

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Abstract. *Botulinum* C3 exoenzyme specifically ADP-ribosylates a group of *ras*-related small molecular weight GTP-binding proteins, *rho*, and inhibits their biological activity. Using this enzyme, we examined the function of *rho* in PMA-induced activation of lymphocyte function-associated antigen-1 (LFA-1) in a B lymphoblastoid cell line, JY. Northern blot analysis revealed that among the three *rho* genes, *rhoA* mRNA was predominantly expressed in JY cells. Consistently, only one [^{32}P]ADP-ribosylated band was found when the lysate of the cells was subjected to ADP ribosylation by C3 exoenzyme. When the cells were cultured with C3 exoenzyme, this substrate was ADP-ribosylated in situ in a time- and concentration-dependent manner. Concomitant with this ADP ribosylation, PMA-induced LFA-1/intercellular adhesion molecule (ICAM)-1-dependent aggregation of JY cells was inhibited. This inhibition was blocked by prior treatment of the enzyme with an anti-C3 monoclonal antibody, and overcome by stimulation with higher concentrations of PMA. The C3 exoenzyme-induced inhibition was not affected by shaking of the cell suspension, while inhibition of aggregation by cytochalasin B was abolished by this procedure, suggesting that the inhibitory effect of the C3 exoenzyme treatment was not due to decrease in cell motility. The C3 exoenzyme treatment affected neither protein phosphorylation in JY cells before and after PMA stimulation, nor affected surface expression of LFA-1 and ICAM-1. These results suggest that *rhoA* protein works downstream of protein kinase C activation linking PMA stimulation to LFA-1 activation and aggregation in JY cells.

Ras and *ras*-related genes constitute a gene family encoding a series of closely related proteins with guanine nucleotide-binding activities. To date, ~40 *ras* and *ras*-related GTP-binding proteins are known, and they are divided into four subfamilies: *ras*, *rho*, *rab*, and others (Hall, 1990; Bourne et al., 1991). These proteins exist in two interconvertible functional states; one in an inactive GDP-bound form and the other in an active GTP-bound form. In resting cells, they are present in an inactive GDP-bound form, and upon cell stimulation, converted to the active GTP-bound form and work as molecular switches linking external stimuli to various cellular responses such as growth, differentiation, and secretion. Among the *ras*-related GTP-binding proteins, *rho* proteins are believed to be involved in organization of cytoskeleton and maintenance of cell shape. There are at least three members in this family in human, which are called *rhoA*, *B*, and *C* (Yeramian et al., 1987; Chardin et al., 1988). *rho* proteins are unique among the small GTP-binding proteins in being substrates for ADP ribosylation by the exoenzyme C3 from *Clostridium botulinum* (Aktories et al., 1987; Morii et al., 1988; Narumiya et al., 1988; Kikuchi et al., 1988). This enzyme ADP-ribosylates the proteins at an asparagine residue in the putative effector domain and inhibits their biological activities presumably by interfering with their interaction with the putative downstream effector molecules (Sekine et al., 1989; Morii et al., 1991). ADP ribosylation of the *rho* proteins by C3 exoenzyme and their activation by GTP result in opposite phenotypes in cultured cells. A typical change induced by the ADP ribosylation is rounding-up of cell bodies associated with disassembly of actin filaments (Rubin et al., 1988; Chardin et al., 1989; Paterson et al., 1990), whereas microinjection of the activated *rhoA* mutant protein into fibroblasts enhances actin filament organization and induces fingerlike cell processes (Paterson et al., 1990). These results suggest that *rho* proteins are involved in the organization of actin filament and the formation of adhesion plaques, and that the ADP ribosylation abolishes their activities. However, it remains unclear how and under what physiological conditions the *rho* protein exerts such action.

The integrin family of adhesion molecules plays a major role in the formation of adhesion plaques and also mediates

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some of the cell to cell adhesions. Lymphocyte function–associated antigen-1 (LFA-1)[1] is a member of the leukocyte integrins, and suggested to be involved in such processes as target cell recognition by cytolytic T lymphocytes and natural killer cells, binding of T lymphocytes to antigen presentation cells, and leukocyte adhesion to endothelial cells, fibroblasts, and epithelial cells (Springer, 1990). The counter-receptors for LFA-1 are intercellular adhesion molecules (ICAMs)-1 and -2. LFA-1 is not constitutively avid for ICAMs but interaction with the ICAMs requires activation of LFA-1 (Dustin and Springer, 1989; van Kooyk et al., 1989). This activation event can be triggered by stimulation of T cell receptor or CD2 on T lymphocytes or by the addition of phorbol esters (Rothlein and Springer, 1986; van Kooyk et al., 1989). However, the signal transduction pathway from these stimuli to the activation of LFA-1 remains unclarified. In this study, we examined the possible role of the *rho* protein in signal transduction in stimulus-evoked LFA-1 activation and cell aggregation. We chose JY Epstein-Barr virus–transformed B lymphoblastoid cells (Rothlein and Springer, 1986), and examined the effects of ADP ribosylation of *rho* protein by C3 exoenzyme on the PMA-induced aggregation of JY cells.

Materials and Methods

Materials

Recombinant *botulinum* C3 exoenzyme was prepared essentially as described (Nemoto et al., 1991). Mouse IgG1 monoclonal anti-C3 exoenzyme antibody, C302-1, was prepared as described previously (Morii et al., 1990). [α-^{32}P]NAD$^+$ (800 Ci/mmol) was obtained from Du Pont-New England Nuclear (Boston, MA). PMA was obtained from Sigma Chem. Co. (St. Louis, MO), and mouse IgG was from ICN Biomedicals, Inc. (Costa Mesa, CA). Human *rhoA* cDNA was isolated from human placenta cDNA library by cross hybridization with bovine *rhoA* clone (Ogorochi et al., 1989). Human *rhoB* and *rhoC* cDNA are gifts from A. Tavitian (Institut National de la Sante et de la Recherche Medicale, Paris, France) and K. Shinjo (Kanegafuchi Chemical Industry Co., Osaka, Japan), respectively.

Cell Line

An Epstein-Barr virus–transformed B lymphoblastoid cell line JY was generously provided by Drs. K. Okumura and H. Yagita of Juntendo University School of Medicine (Tokyo, Japan), and maintained in RPMI 1640 medium supplemented with 10% FCS.

Northern Blot Analysis

RNA was extracted from JY cells using the guanidine thiocyanate homogenization procedure and polyadenylated, or poly(A)$^+$, RNA was prepared by oligo(dT) cellulose column (Pharmacia LKB, Uppsala, Sweden). RNA (5 μg) were separated on a 1% agarose-formaldehyde gel and were transferred to a Hybond-N$^+$ membrane (Amersham Corp., Arlington Heights, IL). A SacII-VspI fragment (900 bp) from *rhoA* cDNA, a PstI-SmaI fragment (550 bp) from *rhoB* cDNA, and a BalI-PstI fragment (321 bp) from *rhoC* cDNA were radiolabeled by random-priming and used as probes. They contain fragments of the 3′-noncoding regions and the 3′-end of the coding regions. Probes used detected their own cDNA but did not crosshybridize with the others. Filters were hybridized overnight at 56°C in 5× SSC–0.5% SDS–5× Denhardt's solution–100 μg/ml of denatured salmon sperm DNA. Filters were washed with 1× SSC–0.1% SDS at 60°C, and then with 0.2× SSC–0.1% SDS at 60°C, and exposed to an x-ray film at −70°C for 56 h with an intensifying screen.

C3 Exoenzyme Pretreatment, Cytochalasin B Treatment, and Aggregation Assay of JY Cells

Logarithmically growing JY cells were taken and plated at 7.5 × 10^5/well in 1.5 ml of the complete medium in a 6-well plate and cultured with various concentrations of C3 exoenzyme for indicated periods. Treatment with C3 exoenzyme affected neither cell viability nor cell size. The cells were then washed twice with RPMI 1640, and suspended at a density of 2 × 10^6 cells/ml in RPMI 1640 containing 5 mM Hepes-NaOH, pH 7.4 (Sigma Chem. Co.). 100 μl of the cell suspension was added to a well of 96-well plate. PMA was then added, and the final volume of the suspension was adjusted to 200 μl/well. They were placed in a CO_2 incubator and aggregations of the cells were observed at 30 min, 3 h, and 16 h after the PMA addition. Aggregation was also quantified according to the method of Rothlein and Springer (1986). Briefly, cells were added to polystyrene tubes at 4 × 10^5 cells in 200 μl. One group of control cells received cytochalasin B at 2 μM. PMA was added and tubes were shaken at 200 rpm in a CO_2 incubator for 2 h. The sample was subjected to analysis on a Coulter Multisizer counter (Coulter Scientific Instruments, Hialeah, FL), and free cells in the suspension were measured. The percentage of aggregation of cells was calculated using the formula: 100 × [1 − (number of free cells/number of input cells)].

Effects of cytochalasin B on JY cell aggregation was further examined by pretreating the cells with 2 μM cytochalasin B for 5, 30, and 60 min before PMA addition and by incubating them with various concentrations (0.2, 0.7, 2, 7, and 20 μM) of this compound with PMA. Aggregation was determined at 2 h after the PMA addition by the quantitative method as described above.

ADP Ribosylation

Aliquots of cells were taken after the C3 exoenzyme treatment, washed, and spun down by brief centrifugation. Cell pellets were suspended in 50 μl of 0.25 M sucrose containing 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 2 mM benzamidine hydrochloride, and 0.5 mM PMSF. The suspension was homogenized by sonication and the homogenate was centrifuged at 1,000 *g* for 5 min. Supernatants (50 μg protein) were incubated at 30°C for 1 h with 50 ng C3 exoenzyme and 10 μM [^{32}P]NAD$^+$ (specific radioactivity, 2,000 cpm/pmol) in 100 mM Tris-HCl, pH 8.0, containing 20 mM nicotinamide, 10 mM thymidine, 10 mM DTT, and 5 mM $MgCl_2$ in a total volume of 100 μl. After the reaction, the mixture was subjected to SDS-PAGE and the ADP ribosylation was determined as described previously (Morii et al., 1990).

Analysis of Protein Phosphorylation by Two-Dimensional Polyacrylamide Gel Electrophoresis

4 × 10^6 cells, pretreated with or without C3 exoenzyme, were washed twice with the RPMI medium and then twice with phosphate-free medium (30 mM Hepes-NaOH, pH 7.4, 110 mM NaCl, 11 mM glucose, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mg/ml BSA), and incubated for 120 min in 50 μl of the phosphate-free medium supplemented with 0.15 mCi of [^{32}P]orthophosphate (Du Pont-New England Nuclear) in a CO_2 incubator at 37°C. Labeled cells were then stimulated with 50 ng/ml of PMA for 20 min, chilled, and collected by centrifugation. Cell pellets were lysed with 500 μl of the solubilizing buffer containing 10 mM Tris-HCl, pH 7.4, 1% NP-40, 66 mM EDTA, 0.4% sodium deoxycholate, 1% aprotinin, 1 mM PMSF, 1 mM sodium orthovanadate, and 0.05% sodium azide. After centrifugation at 10,000 *g* for 10 min, 2.5-μl aliquots were taken from supernatants to determine TCA-insoluble radioactivity, and the same volume of aliquots were used for the two-dimensional PAGE analysis. The samples were first subjected to NEPHGE (pH 3.5–10) (O'Farrell et al., 1977) at 400 V for 4 h, and then to SDS-PAGE on a 10% gel (Laemmli, 1970). After the electrophoresis, the gel was dried and the electrophoretic patterns of radiolabeled polypeptides were analyzed by the use of a Fuji BAS 2000 image analyzer (Fuji Film Co., Tokyo, Japan).

Flow Cytometry

Cells cultured with C3 exoenzyme for 24 h were washed with the RPMI medium twice and collected by centrifugation. 10^5 cells were incubated with 20 μg of mouse monoclonal anti–LFA-1α antibody (G-25.2) or anti–ICAM-1 antibody (LB-2) for 1 h at 4°C. The cells were then washed twice with 1 ml of ice-cold HBSS containing 0.75% BSA and 0.05% sodium azide (Hanks'-BSA), and incubated with 10 μg of FITC-conjugated

1. *Abbreviations used in this paper*: ICAM, intercellular adhesion molecule; LFA-1, lymphocyte function–associated antigen-1.

anti-mouse IgG F(ab')2 fragment (Cappel Laboratories, Malvern, PA) for 30 min on ice. The cells were washed again three times with 1 ml of Hanks'-BSA, suspended in 1 ml of the same solution, and subjected to the analysis on a Cytoron flow cytometer (Ortho Diagnostic Systems Co., Tokyo, Japan).

Results

Expression and ADP Ribosylation of rho *Proteins in JY Cells*

There are three *rho* genes in human: *rhoA*, *B*, and *C*. Using Northern blot analysis, we examined which *rho* gene was expressed in cultured JY cells. As shown in Fig. 1 *A*, *rhoA* was highly expressed in JY cells, whereas expression of *rhoB* and *rhoC* was observed at very low levels. *rhoA* mRNA was present as a doublet as reported previously (Olofsson et al., 1988; Jähner and Hunter, 1991). Consistent with these findings, only one [^{32}P]ADP-ribosylated band was observed when lysates of the cells were ADP-ribosylated by C3 exoenzyme and subjected to SDS-PAGE (Fig. 1 *B*).

We incubated the JY cells with various concentrations of C3 exoenzyme for various lengths of time. After incubation, we washed cells, homogenized and subjected the homogenate to ADP ribosylation to determine the amount of the ADP ribosylation substrate remaining in the cells. Fig. 2 shows typical autoradiograms of these analyses. The amount of the ADP ribosylation substrate in cells treated with 20 μg/ml of C3 exoenzyme decreased significantly with incubation time (Fig. 2 *A*). Observed decreases were 52.2, 67.1, and 77.8% with 8-, 16-, and 24-h incubation, respectively. As shown in Fig. 2 *B*, the decrease in the amount of the ADP ribosylation substrate occurred in a manner dependent on the concentration of C3 exoenzyme used in the treatment. The decrease was 61.0, 78.1, and 83.2% in cells treated with 3, 10, and 30 μg/ml of C3 exoenzyme for 24 h, respectively. These results suggest that the majority of *rhoA* protein in the cells had undergone ADP ribosylation in situ during the C3 exoenzyme treatment and became unable to be modified further with [^{32}P]NAD$^+$.

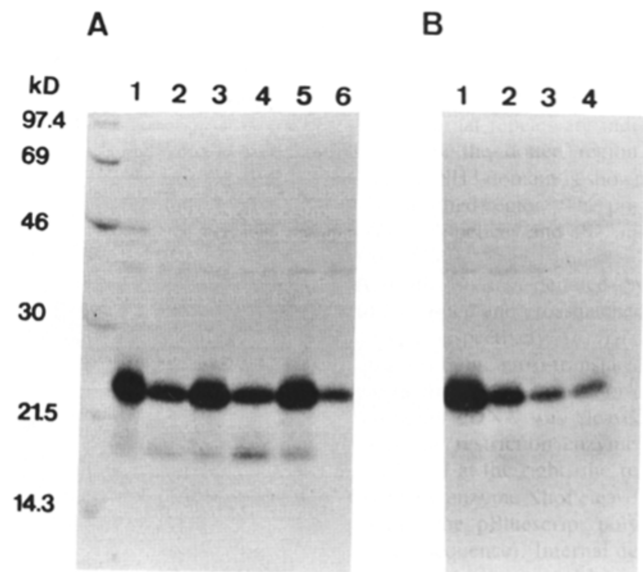

Figure 2. Time- and concentration-dependent decrease in ADP ribosylation substrate in C3 exoenzyme-treated cells. (*A*) Time course. Cells were cultured with (lanes *2*, *4*, and *6*) or without (lanes *1*, *3*, and *5*) 20 μg/ml of C3 exoenzyme for 8 (lanes *1* and *2*), 16 (lanes *3* and *4*), and 24 h (lanes *5* and *6*). Cells were homogenized and centrifuged at 1,000 *g*. The remaining ADP ribosylation substrate (unmodified *rhoA* protein) in the cells was ADP-ribosylated in vitro with C3 exoenzyme and [^{32}P]NAD$^+$, and analyzed by SDS-PAGE and autoradiography as described in Materials and Methods. (*B*) Concentration dependency. Cells were treated with 0 (lane *1*), 3 (lane *2*), 10 (lane *3*), and 30 (lane *4*) μg/ml of C3 exoenzyme for 24 h. The remaining ADP ribosylation substrate in the cells was analyzed as described above.

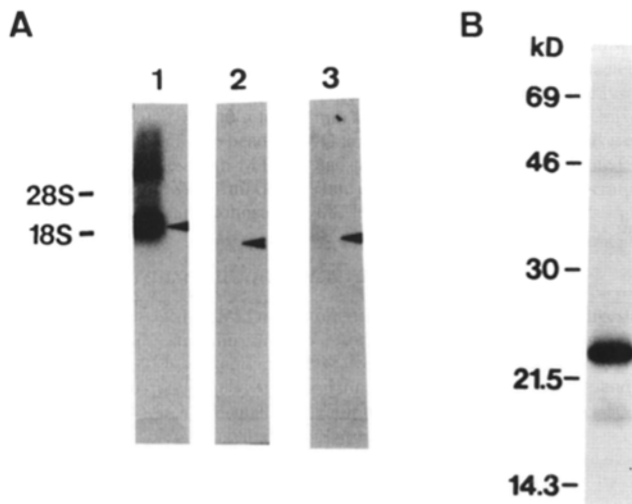

Figure 1. Northern blot analysis of *rhoA*, *B*, and *C* mRNA expression in cultured JY cells (*A*) and [^{32}P]ADP ribosylation of *rho* proteins (*B*). (*A*) Poly(A)$^+$ RNA (5 μg/lane) from JY cells was separated in a 1% formaldehyde-agarose gel. The Northern blot was hybridized with ^{32}P-labeled cDNA probes of *rhoA* (lane *1*), *rhoB* (lane *2*), and *rhoC* (lane *3*) as described in Materials and Methods. Arrowheads indicate specific bands hybridized with each probe. (*B*) Postnuclear homogenate of cells (50 μg) was subjected to ADP ribosylation by C3 exoenzyme in the presence of [^{32}P]NAD$^+$. Autoradiogram of SDS-PAGE of the sample is shown. Only the radioactive band at the 22 K was due to the catalysis of C3 exoenzyme, and other radioactive bands were also seen without the enzyme.

Inhibition by C3 Exoenzyme Treatment of PMA-Induced Aggregation of JY Cells

As previously reported by Rothlein and Springer (1986), JY cells aggregated in response to PMA and this aggregation was inhibited by anti-LFA-1 or anti-ICAM-1 antibody (data not shown). This PMA-induced cell aggregation was inhibited by the prior treatment of the cells with C3 exoenzyme, and this occurred in a manner dependent on the treatment time and the enzyme concentration used in the treatment. As shown in Fig. 3 *A*, aggregation of control cells was observed from a 30-min incubation with PMA and the aggregates became larger and more compact thereafter. There were very few cells remaining free (panel *a*). Cells pretreated with C3 exoenzyme for 8 h made loose aggregates and significant number of cells remained free (panel *b*). In cells with longer C3 exoenzyme treatment, PMA induced only small aggregates and the increasing number of cells

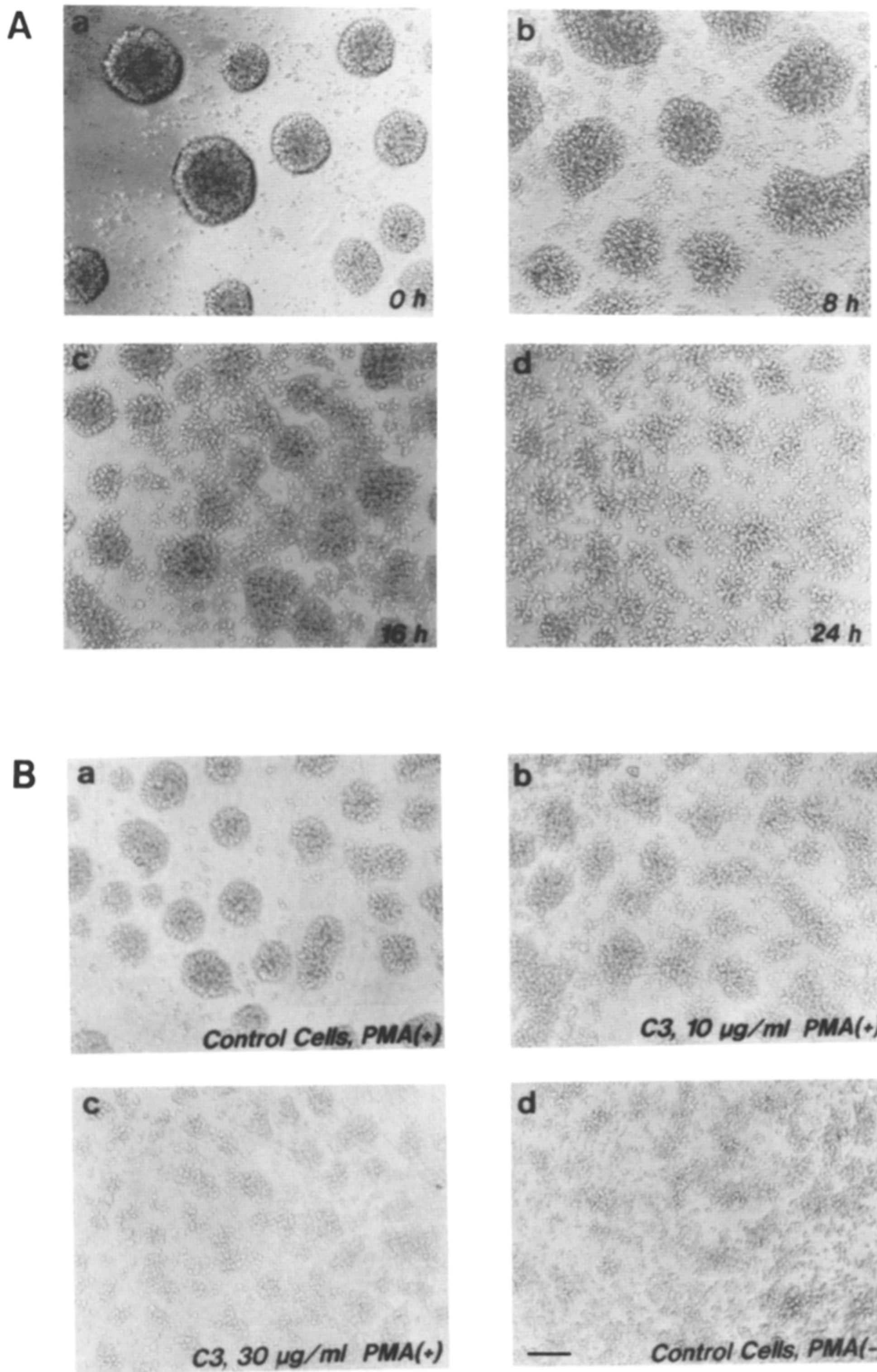

Figure 3. Inhibition of PMA-induced aggregation of JY cells by C3 exoenzyme treatment. (*A*) Time dependency. Cells were treated with 20 μg/ml of C3 exoenzyme for 0 (*a*), 8 (*b*), 16 (*c*), and 24 (*d*) h. Cell aggregation was then induced by 5 ng/ml of PMA as described in Materials and Methods. (*B*) Concentration dependency. Cells were treated without (*a* and *d*) or with 10 (*b*) or 30 (*c*) μg/ml of C3 exoenzyme for 24 h. Aggregation was induced by 5 ng/ml of PMA (*a–c*). Control cells without PMA were shown in *d*. Photographs were taken 16 (*A*) or 3 h (*B*) after the addition of PMA. The effect of C3 exoenzyme treatment was observed at 30 min, 3 h, and 16 h after the addition of PMA and the inhibition was found at any of the three observation points. Bar, 100 μm.

were present either free or as clusters of a few cells (panels *c* and *d*). Fig. 3 *B* shows dependency of the inhibition on the enzyme concentration. In cells treated with 10 μg/ml of the enzyme for 24 h, aggregates were loose and many cells remained free (panel *b*). In cells treated with 30 μg/ml of the enzyme, aggregation was not observed clearly and their appearance was similar to that of the control cells without PMA stimulation (panels *c* and *d*). Thus, the time course and dose dependency of C3 exoenzyme-induced inhibition correlated well with the finding on the ADP ribosylation in Fig. 2.

To confirm that this inhibition is due to C3 exoenzyme itself and not to any contaminants in the preparation, we incubated the enzyme with anti-C3 exoenzyme mAb at 4°C

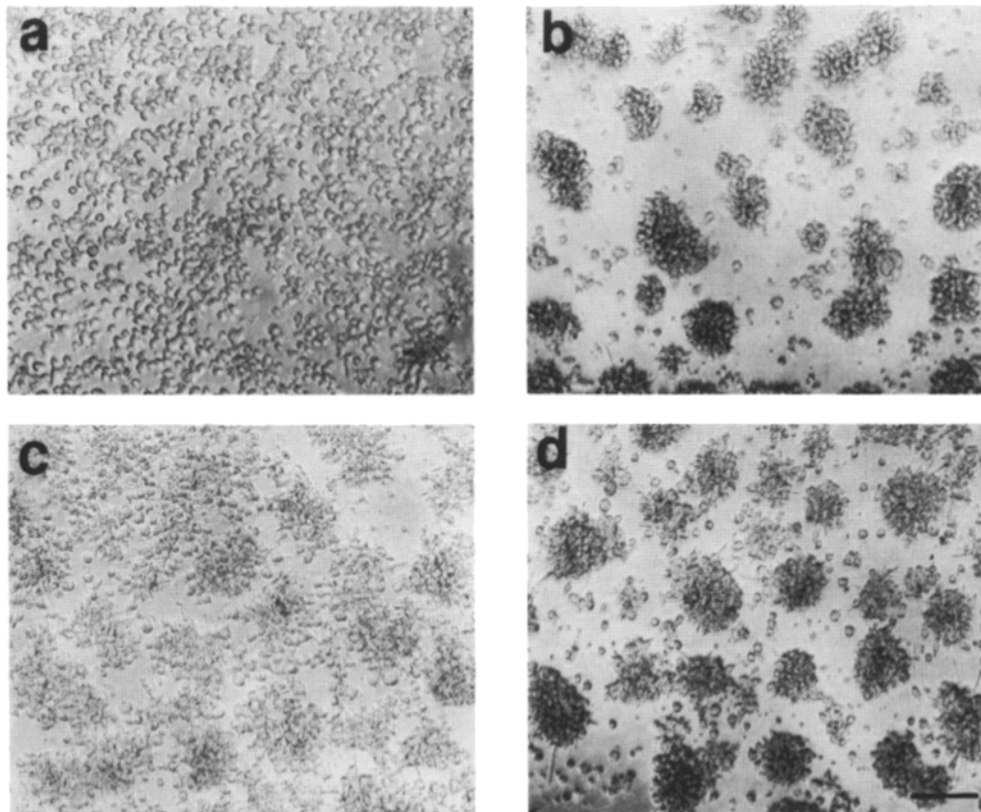

Figure 4. Blocking of the inhibitory activity of C3 exoenzyme by monoclonal anti–C3 exoenzyme antibody. 20 μg C3 exoenzyme was incubated overnight at 4°C with or without 600 μg of mouse monoclonal anti–C3 exoenzyme antibody in 500 μl of PBS. These mixtures were then added to JY cells, and the cells were incubated for 24 h. The cells were washed and aggregation was evoked with 5 ng/ml of PMA. (*a*) Control cells without PMA stimulation; (*b*) control cells stimulated with PMA for 30 min; (*c*) cells pretreated with C3 exoenzyme and stimulated with PMA for 30 min; and (*d*) cells pretreated with a mixture of C3 exoenzyme and anti–C3 mAb and stimulated with PMA for 30 min. Bar, 100 μm.

overnight, and added the mixture to JY cells. After 24 h, aggregation was evoked by PMA in the washed cells. As shown in Fig. 4, pretreatment with anti–C3 exoenzyme mAb abolished the inhibition of aggregation by C3 exoenzyme. Treatment of C3 exoenzyme with normal mouse IgG did not affect the inhibitory effect of C3 exoenzyme on PMA-induced cell aggregation (data not shown).

In the studies shown in Figs. 3 and 4, the inhibitory effect of C3 exoenzyme pretreatment was examined in JY cells settled on culture dishes. In this assay the cells locomote on a dish to contact each other to aggregate. This assay, therefore, may not distinguish inhibition of cell adhesion from impairment of cell motility. To clarify whether the inhibitory effect of C3 exoenzyme was due to the decrease in cell adhesion or to that in cell motility, we shook the cells in tubes and examined the effect of C3 exoenzyme. We also compared it with that of cytochalasin B because this drug disrupts actin filament to inhibit various functions of it including cell motility (Yahara et al., 1982). A previous study (Rothlein and Springer, 1986) showed that this compound inhibited PMA-induced aggregation of JY cells on a culture dish. Cells were treated with 20 μg/ml C3 exoenzyme for 24 h, and subjected to the experiment. As shown in Table I, the control cells without the enzyme treatment aggregated by more than 60%. In contrast, only 27% of total cells aggregated after the C3 exoenzyme treatment. Cytochalasin B (2 μM) was then added to the control cells and its effect on aggregation was examined. Although this concentration of the drug inhibited aggregation completely in experiments in a 96-well plate (data not shown), it showed no inhibition of aggregation when the cell suspension was shaken in a tube, and ∼80% of the cells aggregated. These results suggest that C3 exoenzyme-induced inhibition is not secondary to impairment of cell motility but due to the inhibition of the induction of adhesive property of the cells. These results also revealed that inhibition of cell aggregation with cytochalasin B reported in earlier studies is due to the inhibition of cell motility and not to that of cell adhesion per se.

Effects of cytochalasin B on JY cell aggregation were also examined by changing its concentration and the treatment time. When the cells were treated with 0, 0.2, 0.7, 2, 7, and 20 μM cytochalasin B, aggregation induced by PMA was 55, 58, 62, 70, 57, and 40%, respectively. When 2 μM cytochalasin B was added to the cells at 5 and 30 min, and 1 h before the addition of PMA, 79, 71, and 60% aggregation was observed, respectively (control value = 55%). Thus,

Table I. Comparison of the Effect of C3 Exoenzyme and Cytochalasin B on PMA-induced Aggregation of JY Cells under the Shaking Conditions

Cells	% Aggregation*
Control cells	62.7 ± 3.9
C3-treated cells	27.3 ± 3.4§
Cytochalasin B–treated cells	79.3 ± 2.1‡

JY cells were treated with or without 20 μg/ml of C3 exoenzyme for 24 h. Cytochalasin B (2 μM) was added to one group of control cells 5 min before the addition of PMA. Cell aggregation was evoked and calculated as described under Materials and Methods.
* Values are mean ± SEM of three different experiments.
‡ $p < 0.05$.
§ $p < 0.01$ for control cells.

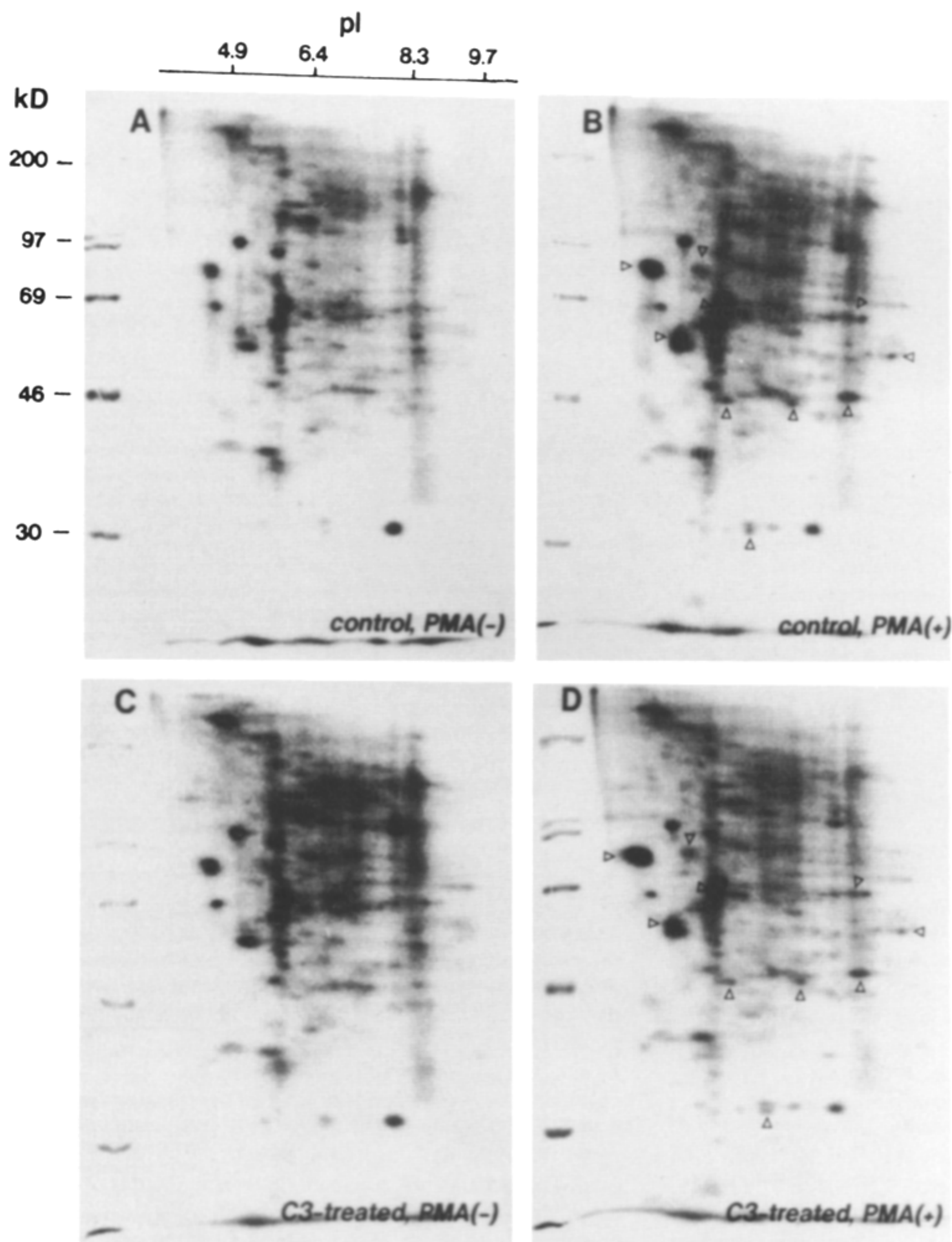

Figure 5. Two-dimensional PAGE analysis of protein phosphorylation in control and C3 exoenzyme-treated JY cells. JY cells preincubated with or without C3 exoenzyme were labeled with [^{32}P]orthophosphate. The control and C3 exoenzyme-treated cells were incubated with or without 50 ng/ml of PMA for 20 min and lysed as described in Materials and Methods. Each cell lysate (the NP-40-soluble fractions) was subjected to an analysis on a two-dimensional gel electrophoresis. (*A*) Control cells without PMA; (*B*) control cells with PMA; (*C*) C3 exoenzyme-treated cells without PMA; and (*D*) C3 exoenzyme-treated cells with PMA. Open arrowheads in *B* and *D* indicate proteins further phosphorylated after PMA stimulation.

cytochalasin B up to 7 μM concentration or added at 2 μM at 1 h before PMA addition did not inhibit the JY cell aggregation, whereas it was partially inhibited with 20 μM concentration of the compound.

Effects of C3 Exoenzyme Treatment on Protein Phosphorylation and Expression of LFA-1 and ICAM-1 in JY Cells

It was recently reported that PMA-induced aggregation of JY cells was blocked by protein kinase C inhibitors (Haverstick et al., 1992), suggesting that protein kinase C is implicated in activation of the LFA-1/ICAM-1-mediated cell adhesion. We examined, therefore, whether the pattern of protein phosphorylation was affected by the treatment with C3 exoenzyme. Cells were labeled with [^{32}P]orthophosphate, incubated with or without PMA for 20 min, and lysed. Radioactivities in TCA precipitates of 2.5-μl aliquots of the lysates were 12,339, 12,956, 14,553, and 13,678 cpm for control cells without and with PMA, and C3 exoenzyme-treated cells without and with PMA, respectively. Thus, there was no significant difference in incorporation of the radioactivity between the control and C3 exoenzyme-treated cells, indicating that the cells treated with C3 exoenzyme were metabolically intact. Fig. 5, *A*-D, presents the results of the gel electrophoresis of the NP-40-soluble cell lysates. Phosphorylation of a number of proteins was already observed in control cells without the PMA stimulation (Fig. 5 *A*). After the PMA stimulation, several proteins were further phosphorylated and some proteins were newly phosphorylated (Fig.

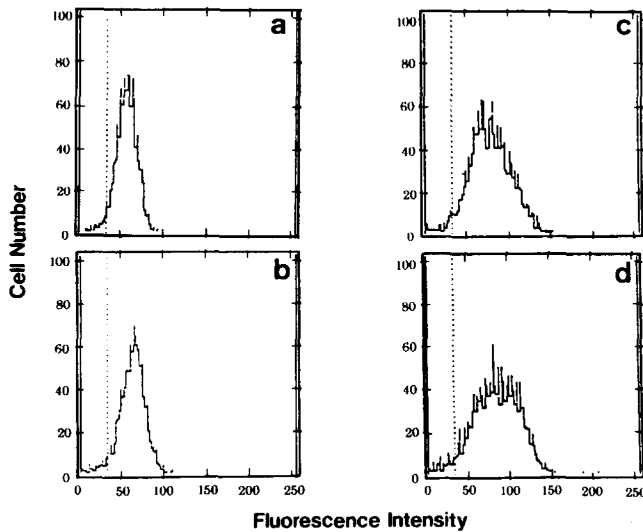

Figure 6. Effect of C3 exoenzyme treatment on expression of LFA-1 and ICAM-1 on JY cells. JY cells were incubated with or without 50 μg/ml of C3 exoenzyme for 24 h. The cells were then stained with anti-LFA-1α mAb or anti-ICAM-1 mAb and with FITC-conjugated goat anti-mouse IgG, and subjected to a flow cytometric analysis as described in Materials and Methods. (*a*) LFA-1 expression on control cells; (*b*) LFA-1 expression on C3 exoenzyme-treated cells; (*c*) ICAM-1 expression on control cells; and (*d*) ICAM-1 expression on C3 exoenzyme-treated cells.

5 *B*). In the C3-treated cells, the phosphorylation pattern at resting was not different from the control cells (Fig. 5 *C*), and the proteins further phosphorylated by PMA were identical to those seen in the control cells (Fig. 5 *D*). There was no difference, either, in the phosphorylation pattern of the NP-40-insoluble residues between the control and C3 exoenzyme-treated cells with or without PMA stimulation (data not shown).

We next examined the expressions of LFA-1 and ICAM-1 on JY cells. Immunofluorescence flow cytometry showed no change in LFA-1 (Fig. 6, *A* and *B*) or ICAM-1 (Fig. 6, *C* and *D*) expression on JY cells by the treatment with C3 exoenzyme.

Antagonism of C3 Exoenzyme-induced Inhibition of Aggregation by Stimulation with Higher Concentrations of PMA

The above results suggest that ADP ribosylation by C3 exoenzyme is responsible for the inhibition of aggregation and the degree of inhibition depends on the extents of the ADP ribosylation in JY cells. In such ADP ribosylation experiments, function of *rho* protein is presumed to depend on the balance of the stimulus-induced active GTP-bound form and the inactive ADP-ribosylated form which is supposed to be dominant negative. As shown in Fig. 2 *B*, ~20% of the *rhoA* protein in the cell was not ADP-ribosylated and remained intact even after the treatment with 30 μg/ml of the enzyme for 24 h. In the case of *ras* p21, the percentage of the GTP-bound active form increased by stimulation with higher concentrations of phorbol ester (Downward et al., 1990). We, therefore, examined whether the higher concentrations of PMA could overcome the inhibitory effect of C3 exoenzyme. After the treatment with 20 μg/ml of C3 exoenzyme for 24 h, the cells were stimulated by 0.5–500 ng/ml of PMA (Fig. 7). In control cells, aggregation was induced by the addition of 0.5 ng/ml of PMA and aggregates became more compact with the addition of 50 and 500 ng/ml of PMA. In the C3 exoenzyme-treated cells, aggregation was not induced by the addition of 0.5 and 5 ng/ml of PMA. However with 50 ng/ml of PMA, irregular and loose aggregates were induced and with 500 ng/ml of PMA, the aggregate formation became more apparent. Thus, the C3 exoenzyme-induced inhibition of aggregation was antagonized by stimulation with higher concentrations of PMA.

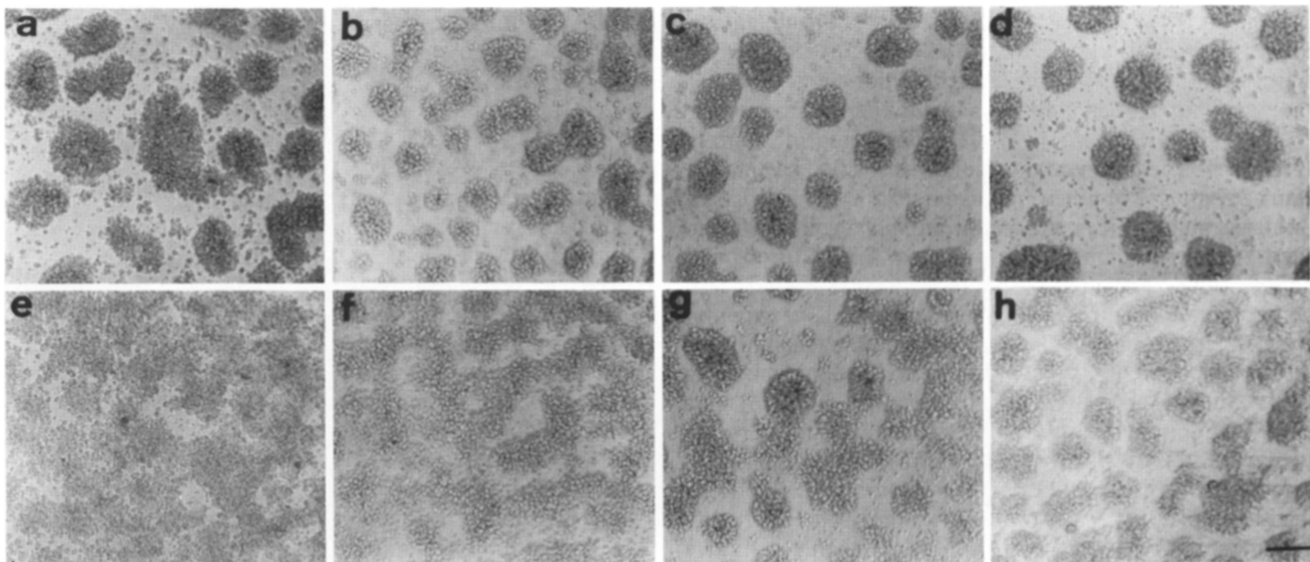

Figure 7. Antagonism of C3 exoenzyme-induced inhibition of aggregation by higher concentrations of PMA. JY cells were treated with (*e–h*) or without (*a–d*) C3 exoenzyme (20 μg/ml) for 24 h and aggregation was evoked by 0.5 (*a* and *e*), 5 (*b* and *f*), 50 (*c* and *g*), and 500 (*d* and *h*) ng/ml of PMA. Photographs were taken 3 h after incubation with PMA. Bar, 100 μm.

Discussion

Aggregation and adhesion of lymphocytes can be evoked by stimuli such as T cell receptor cross-linking or phorbol ester treatment, and this is due to activation of lymphocyte integrin, LFA-1. Avidity of LFA-1 for ICAMs is increased by lymphocyte activation, whereas avidity of ICAMs for activated LFA-1 is constitutively high (Dustin and Springer, 1989). The intracellular signal transduction pathway between the stimuli and LFA-1 activation has remained unknown. The present study used PMA-induced aggregation of B-lymphoblastoid JY cells as a model to elucidate possible involvement of the *rho* protein in this pathway. Our results demonstrated that C3 exoenzyme preincubated with the cells caused in situ ADP ribosylation of *rhoA* protein and inhibited cell aggregation induced by PMA. The two events correlated well in their time course and its dependency on the enzyme concentration. The inhibition was abolished by prior treatment of the enzyme with the specific anti-C3 enzyme monoclonal antibody, and overcome by stimulation with higher concentrations of PMA. These results suggest that the *rho* protein works as an intracellular transducer of the signal to integrin activation and cell aggregation. Very recently, a similar intermediate role of *rhoA* protein in a stimulus-evoked cell adhesion process has been shown in the two systems. Ridley and Hall (1992) used C3 exoenzyme, or ADP-ribosylated or mutant *rhoA* protein, and examined a role of *rho* protein in growth factor-induced stress fiber formation in serum-starved Swiss 3T3 cells. They found that C3 exoenzyme treatment as well as microinjection of the ADP-ribosylated *rhoA* protein inhibits the serum-induced formation of stress fiber and focal adhesion, suggesting that the *rho* protein works as a link between the stimulus and adhesion. We (Morii et al., 1992) examined the role of *rhoA* protein in aggregation of human blood platelets and found that ADP ribosylation of platelet *rhoA* protein inhibits aggregation caused by thrombin. This work demonstrated that the *rho* protein works downstream of the receptor–G protein coupling and phospholipase C activation in platelets. The present study showed that ADP ribosylation of *rhoA* inhibited PMA-induced, protein kinase C–mediated JY cell aggregation (Haverstick et al., 1992), suggesting that the *rhoA* protein works downstream of protein kinase C in lymphocytes. It further revealed that the C3 exoenzyme treatment did not affect protein phosphorylation pattern in the cells not only at resting but also after stimulation. Recently, Hirata et al. (1992), using a skinned smooth muscle preparation, showed that the C3 exoenzyme treatment abolished GTP-induced enhancement of its contraction to free calcium ion, and that the GTP-bound form of *rhoA* protein restored this response. On the basis of the previous analysis on this "GTP-induced calcium sensitization" phenomenon (Kitazawa et al., 1991), they proposed that *rhoA* protein exerts this action by modifying phosphorylation of myosin light chain. Our results on protein phosphorylation does not appear to support their hypothesis, and protein phosphorylation, if significant in the *rho* protein action, would be too subtle to detect by the present autoradiographic analysis.

Then, how does the *rho* protein activate cell adhesion? In the experiments using Swiss 3T3 cells, activated *rho* protein has been shown to induce stress fiber formation and organization of adhesion plaques (Chardin et al., 1989; Paterson et al., 1990; Ridley and Hall, 1992). They reported that the two events occurred in parallel, and it is not known which of the two events is primarily induced by the *rho* protein. In the present study, we compared the effects of C3 exoenzyme and cytochalasin B on PMA-induced aggregation. As shown in Table I, cytochalasin B did not inhibit PMA-induced JY cell aggregation under the condition that it inhibited motility of JY cells, and inhibition of aggregation was only seen partially at the highest concentration (20 μM) of the compound. These results suggest that the new organization of actin filament may not be essential to the activation of integrin and cell adhesion, though preexisting actin skeleton more resistant to the compound (Yahara et al., 1982) may have some role. On the other hand, inhibition of aggregation by C3 exoenzyme treatment was still seen when the cell contact was increased by shaking. It thus clearly showed that the C3 exoenzyme–induced inhibition of aggregation could be dissociated from impairment of cell motility by the enzyme, which was reported in other systems (Stasia et al., 1991; Takaishi et al., 1993). Our results are consistent with the previous findings on blood platelets that cytochalasin B treatment inhibited pseudopodal development and organization of actin bundles but not platelet aggregation (Carroll et al., 1982). Thus, these results taken together suggest that C3 exoenzyme exerts inhibition of aggregation not by affecting induction of actin polymerization but by affecting primarily the process of integrin activation.

Then, what is the primary event in the activation of integrins? LFA-1 binding to ICAM-1 was found to be regulated by the cytoplasmic domain of the β subunit of LFA-1 (Hibbs et al., 1991*b*) and previous studies suggested that phosphorylation of the β subunit may induce the high avidity state of the leukocyte integrin (Chatila et al., 1989; Buyon et al., 1990). However, Hibbs et al. (1991*a*) made mutations at all potential phosphorylation sites of this subunit and showed that phosphorylation of the β subunit is not required for integrin activation. In fact, we observed that both the α and β subunits of LFA-1 in JY cell were constitutively phosphorylated (Tominaga, T., unpublished observation). Thus, it is unlikely that the β subunit phosphorylation is responsible for the integrin activation. An alternative possibility has been suggested by other studies. It is known that several cytoskeletal proteins such as talin, vinculin, and α-actinin localize in close association with integrins in focal contacts (Burridge et al., 1988). Burn et al. (1988) and Kupfer and Singer (1989) showed associations of LFA-1 and talin in T lymphocytes after the phorbol ester stimulation or the activation by antigen-presenting cells. In vitro association of integrins with proteins such as α-actinin and talin has also been shown (Horwitz et al., 1986; Otey et al., 1990). These results suggest that avidity of LFA-1 is increased by its association with these cytoskeletal proteins and it is likely that the *rho* protein catalyzes this association. Further study is required to test this hypothesis.

Recent studies have demonstrated that activation of integrins and resultant cell adhesion release other signals or mediate some cell actions. C3 exoenzyme treatment may, therefore, secondarily inhibit such processes. Lang et al. (1992) reported that ADP ribosylation of *rhoA* by C3 exoenzyme inhibits natural killer cells or cytotoxic T lymphocytes–mediated cytotoxicity. Although the authors suggested that this effect is due to inhibition of exocytosis, it may be secondary

to inhibition of cell–cell adhesion as shown in the present study.

In summary, we have shown that ADP ribosylation of *rhoA* protein by C3 exoenzyme inhibits the PMA-induced aggregation of JY cells. This is the first report to indicate that *rhoA* protein works as a transducing protein in the stimulus-evoked activation of LFA-1 in lymphocytes.

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