ADP-ribosylation of rho p21 Inhibits Lysophosphatidic Acid-induced Protein Tyrosine Phosphorylation and Phosphatidylinositol 3-Kinase Activation in Cultured Swiss 3T3 Cells*

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Naokazu Kumagai, Narito Mori, Kazuko Fujisawa, Yasuo Nemoto, and Shuh Narumiya†

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

Botulinum C3 exoenzyme was used to specifically ADP-ribosylate and inactivate rho p21, and the effects of rho p21 inactivation on lysophosphatidic acid (LPA)-induced tyrosine phosphorylation were examined in cultured Swiss 3T3 cells. LPA induced a rapid increase in the tyrosine phosphorylation of a number of proteins. Pretreatment of the cells with the C3 exoenzyme caused ADP-ribosylation of rho p21 in the cells and selectively attenuated the phosphorylation of several proteins, including p43 mitogen-activated protein kinase, p125 focal adhesion kinase, and two proteins of 72 and 88 kDa. C3 exoenzyme pretreatment did not block the initial phosphorylation and activation of mitogen-activated protein kinase but suppressed its subsequent rise. In contrast, the enzyme treatment inhibited the induction of phosphorylation of the 72- and 88-kDa proteins and suppressed the basal and LPA-induced tyrosine phosphorylation of p125 focal adhesion kinase. In addition, immunoprecipitation of cell lysates with an antibody directed against the 85-kDa subunit of phosphatidylinositol 3-kinase (P1 3-kinase) co-precipitated a tyrosine-phosphorylated band of 180 kDa. C3 exoenzyme pretreatment suppressed both the phosphorylation of this band and P1 3-kinase activation associated with LPA stimulation. These findings suggest that rho p21 works as a link between the LPA receptor signal and the subsequent tyrosine phosphorylation and P1 3-kinase activation in these cells.

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† Present address: Mitsubishi Kasei Institute of Life Sciences, Machida, Tokyo 194, Japan.
‡ To whom correspondence should be addressed: Dept. of Pharmacology, Kyoto University Faculty of Medicine, Yoshida, Sakyō-ku, Kyoto 606, Japan. Tel.: 81-75-753-4392; Fax: 81-75-753-4693

The rho gene products (rho p21s) are members of the ras superfamily of small GTPases (1–3). Following receptor stimulation, these GTPases are converted from the inactive GDP-bound form to the active GTP-bound form and link external stimuli to cellular responses such as growth, differentiation, and secretion. rho p21s are unique among the ras-related GTPases, since they are substrates for botulinum C3 ADP-ribosyltransferase (3–5). This enzyme specifically modifies the Asn41 residue of rho p21s, and inhibits their biological activity, presumably by interfering with their interaction with downstream targets (6). Using this exoenzyme and/or mutants of rho p21, the cellular functions of the rho p21s have been investigated. These studies have revealed that rho p21 mediates such cellular processes as stimulus-evoked cell adhesion (7–9) and motility (10), regulation of smooth muscle contraction (11), G1 to S phase progression in cell cycle (12), and cytokinesis of the fertilized egg (13, 14).

LPA acts on a cell surface receptor coupling to G-proteins, G, and G, and evokes a variety of biological responses (15, 16). It is a normal constituent of serum and responsible for the majority of its growth promoting activity (7, 17). Ridley and Hall (7) reported that LPA characteristically induces focal adhesion and stress fibers in quiescent Swiss 3T3 cells, and that this response was mediated by rho p21. These findings suggest that rho p21 receives a signal from LPA in the cell and transduces it to a system regulating cell adhesion. However, little is known about this transduction mechanism. We recently found that LPA rapidly induced the tyrosine phosphorylation of a number of proteins in Swiss 3T3 cells, including the MAP kinases and p125 FAK (18). In the present study, we used botulinum C3 exoenzyme to examine the involvement of rho p21 in this process.

EXPERIMENTAL PROCEDURES

Materials—LPA (oleoyl-sn-glycero-3-phosphate), PI, PI 4-monophosphate, PI 4,5-diphosphate, and l-ω-phosphadithiol-serine were obtained from Sigma. Antibody against MAP kinase 2Y for ERK2 (19) was a gift of Dr. T. Yoshimasa and Professor K. Nakao of Kyoto University. Mouse monoclonal antibody CA3 against the p85 subunit of human PI 3-kinase (20) was purchased from MBL (Nagoya, Japan). Rabbit polyclonal anti-phosphotyrosine antibody was obtained from Zymed Laboratories. Monoclonal anti-p125 (FAK) antibody was purchased from Upstate Biotechnology. 125I-Protein (967 mCi/mg) and [γ-32P]ATP (3,000 Ci/mmol) were purchased from ICN Biomedicals and DuPont NEN, respectively. Insulin-transferrin-sodium selenite tissue culture supplement was obtained from Boehringer Mannheim.

Preparation of Recombinant C3 Exoenzyme—C3 exoenzyme gene (5) was modified by a PCR-mediated site directed mutagenesis to produce a recombinant C3 exoenzyme that lacks the signal peptide and has dipetide Met-Ala attached to Ser of the mature exoenzyme. PCR was performed with the cloned gene as template and synthetic oligonucleotides (5'-ACTGTTCATATGGCTAGCTATGCAGATACT'C-ACA-3') and 5'-TTATTGGATCCTATTA'AATATCATTGCTGTA3') as primers. The amplified fragment was cleaved with NdeI and BamHI and ligated with a pET-3a vector (21). After confirming DNA sequence, the recombinant plasmid, pET-3a/C3, was introduced into Escherichia coli BL2UDE3)pLysE and expressed (5, 21, 22).

The rho gene product (rho p21) is a heterotrimeric GTP-binding protein, LPA, lysophosphatidic acid; MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; C3 exoenzyme, botulinum C3 ADP-ribosyltransferase; FAK, focal adhesion kinase; PCR, polymerase chain reaction; PI, l-ω-phosphatidylinositol; p85 the 85-kDa subunit of PI 3-kinase; PAGE, polyacrylamide gel electrophoresis.
Cell Culture and LPA Stimulation—Swiss 3T3 mouse fibroblasts were maintained and cultured as described previously (12, 18). The cells were seeded at a density of 1.0 × 10^4/well in six-well culture plates (Falcon) and cultured in complete medium for 3 days. The cells were then cultured with or without 5 μg/ml recombinant C3 exoenzyme, first for 48 h in the complete medium and then for 12 h in serum-free medium (a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium containing 5 mg/ml bovine serum albumin and 0.05% insulin-transferrin-selenium supplement). LPA at 0.2 μM was then added to the culture. The cells were incubated at 37°C in a CO2 incubator for the indicated times and extracted (18). LPA-induced inhibition of cAMP accumulation was studied by incubating the cells with 3 μM forskolin for 15 min in the presence of 1 μM isobutylmethylxanthine added 10 min prior to the forskolin addition. LPA at 2 μM was added at 5, 10, and 14 min after the addition of forskolin. The reaction was stopped by adding 10% ice-cold trichloroacetic acid, and CAMP content was determined using 125I-cAMP assay system from Amersham Corp.

Immunoprecipitation and Immunoblotting—Immunoprecipitation of FAK, ERK-2 and PI 3-kinase, SDS-PAGE, and immunoblotting were all carried out as described previously (18). Bands immunoreactive with the anti-phosphotyrosine antibody were visualized by incubation with 125I-protein A diluted 10,000-fold in Tris-buffered saline, 0.05% Tween 20, and were analyzed by autoradiography or by a Bioimage BAS2000 analyzer (Fuji, Tokyo). Bands immunoreactive with anti-MAP kinase antibody were visualized by a Vecta Stain ABC kit.

Assays—MAP kinase activity was measured as described previously (19). The activity of PI 3-kinase was assayed as described previously (23, 24) except that thin layer chromatography was carried out with a solvent of chloroform:methanol:acetic acid:water (40:28:12:8). ADP-ribosylation was carried out as previously described (12). Protein contents were determined according to the method of Bradford with bovine serum albumin as a standard (25).

RESULTS AND DISCUSSION

Swiss 3T3 cells were incubated with 5 μg/ml C3 exoenzyme first in complete medium for 48 h and then in serum-free medium for 12 h. Fig. 1A shows an autoradiogram of the ADP-ribosylation reaction of lysates from the control and C3 exoenzyme-treated cells. The control lysates yielded a single [32P]ADP-ribosylated band at M_r 23,000 (lane 1), previously identified as rhoA p21 (12). The corresponding band was reduced in lysates from C3 exoenzyme-treated cells (lane 2), suggesting that the rhoA p21 in the C3 exoenzyme-treated cells had undergone in situ ADP-ribosylation. The ADP-ribosylation and, consequently, the inactivation of rho p21 did not affect the initial signal transduction of LPA, because LPA-mediated inhibition of cAMP generation occurred equally in control and C3 exoenzyme-treated cells (data not shown). Using these cells, we examined the effects of this treatment on protein tyrosine phosphorylation. As shown in Fig. 1B, LPA induced a rapid tyrosine phosphorylation of multiple proteins, and the C3 exoenzyme treatment attenuated some of the phosphorylation. For example, phosphorylation of a protein designated p72 occurred rapidly at 1 min after LPA addition and remained high during the entire 60-min incubation. This response was suppressed by the C3 exoenzyme pretreatment. A similar suppression was observed for p88. On the other hand, C3 exoenzyme pretreatment did not affect the phosphorylation of a protein designated p64. We previously identified the tyrosine-phosphorylated protein p43 as the MAP kinase, ERK-2 (18). Tyrosine phosphorylation of this protein was detected at 1 min after LPA addition, increased to a maximum at 5 min, and declined thereafter. The C3 pretreatment did not affect the initial phosphorylation observed at 1 min (lanes 2 and 3), but significantly attenuated phosphorylation by 5 min (lanes 3 and 3'). To confirm this effect, we measured MAP kinase activity using myelin basic protein as a substrate. As shown in Fig. 2, MAP kinase was rapidly and transiently activated by LPA addition; the activity increased about 3-fold at 1 and 5 min but declined quickly. In the C3 exoenzyme-treated cells, activation was also observed at 1 min, but the activity had already decreased by 5 min. These results are consistent with the findings on tyrosine phosphorylation in Fig. 1B. In order to identify p88, immunoprecipitation of the cell extracts from both control and C3 exoenzyme-

![Fig. 1. C3 exoenzyme treatment and LPA-induced tyrosine phosphorylation. A, ADP-ribosylation of cell lysates. Cell lysates from control (lane 1) and C3 exoenzyme-treated 3T3 cells (lane 2) were subjected to in vitro ADP-ribosylation using [32P]NAD. B, LPA-induced tyrosine phosphorylation in control and C3 exoenzyme-treated cells. Control (lanes 1–6) or C3 exoenzyme-treated (lanes 1′–6′) cells were incubated with 0.2 μM LPA at 37°C for 0 (lanes 1 and 1′), 1 (lanes 2 and 2′), 5 (lanes 3 and 3′), 10 (lanes 4 and 4′), 30 (lanes 5 and 5′) and 60 min (lanes 6 and 6′). The cell extracts were prepared and subjected to immunoblot analysis as described under “Experimental Procedures.” A typical result of at least five experiments is shown.](https://example.com/fig1.jpg)

![Fig. 2. Activation of MAP kinase in the control and C3 exoenzyme-pretreated cells. Control (○) and C3 exoenzyme-treated (●) cells were incubated with 0.2 μM LPA for the indicated times. The cells were then extracted, and the MAP kinase activity in the extracts was assayed as described (17). Portions of the extracts were subjected to SDS-PAGE and immunoblotting. The inset shows immunoblots with an anti-phosphotyrosine antibody (above) and with an anti-ERK 2 antibody (below). The numbers above the lanes correspond to those described in the legend for Fig. 1B. Experiments were repeated three times with reproducible results.](https://example.com/fig2.jpg)
treated cells was carried out using the CA3 monoclonal antibody specific for the p85 subunit of PI 3-kinase (19). Although this procedure precipitated the immunoreactive p85 protein, no precipitation of p88 was observed (Fig. 3A). Instead, a tyrosine-phosphorylated protein of 180 kDa, p180, was precipitated. This precipitation appeared specific, because no precipitation was found without anti-p85 antibody or with other antibodies such as anti-p125 FAK (data not shown and Fig. 4). Since its phosphorylation was suppressed by C3 exoenzyme, no phosphorylation was suppressed by C3 exoenzyme pretreatment. As shown in Fig. 3B, the activity of PI 3-kinase as determined by the 32P-phosphorylation of PI 4-monophosphate and PI 4,5-diphosphate was elevated at 3 min after LPA addition, and this activation was suppressed by C3 exoenzyme pretreatment. The immunoblot shown in Fig. 3A showed that electrophoresis on an 8% polyacrylamide gel separated p72 into at least three proteins, tyrosine phosphorylation of all of which was significantly suppressed by C3 exoenzyme treatment. It also revealed that the enzyme treatment suppressed the phosphorylation of a group of proteins between 110 and 130 kDa. p110-

**Fig. 3. Immunoprecipitation of the cell extracts with anti-p85 antibody and the PI 3-kinase activity associated with the immunoprecipitates.** A, control (lanes 1–3) and C3 exoenzyme-treated cells (lanes 4–6) were stimulated with 0.2 µM LPA for 0 (lanes 1 and 4), 1 (lanes 2 and 5), and 5 min (lanes 3 and 6). The cell extracts prepared were subjected to immunoprecipitation with a CA3 anti-p85 mouse monoclonal antibody. The immunoprecipitates were subjected to SDS-PAGE and immunoblotting with an anti-phosphotyrosine antibody. Lanes 1–6 show the immunoblots for total cell lysates corresponding to lanes 1–6. B, PI 3-kinase activity in immunoprecipitate from control (open columns) and C3 exoenzyme-treated cells (hatched columns) stimulated with 0.2 µM LPA for the indicated times was determined as described under "Experimental Procedures." The inset shows autoradiogram of thin layer chromatography of PI 3-kinase assay on the samples prepared from the control (lanes 1, 3, and 5) and C3 exoenzyme-treated cells (lanes 2, 4, and 6) incubated with LPA for 0 (lanes 1 and 2), 5 (lanes 3 and 4), and 10 min (lanes 5 and 6). Experiments were repeated twice with essentially identical results.

**Fig. 4. Tyrosine phosphorylation of p125 FAK in control and C3 exoenzyme-treated cells.** Control (lanes 1, 3, and 5) and C3 exoenzyme-treated (lanes 2, 4, and 6) cells were incubated with 2 µM LPA at 37 °C for 5 min after LPA addition but increased at 5 and 10 min after the addition. C3 exoenzyme treatment suppressed both basal and LPA-induced phosphorylation. The present study demonstrates that C3 exoenzyme pretreatment and consequently inactivation of rho p21 by ADP-ribosylation inhibited some of the LPA-induced tyrosine phosphorylation of the proteins. Because the initial transduction of LPA signaling as determined as inhibition of adenylate cyclase was not inhibited by C3 exoenzyme treatment, rho p21 most probably works downstream of second messenger generation. Recent studies have revealed that many agonists acting on G-protein-coupled receptors can induce tyrosine phosphorylation in Swiss 3T3 cells (26, 27) and activate some enzymes activated by tyrosine phosphorylation such as PI 3-kinase (28). The present study indicates that the activation of rho p21 may be a link between the stimulation of such receptors and some of the intracellular protein tyrosine phosphorylation events. It also suggests that the activation of PI 3-kinase by LPA is regulated at least in part by a rho p21-dependent pathway. One contentious point is the relationship of these tyrosine phosphorylations to the site of rho p21 action. Tyrosine phosphorylation of p125 FAK is induced similarly by several agonists (29) and by cell adhesion to the substrate matrix (30–32). Since the activation of rho p21 leads to integrin activation and cell adhesion (7–9), it is conceivable that the observed phosphorylations are secondary to the LPA-induced cell adhesion. However, cytochalasin B treatment did not affect some of the phosphorylations (e.g., the phosphorylation of MAP kinase), although it caused cell rounding and suppressed the phosphorylation of p125 FAK. In addition, C3 exoenzyme treatment suppressed the tyrosine phosphorylation of other proteins such as p72 and p88, which responded much faster to LPA than p125. It is therefore likely that rho p21 activates a tyrosine kinase cascade in these cells which eventually leads to cell adhesion. Recently, Volberg et al. (33) reported that the treatment of cultured Madin-Darby canine kidney cells with specific tyrosine phosphatase inhibitors induces focal adhesion and stress fiber formation, suggesting involvement of tyrosine phosphorylation in the induction of cell adhesion. Two modes of action for the ras-related small GTPases are now coming to focus. One is kinase regulation exemplified by ras p21, which complexes with c-raf and/or MAP kinase kinase kinase and regulates their activity (34). Recently Manser et al. (35) reported that CDC42 p21 can complex with a nonreceptor tyrosine kinase in a GTP-dependent fashion. The other is the regulation of translocation (36) shown by the ras family of GTPases, 2N. Kumagai, N. Morii, and S. Narumiya, unpublished observation.
which promote unidirectional vesicular transport between the two compartments. Thus far rho p21 action has been analyzed by following changes in the cell phenotype, and there is no definitive evidence as to the mode of action of rho p21. This is the first study showing that rho p21 can change functional parameters in the cell, which suggests that rho p21 can directly regulate an intracellular signaling molecules such as a tyrosine kinase.

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