LIM Kinase 1 Modulates Opsonized Zymosan-triggered Activation of Macrophage-like U937 Cells

POSSIBLE INVOLVEMENT OF PHOSPHORYLATION OF COFILIN AND REORGANIZATION OF ACTIN CYTOSKELETON*

We have previously reported that cofilin, an actin-binding protein, plays an important role in phagocyte functions, such as respiratory burst, phagocytosis, and chemotaxis. On the other hand, it was recently found that LIM motif-containing kinase (LIMK) phosphorylates cofilin. In this work, we investigated the roles of LIMK in activated phagocytes. The results of immunostaining showed that in dormant phagocytes the endogenous LIMK1 was diffusely distributed in the cytosol of macrophage-like U937 cells, and when activated by opsonized zymosan (OZ), it was translocated to plasma membranes. Green fluorescence protein (GFP)-conjugated LIMK was expressed in the phagocytes, and the GFP-positive cells were isolated by a fluorescence-activated cell sorter. The isolated wild-type LIMK-overexpressing cells produced superoxide at a rate that was 3.2-fold higher than that of only GFP-expressing control cells, whereas the respiratory burst of dominant negative mutant LIMK1(D460A)-expressing cells decreased to 31% of that of the control cells. Phagocytic activity monitored by using Texas Red-labeled OZ was also decreased in the D460A-expressing cells. By immunoblotting using a specific anti-phosphorylated cofilin antibody, it was revealed that in the OZ-activated wild-type LIMK1-GFP-expressing cells, the phosphorylated cofilin increased by 2.3-fold, and that in the OZ-activated D460A-GFP-expressing cells, the phosphorylated cofilin decreased to 47% of that of only GFP-expressing cells (mock control). Furthermore, in the wild-type LIMK1-expressing cells, OZ-evoked increase in filamentous actin was markedly enhanced, whereas in the dominant negative LIMK1-expressing cells, the total level of F-actin was strongly suppressed. These results suggest that LIMK1 regulates the functions of phagocytes through phosphorylation of cofilin and enhances the formation of filamentous actin.

**Phagocytes including neutrophils and macrophages rest**

without any stimuli, however, when activated by bacteria or other various harmful substances, they play a crucial role in host defense through various functions, such as chemotaxis, phagocytosis, and superoxide production. The activation mechanisms have been studied in terms of protein phosphorylation and cytoskeletal reorganization (1). Recently, we found that cofilin, an actin/phosphatidylinositol 4,5-bisphosphate-binding protein, is rapidly dephosphorylated in the phagocytes upon activation and translocated to plasma membranes (2). These findings were confirmed by other groups (3–5). It has been generally thought that among many actin-binding proteins, cofilin plays an essential role in control of the reorganization of actin in various types of cells (6). Only the unphosphorylated form of cofilin can bind actin and depolymerize the actin filament, and it was recently proposed that cofilin is involved in the turnover of actin filament. In the phagocytes, cofilin seemed to be involved in many important functions, including superoxide production (7, 8), phagocytosis (9), and chemotaxis (10). Recently, we also reported that opsonized zymosan (OZ), a complement C3bi-coated insoluble polysaccharide, binds to its specific receptor of the β2 integrin family (CR3, CD11b/CD18) to trigger the dephosphorylation of cofilin, and it occurs downstream of a tyrosine kinase and a phospholipase C (9, 11). However, in the phagocytes, the regulatory mechanisms of the phosphorylation of cofilin remain unclear. On the other hand, we and another group elucidated that LIM kinases phosphorylate cofilin (12, 13). In this paper, we investigated the roles of LIMK in the phagocytes. We found that OZ activation evoked changes in the intracellular distribution of endogenous LIMK1 and that expression of the protein kinase or its kinase-dead mutant modulate the functions of phagocytes. This is the first paper describing the roles of LIMK in immunological phagocytes.

**MATERIALS AND METHODS**

Cells and Vectors—Human monocytic leukemia U937 cells were obtained and maintained as described previously (9). DNA coding full-length LIM kinase 1 (wild-type or dominant negative mutant D460A) was inserted into a GFP expression vector pC1 (CLONTECH Inc., Palo Alto, CA) or into an expression vector pUC8 having a promoter sequence of SRα (14) and purified by using an EndFree kit (Qiagen, Hilden, Germany).

Antibodies and Reagents—An anti-PDZ domain of LIMK1 antibody that binds LIMK1 specifically was prepared by injection of a peptide of

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1 The abbreviations used are: OZ, opsonized zymosan; FACS, fluorescence-activated cell sorter; GFP, green fluorescence protein; LIMK, LIM motif-containing protein kinase; PBS, phosphate-buffered saline.
FIG. 1. *Time-dependent change in the intracellular distribution of endogenous LIMK1.* Macrophage-like U937 cells were stimulated with OZ for the indicated times, and the endogenous LIMK1 was visualized by immunostaining. *Upper panels* are photographs of the cells in light fields, and *lower panels* show the fluorescence-stained LIMK1 of the cells of the *upper panels*. Incubation times were 0 min (*a*), 3 min (*b*), 10 min (*c*), 20 min (*d*), and 30 min (*e*).

FIG. 2. *Flow cytometric analysis of LIMK1-GFP-expressing cells and their purification.* The morphologies of untransfected cells and dominant negative LIMK1-GFP (*D460A*), wild-type LIMK1-GFP (*WT*), or GFP only mock control-expressing cells were analyzed by forward and side scatters of fluorescence, and their fluorescence intensities were measured (*upper panels*). R2 indicates the region of untransfected cells. *Lower panels* show the fluorescence photographs of the cells before sorting (unfractionated) and of the cells sorted from regions R3 and R4 of the *upper panels* (purified).
the PDZ (amino acids 159–263 of LIMK1) to rabbits and purified by affinity chromatography. Fluorescein-conjugated donkey anti-rabbit IgG was obtained from Chemicon (Temecula, CA). The anti-phosphorylated cofilin antiserum was derived from a rabbit that was immunized with a phosphorylated N-terminal peptide (AcNH₂-pS/pGAVSVDCC)-conjugated keyhole limpet hemocyanin (15). The anti-phosphopeptide antibody reacts only with phosphorylated cofilin and not with unphosphorylated cofilin. Monoclonal anti-cofilin antibody was generously donated by Drs. H. Abe and T. Obinata (Chiba University, Chiba, Japan). The monoclonal antibody reacts with the C-terminal regions of both phosphorylated and unphosphorylated cofilin. Donkey IgG was purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Zymosan (Sigma) and Texas Red-labeled zymosan (Molecular Probes, Eugene, OR) were opsonized as described previously (7). Alexa Fluor 488-labeled phalloidin was purchased from Wako Chemical Co. (Osaka, Japan). All other chemicals used were commercial preparations of the highest purity.

**FIG. 3.** Superoxide production of LIMK-transfected U937 cells. The FACS-purified cells were stimulated with OZ, and the superoxide production was monitored by chemiluminescence. The graph shows the accumulated superoxide produced by wild-type LIMK1-GFP-transfected cells (a), GFP-transfected control cells (b), and dominant negative LIMK1-transfected cells (c). The photographs on the right show the superoxide-dependent reduction of nitro blue tetrazolium by the representative cells that were activated with OZ for 90 min. The letters a–c marked on the photographs represent the same cells as those in the graph on the left. Experimental details are described under “Materials and Methods.”

**FIG. 4.** Phagocytic activities of LIMK1-expressing U937 cells. The cells that expressed GFP mock control (A), wild-type LIMK1-GFP (B), or dominant negative LIMK1(D460A)-GFP (C) were incubated with Texas Red-labeled OZ for 15 min, and dual staining images were obtained by confocal laser-scanning microscopy. The GFP-expressing cells are green, and the particles of Texas Red-labeled OZ are red. The OZ particles engulfed by the cells are yellow as a merged color.
reaction was terminated by the addition of paraformaldehyde (final 2%), and F-actin in the cells was stained as described previously (8) using AlexaFluor 488-labeled phalloidin. The stained cells were observed by a confocal laser microscope as described above, and the intensities of fluorescence were determined by a flow cytometer (Facs-Calibur, BD Co.).

RESULTS

Translocation of Endogenous LIMK1—We have described that when macrophage-like U937 cells were activated with OZ, cofilin was translocated to plasma membranes, and F-actin was formed around the OZ-engulfing phagosomes (9). We then investigated the changes in intracellular distribution of LIMK1, a cofilin kinase. LIMK1 was visualized by a specific antibody that reacts with the PDZ domain of LIMK1. When exposed to OZ, the adherent cells moved on the surface of the dish and were sometimes aggregated. OZ was trapped specifically by its receptor CR3 and engulfed. As shown in Fig. 1, in the resting cells endogenous LIMK1 is diffusely distributed in the cytosol, and when the cells were stimulated with OZ, the LIMK1 was time-dependently translocated to plasma membranes especially around the deformed membranes engulfing OZ. The translocated LIMK1 is presumed to be involved in cytoskeletal remodeling through the phosphorylation of cofilin beneath the plasma membranes.

Expression of LIMK1 in Macrophage-like U937 Cells—To elucidate the roles of LIMK1 in the phagocytes, a wild-type or dominant negative LIMK1(D460A)-coding vector was transfected into the cells. The kinase was expressed as a GFP-fused protein whose kinase activity had been confirmed. Among various transfection reagents, Effectene was the most effective, although its efficiency was approximately 20%. In the preparation of the GFP-LIMK-expressing cells, there were many untransfected cells, and the debris derived from the dead or fragmented cells was also present. As shown in Fig. 2, the untransfected cells in their region (R2) showed weak fluorescence and were hardly ever present in the higher fluorescence region (R4). Therefore, the viable GFP-positive cells in region R4 were purified by a flow cytometer to perform the experiments described below.

Assay of Superoxide Production—The purified LIMK-transfected cells were stimulated with OZ, and the generated superoxide was assayed by the chemiluminescence method. The accumulated superoxide was calculated by integration, the obtained curves are shown in the graph in Fig. 3. The wild-type LIMK1-overexpressing cells produced superoxide at a rate of 3.2 times higher than the mock control cells, whereas the dominant negative LIMK1-expressing cells produced superoxide at a rate of approximately 31% of that of the control cells. The experiments were replicated seven times, and essentially the same results were obtained. Furthermore, the same tendency was observed when nitro blue tetrazolium was used as a superoxide-specific indicator. As shown in the photographs in Fig. 3, the wild-type LIMK1-expressing cells and the control cells engulfed OZ, and the insoluble precipitate of diformazan was generated in the phagosomes. However, such precipitates were hardly observed in the dominant negative LIMK1 (D460A)-expressing cells, although OZ attached to their cell surfaces. These data indicate that LIMK enhances the respiratory burst, whereas dominant negative LIMK1 inhibits it.

Phagocytosis—The phagocytic activities of the transfected cells were monitored by fluorescence microscopy using Texas Red-labeled OZ. The GFP-expressing cells are green, free Texas Red OZ are red, and the engulfed OZ are yellow as a merged color. As shown in Fig. 4, wild-type LIMK1-expressing cells and the control cells showed similar phagocytic activities, whereas dominant negative LIMK1-expressing cells had almost no activity. The loss of phagocytic activity of dominant negative LIMK1-expressing cells may be a result of their inability to form F-actin that is required for phagocytosis as described below.

Phosphorylated States of Cofilin—Because LIMK1 phosphorylates cofilin in vitro, the state of phosphorylation of cofilin in the kinase-expressing cells was examined using anti-phosphorylated cofilin antibody. As shown in Fig. 5, cofilin in the wild-type LIMK1-overexpressing cells was phosphorylated 2.3 times more than the mock control, but cofilin in the dominant negative LIMK1-transfected cells was less phosphorylated than in the control cells.

Activation-induced Changes in F-actin—Because F-actin-depolymerizing and severing activities of cofilin are dependent on the degree of its phosphorylation, dynamic changes in F-actin in the LIMK1-transfected cells were investigated. As shown in the photographs in Fig. 6, the fluorescent phalloidin-stained intracellular F-actin was transiently increased in the phagocytes upon activation by OZ. The rapid increase in F-actin was markedly enhanced in wild-type LIMK1-expressing cells. In the dominant negative LIMK1-expressing cells, the level of F-actin was totally suppressed. This tendency was quantitatively confirmed by flow cytometric analysis (Fig. 6, graph).

These results suggest that LIMK-dependent phosphorylation of cofilin enhanced the formation of stable F-actin, a major component of cortical cytoskeleton, which supports the respiratory burst and phagocytosis.

DISCUSSION

In this paper, we first examined the roles of LIMK1 in the phagocytes in the immune system and concluded that LIMK1 is deeply involved in their OZ-triggered functions based on the following observations. 1) Upon activation, the endogenous LIMK1 translocated from cytosol to the plasma membrane region where phagocytosis and superoxide production occurred. 2) Wild-type LIMK1-overexpressing cells showed increased OZ-production, whereas in the dominant negative LIMK1-expressing cells, the respiratory burst was suppressed. 3) The phagocytic activity of the dominant negative LIMK1-expressing cells was much lower than that of the control cells. These phenomena seem to be mediated not only by a change in the phosphorylated state of cofilin, because cofilin is the substrate of LIMK1 and because the result of immunoblotting indicated that cofilin was highly phosphorylated in the wild-type LIMK1-expressing cells and much less phosphorylated in dominant negative LIMK1-expressing cells.

In general, it is known that stimulation triggers an increase in F-actin in phagocytes including neutrophils (16) and macrophages (9, 17). The data of fluorescence microscopy and flow...
cytometry clearly show that the OZ-evoked increase in F-actin was remarkably enhanced in wild-type LIMK1-expressing cells, whereas it was suppressed in the dominant negative LIMK1-expressing cells. It is possible to consider that in the wild-type LIMK1-expressing cells F-actin depolymerizing activity of cofilin was inhibited by phosphorylation, leading to the enhancement of the OZ-triggered formation of F-actin, and in the dominant negative LIMK1-expressing cells, the increased amount of unphosphorylated cofilin sequestered monomeric actin and severed F-actin (6), resulting in the inhibition of the formation of F-actin.

It has been elucidated that the superoxide-generating enzyme NADPH oxidase is integrated into the plasma membranes of phagocytes and is activated by assembly with cytosolic factors p47phox, p67phox, p40phox, and rac (1). The translocation mechanisms of these factors have not been determined, whereas it has been reported that some of those factors interact with actin cytoskeleton (18, 19) or actin-binding proteins (7, 20), and that the activated oxidase is associated with Triton X-100-insoluble cytoskeleton (21). It is probable that the actin-cytoskeleton plays a crucial role in the translocation of the cytosolic factors and supports the activation of oxidase (1).

By using neutrophil-like HL-60 cells, we originally identified cofilin as a phosphoprotein, which was rapidly dephosphorylated upon activation (2). This observation was confirmed by other groups (3–5). However, the results of this study apparently contradict previous observations, because the LIMK1-dependent phosphorylation of cofilin seems to be an enhancing signal for the OZ activation of the macrophage-like cells. Based on all the data described here and in previous reports (2–5, 6–11), the following considerations are possible. At a very early stage of activation, the preexisting intracellular actin network, which supports the dormant stage of the phagocytes, should decay once, and dephosphorylated cofilin is required for the depolymerization of the preexisting filamentous actin. At the next stage, the monomeric actin must be reorganized to form new actin filaments, which are necessary to express the various functions, namely phagocytosis, respiratory burst, migration, and degranulation. Newly reconstructed F-actin may support these functions, and an excess amount of unphosphorylated cofilin seems to impair the reorganization of actin, leading to suppression of the phagocyte functions.

It has been reported that in other cells both p21(rac)-activated kinase (22) and Rho-activated kinase (23) phosphorylate the threonine of the activation loop of LIMK to phosphorylate cofilin. Rac may participate not only in the respiratory burst as mentioned above but also in the phosphorylation of cofilin. At present, cofilin is the only known substrate of LIMK1, however, there may be other substrates that could affect the reorganization of actin cytoskeleton. Further studies should be performed to resolve the signaling pathway in detail.

REFERENCES
1. Babior, B. M. (1999) Blood 93, 1464–1476
2. Suzuki, K., Yamaguchi, T., Tanaka, T., Kawanishi, T., Nishimaki-Mogami, T., Yamamoto, K., Tsuji, T., Irimura, T., Hayakawa, T., and Takahashi, A. (1995) J. Biol. Chem. 270, 19551–19556
3. Okada, K., Takano-Ohmuro, H., Obinata, T., and Abe, H. (1996) Exp. Cell Res. 227, 116–122
4. DiIaurazadeh, S., and Niggli, V. (1997) Exp. Cell Res. 236, 427–435
5. Heyworth, P. G., Robinson, J. M., Ding, J., Ellis, B. A., and Badwey, J. A. (1997) Histochem. Cell Biol. 108, 221–233
6. Bamburg, J. R. (1999) Annu. Rev. Cell Dev. Biol. 15, 185–230
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7. Suzuki, K., Yamaguchi, T., Oshizawa, T., Yamamoto, Y., Nishimaki-Mogami, T., Hayakawa, T., and Takahashi, A. (1995) Biochem. Biophys. Acta 1266, 261–267.

8. Nagaishi, K., Adachi, R., Kawanishi, T., Yamaguchi, T., Kasahara, T., Hayakawa, T., and Suzuki, K. (1999) J. Biochem. 125, 891–898.

9. Nagaishi, K., Adachi, R., Matsui, S., Yamaguchi, T., Kasahara, T., and Suzuki, K. (1999) J. Cell. Physiol. 180, 345–354.

10. Adachi, R., Matsui, S., Kinoshita, M., Nagaishi, K., Sasaki, H., Kasahara, T., and Suzuki, K. (2000) Int. J. Immunopharmacol. 22, 855–864.

11. Matsui, S., Adachi, R., Kusui, K., Yamaguchi, T., Kasahara, T., Hayakawa, T., and Suzuki, K. (2001) Cell. Signal. 13, 17–22.

12. Arber, S., Barbayannis, F. A., Hanser, H., Schneider, C., Stanyon, C. A., Bernard, O., and Caroni, P. (1998) Nature 393, 805–809.

13. Yang, N., Higuchi, O., Ohashi, K., Nagata, K., Wada, A., Kangawa, K., Nishida, E., and Mizuno, K. (1998) Nature 393, 809–812.

14. Tsukamoto, T., Miura, S., Nakai, T., Yokota, S., Shimozawa, N., Suzuki, Y., Orri, T., Fujiki, Y., Sakai, F., Bogaki, A., Yasuno, H., and Osumi, T. (1995) Nat. Genet. 11, 395–401.

15. Toshima, J., Toshima, J. Y., Amano, T., Yang, N., Narumiya, S., and Mizuno, K. (2001) Mol. Biol. Cell 12, 1131–1145.

16. Downey, G. P., Chan, C. K., Lea, P., Takai, A., and Grinstein, S. (1992) J. Cell Biol. 116, 695–706.

17. Greenberg, S., el Khoury, J., di Virgilio, F., Kaplan, E. M., and Silverstein, S. C. (1991) J. Cell Biol. 113, 757–767.

18. Nauseef, W. M., Volpp, B. D., McCormick, S., Leidal, K. G., and Clark, R. A. (1991) J. Biol. Chem. 266, 5911–5917.

19. El Benna, J., Ruedi, J. M., and Babior, B. M. (1994) J. Biol. Chem. 269, 6729–6734.

20. Grogan, A., Reeves, E., Keep, N., Wientjes, F., Totty, N. F., Burlingame, A. L., Hsuan, J. J., and Segal, A. W. (1997) J. Cell Sci. 110, 3071–3081.

21. Woodman, R. C., Ruedi, J. M., Jesaitis, A. J., Okamura, N., Quinn, M. T., Smith, R. M., Curtinute, J. T., and Babior, B. M. (1991) J. Clin. Invest. 87, 1345–1351.

22. Edwarde, D. C., Sanders, L. C., Bokoch, G. M., and Gill, G. N. (1999) Nat. Cell Biol. 1, 253–259.

23. Maekawa, M., Ishizaki, T., Boku, S., Watanabe, N., Fujita, A., Iwamatsu, A., Obinata, T., Ohashi, K., Mizuno, K., and Narumiya, S. (1999) Science 285, 895–898.
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