Dynein Light Chain 1 Phosphorylation Controls Macropinocytosis*

Zhibo Yang, Ratna K. Vadlamudi, and Rakesh Kumar†

From the Department of Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Recent studies have identified dynein light chain-1 (DLC1), a component of the dynein motor, as a p21-activated kinase 1 (Pak1)-interacting substrate with binding sites mapped to amino acids 61–89 of DLC1 and phosphorylation site at serine 88. Here we investigated the role of DLC1 phosphorylation by Pak1 upon the process of macropinocytosis. We found that Pak1 associates with dynein motor and that Pak1-DLC1 interaction starts at the initiation of pinosome formation and persists in early and late endosomes. Pak1 phosphorylation of DLC1 on Ser-88 controls vesicle formation and trafficking functions, as Ser-88 substitution for alanine prevents macropinocytosis. A peptide spanning the C-terminal 19-amino acid region of DLC1 efficiently blocked Ser-88 phosphorylation and macropinocytosis. These results suggest that the regulation of DLC1 by Pak1 is a novel mechanism by which a signaling kinase might influence macropinocytosis.

The p21-activated kinases (Paks),1 an evolutionarily conserved family of serine/threonine kinases, are important for a variety of cellular functions, including cell morphogenesis, cell motility, cell survival, angiogenesis, and mitosis (1, 2). Pak1 was identified as one of the targets of the activated Rho GTPases Cdc42 and Rac1, which stimulate Pak autophosphorylation and activity (3). Stimulation of Pak1 activity results in several phenotypic changes reminiscent of those produced by Cdc42 and Rac1 (4–7). Pak1 is widely expressed in numerous tissues and is activated by a number of polypeptide factors and extracellular signals in both a GTPase-dependent (via Rac1 or Cdc42) and GTPase-independent manner via its localization to membrane/focal adhesion (8–10). Pakks are also activated by lipids (11), tyrosine kinases (12, 13), novel substrates such as filamin (2), and G-proteins (14).

The activation of Pak1 by diverse signals leads to its autophosphorylation at multiple sites, including threonine 423 (Thr-423), within the activation loop of the kinase (15). Expression of an activated Pak1 mutant (T423E) triggers the dissolution of stress fibers and focal adhesion complexes, the formation of lamellipodia (4, 16), and reorganization of the actin cytoskeleton. Some effects of Pak1 on the actin cytoskeleton appear to be independent of Pak1 kinase activity but dependent on protein-protein interactions (4, 18).

In addition to cell locomotion, actin-dependent plasma membrane ruffling is essential to the internalization of exogenous macromolecules and fluids by macropinocytosis in stimulated mammalian cells (19, 20). Macropinocytosis is a cell type-specific endocytic pathway involving the formation of endocytic vesicles and the closure of lamellipodia at the sites of membrane ruffling to form the large (0.2–3 μm) irregular vesicles known as macropinosomes (21, 22). The process of macropinocytosis also requires the small GTPases and Pak1 (23, 24). A fraction of Pak1 was observed to be colocalized with the vesicular fraction (25). Macropinocytosis is important in the uptake of macromolecules in epithelial cells, neutrophils and macrophages (26). Macropinocytosis is also important in taking up extracellular antigens into antigen-presenting dendritic cells (27). A close relationship also exists between constitutive ruffling and macropinocytosis in oncogene-transformed cells (28). Furthermore, growth factor stimulation of macropinocytosis has been implicated in directional locomotion, because regulation of membrane flux via pinocytosis may contribute to the membrane flow, therefore generating force of cell locomotion (24, 29, 30). Despite the widely acknowledged roles of Pak1 in macropinocytosis and of macropinocytosis in pathogen entry, the nature of the responsible molecular mechanism remains unknown.

Cytoskeleton remodeling-dependent cellular processes, such as vesicle transport and membrane transport, are influenced by dynein, a multiprotein complex originally shown to regulate chromosome movement, assembly, and orientation of mitotic spindles and nuclear migration (31). Dynein light chain-1 (DLC1), an 8-kDa component of the cytoplasmic dynein complex, is a minus end-directed microtubule-based motor that transports cargo along microtubules (32). Subsequent studies identified DLC1 as a stoichiometric subunit of myosin V motor, essential for short-range transport of vesicles in the actin-rich cortex of the cells (33, 34). DLC1 is highly conserved among species and widely expressed in a number of tissues. In addition to its essential role in dynein motor function, DLC1 interacts with a number of regulatory proteins with diverse functions. For example, DLC1 associates with neuronal nitric oxide synthase and inhibits pro-apoptotic function (35). DLC1 also interacts and interferes with the pro-apoptotic Bcl-2 family protein BimL (36). Recent data suggest that DLC1 is also a Pak1-interacting substrate with binding sites mapped to amino acids 61–89 of DLC1 and phosphorylation at Ser-88 (37). Because DLC1 is a physiologic interacting substrate of Pak1 and because previous studies have implicated Pak1 in macropinocytosis, here we investigated the role of DLC1 phosphorylation upon the process of macropinocytosis.

EXPERIMENTAL PROCEDURES

Cell Cultures, Reagents, and Transfections—MCF-7 and ZR75 breast cancer cells (38) and NIH3T3 cells were maintained in Dulbecco’s modi-
FIG. 1. Association of DLC1 and Pak1 with macropinocytosis markers. Fluid-phase macropinocytosis markers of 0.5 mg/ml FITC-dextran (a, b) or 0.5 mg/ml Lucifer yellow (c) were added to the medium of NIH3T3 cells serum-starved for 24 h and treated with 1 nM lysophosphatidate (b) or 10% serum (a, c) for 30 min. After internalization and incubation at 37 °C for 30 min, cells were fixed with 4% PFA for 20 min, and the internalization of markers and their colocalization with endogenous Pak1 were analyzed by confocal microscopy. The white color (a, c) results from the overlap between Pak1 (blue) and FITC-dextran (green in panel a) or Lucifer yellow (yellow in panel c). The yellow color (b) results from the overlap between Pak1 (red) and FITC-dextran (green in panel b). d, the colocalization (yellow) of internalized FITC-dextran (green) and DLC1 (red) in NIH3T3 cells transfected with red fluorescent protein (RFP-DLC1) and stimulated with LPA was analyzed by confocal microscopy after 30 min of incubation with 0.5 mg/ml FITC-dextran at 37 °C. e, the colocalization (yellow) of FITC-dextran (green) and DLC1 in MCF-7 cells transfected with RFP-DLC1 (red). Twenty-four hours after transfection, 24-h serum-starved cells were stimulated with serum for 15 min, and 0.5 mg/ml FITC-dextran was added to the medium. After incubation at 37 °C for 20 min, cells were fixed with cold methanol for 5 min, and the colocalization was visualized with confocal microscopy. f, the colocalization (pink) of DLC1 (red) and Pak1 (blue) in breast cancer cell line ZR75 cultured in Dulbecco’s modified Eagle medium-F12 (1:1) supplemented with 10% fetal calf serum. F-actin (green) was detected using FITC-conjugated phalloidin.

FIG. 2. Pak1 signaling in macropinocytosis. a, growth factor regulation of macroinosomes. ZR75 cells expressing either RFP or RFP-DLC1 were serum starved for 24 h, FITC-dextran was added for 5 min, and cells were then stimulated with 1 nM epidermal growth factor for 20 min. Localization of RFP-DLC1 (red) and FITC-dextran (green) was imaged using confocal microscopy. b, requirement of Pak1 in DLC1-mediated macropinocytosis. ZR75 cells expressing RFP-DLC1 were incubated with FITC-dextran. After 20 min, cells were fixed with methanol and stained for endogenous Pak1 (blue), RFP-DLC1 (red), and FITC-dextran (green). An inset containing the cytoplasmic region was blown up to visualize the colocalization of Pak1 and RFP-DLC1 on FITC-dextran. Merged images are presented in the upper right panel, and colocalization of Pak1 with RFP-DLC1 can be visualized as white vesicles due to colocalization of pixels from Pak1 (blue), RFP-DLC1 (red), and FITC-dextran (green). c, ZR75 cells expressing RFP-DLC1 were transfected with a dominant negative Pak1 mutant K299RLL (KLL-Pak1), a Myc-tagged kinase-dead and GTPase binding defective construct of Pak1. After 24 h, cells were incubated with FITC-dextran for 20 min. Cells were fixed in methanol and stained for Myc-tagged KLL-Pak1 (blue), RFP-DLC1 (red), and FITC-dextran (green). Myc epitope tag staining (blue) was used to identify cells transfected with KLL-Pak1. Note in blue cells expressing KLL-Pak1, there was no or very little uptake of FITC-dextran.

Pak Assay—In vitro kinase assays using myelin basic protein or glutathione S-transferase (GST)-DLC1 protein (4 μg each) were performed in HEPES buffer (50 mM HEPES, 10 mM MgCl₂, 2 mM MnCl₂, 0.2 mM dithiothreitol) containing 100 ng of purified bacterially expressed GST-Pak1 enzyme, 10 μCi of [γ-³²P]ATP, and 25 μM COLD ATP (2). In some experiments, DIC immunoprecipitates were used as a substrate in lieu of myelin basic protein.

Immunofluorescence and Confocal Studies—The cellular location of proteins was determined using indirect immunofluorescence (2). Briefly, cells grown on glass cover slips were fixed in methanol at −20 °C for 6 min. Cells were incubated with primary antibodies for 2 h at room temperature, washed three times with phosphate-buffered saline, and then incubated with 546 Alexa (red), 633 Alexa (blue), or 488 Alexa (green) labeled secondary antibodies (Molecular Probes). The DNA dye Topro-3 was used to co-stain the DNA, which gives an emission in the far red segment of the light spectrum, and color-coded in blue (Molecular Probes). Confocal analysis was performed with a Zeiss laser-scanning confocal microscope using established methods of processing of the same section for each detector (the two excitations corresponding to 546 and 488, or 633 nm), comparing the sections pixel by pixel. Quantification of vesicle-like structure was done in some experiments as follows. Ten randomly selected fields were analyzed for each condition. The experiment was repeated two to three times. Quantitation of ves-
were treated with LPA for 30 min in the presence of FITC-dextran. The colocalization of RFP-DLC1 (red) with dextran (green) can be seen as a yellow color in merged images (Fig. 1d). Similarly, we further examined the colocalization of DLC1 with macroincotic markers and found that RFP-DLC1 was colocalized with FITC-dextran in more than 85% of transfected MCF-7 breast cancer cells stimulated with serum (Fig. 1e), suggesting a role of DLC1 in macroinocytosis of stimulated epithelial cells. The colocalization of Pak1 with DLC1 in ZR75 breast cancer cells stimulated with serum (Fig. 1f) indicated the potential regulatory role of DLC1-Pak1 pathway in the process of macroinocytosis.

To clarify the role of DLC1 in macroinocytosis, using the ZR75 breast cancer cell line we generated two stable clones expressing either RFP vector only or RFP-DLC1. We found that RFP-DLC1 clone is much more active in uptake of FITC-dextran and also that RFP-DLC1 colocalizes with FITC-dextran upon epidermal growth factor treatment (Fig. 2a). The colocalization was found of transient transfected RFP-DLC1 (red) and FITC-dextran (green), as well as endogenous Pak1 (blue), in ZR75 breast cancer cells (Fig. 2b), suggesting that the DLC1-localized dextran vesicles also contain Pak1. To show the dependence of DLC1-mediated endocytosis on Pak1 activation, we expressed kinase-dead K299RLL-Pak1 (5) into ZR75 breast cancer cells (Fig. 2c), suggesting that the DLC1-localized dextran vesicles also contain Pak1. To show the dependence of DLC1-mediated endocytosis on Pak1 activation, we expressed kinase-dead K299RLL-Pak1 (5) into ZR75 breast cancer cells (Fig. 2c), suggesting that the DLC1-localized dextran vesicles also contain Pak1. To show the dependence of DLC1-mediated endocytosis on Pak1 activation, we expressed kinase-dead K299RLL-Pak1 (5) into ZR75 breast cancer cells (Fig. 2c), suggesting that the DLC1-localized dextran vesicles also contain Pak1. To show the dependence of DLC1-mediated endocytosis on Pak1 activation, we expressed kinase-dead K299RLL-Pak1 (5) into ZR75 breast cancer cells (Fig. 2c), suggesting that the DLC1-localized dextran vesicles also contain Pak1. To show the dependence of DLC1-mediated endocytosis on Pak1 activation, we expressed kinase-dead K299RLL-Pak1 (5) into ZR75 breast cancer cells (Fig. 2c), suggesting that the DLC1-localized dextran vesicles also contain Pak1. To show the dependence of DLC1-mediated endocytosis on Pak1 activation, we expressed kinase-dead K299RLL-Pak1 (5) into ZR75 breast cancer cells (Fig. 2c), suggesting that the DLC1-localized dextran vesicle formation was done using the following scoring system: −, cells containing 0–9 vesicles; +, cells containing 10–20 vesicles; ++, cells containing >20 vesicles.

RESULTS AND DISCUSSION

Emerging data suggest that the functions of DLC1 are regulated by its phosphorylation on Ser-88 by its upstream kinase Pak1 that also interacts with the penultimate C-terminal 19 amino acids of DLC1 (37). Because DLC1 was shown to be an essential component of dynein motors (31, 32), we reasoned that the process of macroinocytosis might utilize cellular motor. To explore the potential role of DLC1-Pak1 pathway in the process of macroinocytosis, we asked whether DLC1 (and also Pak1) colocalizes with macroinocytosis markers. Consistent with the earlier findings (25), we found Pak1 colocalization with detectable endocytosed FITC-dextran (Fig. 1, a and b) and also with endocytosed Lucifer yellow (Fig. 1c), the two commonly used markers of macroinocytosis, in NIH3T3 cells pretreated with either lysophosphatidate (LPA) or serum for 30 min using scanning confocal microscopy. To show the association of DLC1 with macroinocytotic markers, NIH3T3 cells transfected with red fluorescent protein (RFP)-tagged DLC1
possibilities, we colocalized Pak1 and DLC1 with EEA1, a well established marker of early endosome (39). Because both Pak1 and EEA1 antibodies are of rabbit origin, we used GFP-tagged Pak1 and RFP-tagged DLC1 and anti-EEA1 antibodies to detect endogenous EEA1. Results showed the presence of DLC1, Pak1, and EEA1 on small vesicle-like structures. Analysis of merged images showed colocalization (pink) of DLC1 with EEA1 and colocalization (yellow) of DLC1 with GFP-Pak1. Further analysis of the merged image of three colors (white) showed Pak1 and DLC1 colocalization with early endosome marker EEA1 (Fig. 3a).

Because dynein is also known to participate in the transport of late endosomes, we used a late endosomal marker LAMP1 (40) to analyze whether Pak1-DLC1 interaction also occurs on late endosomes. As expected, RFP-DLC1 colocalized with LAMP1 on vesicular structures (Fig. 3b). Interestingly, GFP-Pak1 colocalized with late endosomal marker LAMP1; colocalization of GFP-Pak1, RFP-DLC1, and LAMP1 was observed as well (Fig. 3b, white color). The presence of Pak1 and DLC1 on macropinosytic markers, such as FITC-dextran, and their continued presence on early and late endosomes suggested that Pak1-DLC1 interaction may have multiple roles, including formation, maturation, and trafficking of macropinosomes.

Because DLC1-containing dynein motors have been implicated in vesicle transport using microtubules (32, 33, 41) and because we noticed colocalization of Pak1 and DLC1 with the early and late endosomes, we next examined whether Pak1 also colocalizes with DLC1 in the context of dynein motor, using DIC as a marker of the dynein complex. Confocal analysis revealed colocalization of GFP-Pak1 with RFP-DLC1 (Fig. 4a) and, as expected, DIC was colocalized with DLC1. In some areas, triple colocalization of GFP-Pak1, RFP-DLC1, and DIC (shown by white color) was observed (Fig. 4a). To confirm physiologic interaction of Pak1 with dynein motors, immunoprecipitation experiments were performed using antibodies specific to DIC to precipitate dynein motor complex. Pak1 was found in epidermal growth factor-activated DIC immunoprecipitates (Fig. 4b), suggesting that Pak1 associates with the dynein motor complexes. Furthermore, wild-type Pak1, but not kinase-dead K299R Pak1 enzyme, could phosphorylate DLC1 as a part of native dynein complex precipitated from MCF-7 cells (Fig. 4c).

To delineate the underlying mechanism by which DLC1-Pak1 interaction might regulate macropinocytosis, we next explored the potential involvement of DLC1 phosphorylation by Pak1. We cotransfected SKBR3 breast cancer cell line cells with GFP-Pak1 either together with RFP-DLC1 or with RFP-DLC1-S88A, which lacks the Pak1 phosphorylation site. Vesicle formation and localization of GFP and RFP proteins were analyzed by confocal microscopy. Ten randomly selected fields were analyzed under microscope for quantitation of vesicle-like structure. As compared with the cells transfected with wild-type DLC1, the vesicle-like structures were reduced 90% in cells transfected with DLC1-S88A (Fig. 5a). To further clarify whether Pak1-mediated macropinocytosis requires Pak1-DLC1 interactions, we transfected NIH3T3 cells with RFP-DLC1,
The expression of RFP-DLC1-S88A and RFP-DLC1-S88E substantially interfered with the macropinocytosis of FITC-dextran (Fig. 5b). In contrast, expression of RFP-DLC1-S88E, which mimics the phosphorylated form because of a negative charge, profoundly enhanced the macropinocytosis of FITC-dextran as compared with RFP-DLC1 (Fig. 5b). Quantitative analysis of macropinocytic uptake showed that the DLC1-S88E mutant substantially enhanced macropinocytosis compared with that of wild-type DLC1-transfected cells, whereas the DLC1-S88A mutant showed 50% less uptake of FITC-dextran compared with that of wild-type RFP-DLC1-expressing cells (Fig. 5c). In addition, DLC1-S88A blocked macropinocytosis in the presence of active Pak1 (supplemental Fig. 1), suggesting that Pak1 binding and phosphorylation of DLC1 participates in macropinocytosis.

Because the Pak1 binding region and phosphorylation site are localized to the C-terminal 19 amino acids of DLC1 (37) and because mutants of DLC1 lacking a Pak1 binding/phosphorylation site effectively inhibited macropinocytosis (Fig. 5), we hypothesized that the C-terminal peptide of DLC1 containing the Pak1 interaction site may interfere with Pak1 functions and act as a potent inhibitor of macropinocytosis. To test this hypothesis, we synthesized a peptide consisting of the C-terminal 19 amino acids of DLC1 fused to the TAT protein polybasic sequence (Fig. 6a) to facilitate entry into cells (17). To detect the cellular uptake of this peptide, it was biotinated at the N-terminus. Initially, we used in vitro kinase assays to determine whether the DLC1 peptide interferes with Pak1 phosphorylation of wild-type DLC1. Addition of DLC1 peptide inhibited Pak1-mediated phosphorylation of wild-type DLC1 in a dose-dependent manner, whereas the control TAT peptide had no significant effect (Fig. 6b). Pak1 also efficiently phosphorylated the synthetic DLC1 C-terminal peptide in in vitro kinase assays (Fig. 6c). The TAT-DLC peptide disrupted Pak1-DLC1 complex formation in GST pulldown assay (Fig. 6d). These results suggested that the DLC1 peptide we synthesized con-
tained the Pak1 binding region and phosphorylation site.
Next we visualized entry of the DLC1 peptide into the cells and determined its colocalization with Pak1. Biotin-labeled DLC1 peptide was added into the medium of NIH3T3 cells. 60 min later, cells were fixed and stained for biotin with streptavidin (red) and endogenous Pak1 (blue). The DLC1 peptide efficiently entered cells (Fig. 7, a and b) and colocalized with endogenous Pak1 (Fig. 7a). Confocal analysis revealed that DLC1 peptide treatment prevented interaction of Pak1 with dynemin complex (data not shown). We next tested whether the DLC1 peptide would inhibit macropinocytosis by interfering with Pak1-DLC1 interaction. NIH3T3 cells were stimulated with 10% serum to accelerate the process of macropinocytosis. Cells were pretreated with control peptide (Fig. 7c) or DLC1 peptide (Fig. 7d) for 30 min, and then FITC-dextran was added into the medium. Uptake of FITC-dextran by macropinocytosis was analyzed by confocal microscopy. Quantitation of macropinocytosis by counting the cellular uptake of FITC-dextran showed a 75% reduction in bead uptake in DLC1 peptide-treated cells compared with bead uptake in control peptide-treated cells (Fig. 7e). Together, these results suggested that the C-terminal DLC1 peptide is a potent inhibitor of macropinocytosis.

In brief, results presented here suggested that DLC1 regulation by Pak1 might represent an essential step in the process of macropinocytosis. In this study we showed that Pak1 associates with dynemin motor and that Pak1-DLC1 interaction starts at the initiation of pinosome formation and persists in early and late endosomes. Pak1 phosphorylation of DLC1 on Ser-88 controls vesicle formation and trafficking functions, as Ser-88 substitution for alanine prevents macropinocytosis. A peptide spanning the C-terminal 19-amino acid region of DLC1 efficiently blocked Ser-88 phosphorylation and macropinocytosis. These results suggest that the regulation of DLC1 by Pak1 is a novel mechanism by which a signaling kinase might influence macropinocytosis.

Acknowledgment—We thank Liana Adam for partial confocal microscopy.

REFERENCES
Dynein Light Chain 1 Phosphorylation Controls Macropinocytosis
Zhibo Yang, Ratna K. Vadlamudi and Rakesh Kumar

doi: 10.1074/jbc.M408486200 originally published online October 24, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M408486200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2004/11/17/M408486200.DC1

This article cites 41 references, 20 of which can be accessed free at
http://www.jbc.org/content/280/1/654.full.html#ref-list-1