Inactivation of Cdc42 Is Necessary for Depolymerization of Phagosomal F-Actin and Subsequent Phagosomal Maturation

Maria Lerm, Veronika Patcha Brodin, Iida Ruishalme, Olle Stendahl and Eva Särndahl

*J Immunol* 2007; 178:7357-7365; doi: 10.4049/jimmunol.178.11.7357
http://www.jimmunol.org/content/178/11/7357

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2008/03/13/178.11.7357.DC1

**References**
This article cites 54 articles, 25 of which you can access for free at:
http://www.jimmunol.org/content/178/11/7357.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Inactivation of Cdc42 Is Necessary for Depolymerization of Phagosomal F-Actin and Subsequent Phagosomal Maturation

Maria Lerm, Veronika Patcha Brodin, Iida Ruishalme, Olle Stendahl, and Eva Särndahl

Phagocytosis is a complex process involving the activation of various signaling pathways, such as the Rho GTPases, and the subsequent reorganization of the actin cytoskeleton. In neutrophils, Rac and Cdc42 are activated during phagocytosis but less is known about the involvement of these GTPases during the different stages of the phagocytic process. The aim of this study was to elucidate the role of Cdc42 in phagocytosis and the subsequent phagosomal maturation. Using a TAT-based protein transduction technique, we introduced dominant negative and constitutively active forms of Cdc42 into neutrophil-like HL60 (human leukemia) cells that were allowed to phagocytose IgG-opsonized yeast particles. Staining of cellular F-actin in cells transduced with constitutively active Cdc42 revealed that the activation of Cdc42 induced sustained accumulation of periphagosomal actin. Moreover, the fusion of azurophilic granules with the phagosomal membrane was prevented by the accumulated F-actin. In contrast, introducing dominant negative Cdc42 impaired the translocation per se of azurophilic granules to the periphagosomal area. These results show that efficient phagosomal maturation and the subsequent eradication of ingested microbes in human neutrophils is dependent on a strictly regulated Cdc42. To induce granule translocation, Cdc42 must be in its active state but has to be inactivated to allow depolymerization of the F-actin cage around the phagosome, a process essential for phagolysosome formation. The Journal of Immunology, 2007, 178: 7357–7365.

Phagocytosis of microorganisms requires a complex machinery of membrane reconstitution and rearrangements of the actin cytoskeleton (1, 2). The identification of the Rho family of small GTPases and their functions has largely improved the understanding of the mechanisms by which the actin cytoskeleton is regulated. Rho GTPases, including Rho, Rac, and Cdc42, act as molecular switches by cycling between GDP- and GTP-bound states (3). In their active GTP-bound state, Rho GTPases regulate cellular functions through interaction with downstream effectors, many of which are involved in the dynamic rearrangement of actin filaments.

In phagocytic cells such as neutrophils and macrophages, two main routes of phagocytosis of opsonized particles have been described (4). Particles coated with C3b/C3bi fragments of the complement system are recognized by the complement receptors CR1 and CR3 (also called β2 integrin), whereas particles coated with IgG are recognized by FcyR. In macrophages, phagocytosis of IgG-coated particles requires Rac and Cdc42 (4). The activation of Rac and Cdc42 induces the formation of membrane extensions, i.e., pseudopodia, whereas phagocytosis of C3b/C3bi-opsonized particles, which does not require membrane protrusions, involves activation of RhoA (4). Looking more closely at the involvement of Rac and Cdc42 during IgG-mediated phagocytosis in macrophages, Hoppe and Swanson (5) showed that activated Cdc42 is restricted to the leading edge of the cell and to the tip of the phagocytic cup, whereas activated Rac1 is found evenly distributed throughout the phagocytic cup. Neither protein in its activated form is localized around the phagosome. In neutrophils, Rac and Cdc42 are activated during both CR3- and FcyR-mediated phagocytosis (6). However, the role of these Rho GTPases during the different stages of phagocytosis in neutrophils is, to date, not established (6–9). For phagolysosome formation to occur, the actin network coating the phagocytic cup and the developing phagosome must be reduced to allow proper apposition and the subsequent fusion of granules with the phagosome (10, 11). In fact, certain pathogens, e.g., Leishmania and Salmonella, build up periphagosomal F-actin as a strategy for intracellular survival (12–14).

The present study investigates how Cdc42 modulates F-actin around IgG phagosomes during phagocytic maturation in human neutrophils. Using the TAT transduction technique, we show that constitutively active Cdc42 markedly enhances the amount of F-actin surrounding phagosomes containing IgG-opsonized yeast. Furthermore, we find that Cdc42-mediated accumulation of F-actin at the phagosome results in the prevention of phagolysosome fusion. Previous studies in neutrophils have shown the activation of Rho GTPases during phagocytosis but have not clarified the role of active/inactive Rho GTPases during the formation of the phagosome and the subsequent phagolysosome. This is the first study showing a role for Cdc42 in the remodeling of periphagosomal F-actin, and our results suggest that a tightly regulated Cdc42 is required for an efficient phagolysosome formation in human neutrophils.
Purification of TAT proteins

The pTAT-HA vector carrying dominant negative (N17) and constitutively active (V12) mutants of Cdc42 (N17Cdc42 and V12Cdc42, respectively) was transformed into the E. coli strain B121 (DE3), and well-expressing clones were identified. Large-scale purifications were performed in Luria-Bertani broth containing 50 μg/ml ampicillin and 0.1 mM isomoprol β-D-thiogalactoside. The 6His-tagged TAT fusion proteins were purified from the E. coli lysates using Ni-NTA-agarose columns as previously described (15). Briefly, the bacteria were collected by centrifugation (5,000 × g for 10 min at 4°C), lysed in buffer Z (8 M urea and 10 mM imidazol), sonicated, and centrifuged (18,000 × g for 10 min at 4°C). The supernatants were loaded onto Ni-NTA-agarose for affinity purification of the 6His-tagged TAT-Cdc42. The columns were washed with 25 bed volumes of buffer A. The proteins were eluted with an increasing concentration of imidazole (0.1, 0.25, 0.5, and 1.0 M) in buffer Z. The eluted fractions were checked for protein concentration using Bradford reagent, pooled, and desalted on PD-10 columns equilibrated with buffer B (50 mM Tris (pH 7.4) and 10% glycerol). After elution in the Tris-buffer, the protein preparations were aliquoted and stored at −70°C. The amount of protein in each preparation was estimated on a Coomassie gel. The eluted protein, which migrated as a single band with a molecular mass of ~30 kDa, was checked by Western blotting using anti-HA Abs (rabbit polyclonal) and anti-Cdc42 Abs (mouse monoclonal). The amount of coating LPS, which was determined by means of a Limulus assay, ranged between 5 and 20 ng/ml (final concentration). To prevent unwanted effects due to LPS, 500 μg/ml PMB was added to the protein preparations before use (final concentration of PMB with the cells was 10 μg/ml) (14). Because PMB did not affect the efficiency of the TAT transduction, the survival of the cells, the cell morphology, or phagocytosis, it was used in all samples throughout the study.

Preparation of human neutrophils

Peripheral human blood was drawn from healthy volunteers and collected in heparin-containing Vacutainer tubes, and neutrophils were isolated by means of sucrose gradient centrifugation as previously described (17–19). The obtained cell suspension, consisting of >95% neutrophils, was resuspended in ice-cold Krebs-Ringer phosphate buffer (KRG; 120 mM NaCl, 1.2 mM MgSO4, 7.1 mM KCl, 1.7 mM KH2PO4, 8.3 mM NaHCO3, 2 H2O, 1 mM CaCl2, 4.9 mM KCl, and 10 mM glucose (pH 7.3)).

Cell culture

The promyelocytic human leukemia HL60 cells were grown in suspension culture in RPMI 1640 supplemented with 10% heat-inactivated FCS, t-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (1 mg/ml), as previously described (20). The cells were maintained in a humidified atmosphere of 5% CO2 at 37°C and given fresh medium every third day. The cells were induced to differentiate by the addition of DMSO (1.3%; v/v) and harvested after 6 or 7 days (21). By then, >75% of the cells had differentiated to granulocyte-like cells as measured by NBT reduction.

Transduction of TAT-Cdc42

Transduction of TAT-Cdc42 was generally performed in KRG without Ca2+ to avoid activation of the cells. Ca2+ depletion did not reduce the amount of TAT-Cdc42 found in the cell lysates. Neutrophils and HL60 cells were incubated at 37°C with 200 nM PMB-treated TAT fusion protein for the indicated times (Fig. 1). To test whether the proteins were successfully transduced, the cells were collected by centrifugation (200 × g for 10 min at room temperature), washed three times, and lysed in sample buffer. The samples were treated with Benzonase to cleave DNA and subjected to SDS-PAGE. The proteins were transferred onto nitrocellulose, and the TAT-GTPases were detected by means of ECL using a rabbit polyclonal Ab directed toward the HA epitope and a secondary HRP-conjugated anti-rabbit antibody. To analyze the amount of TAT fusion protein, EDC-conjugated anti-HA Fab’s were incubated (on ice for 10 min) together with the TAT-fusion protein and PMB before being given to the cells (37°C). After washing, the fluorescence was determined by microscopy. Nontransduced cells, i.e. cells treated as described above but not subjected to TAT proteins, were used as control and showed no intracellular fluorescence.

Phagocytosis

After TAT transduction, the cells were washed twice and resuspended in KRG. Phagocytosis on glass slides was performed as previously described (22). In short, TAT-transduced cells were seeded on glass slides covered with IgG-opsonized yeast, and incubated for 15 min at 37°C. Fixing the cells in ice-cold 3% paraformaldehyde (PFA) with 0.05% glutaraldehyde terminated the phagocytosis. The cells were permeabilized with lysophosphatidylcholine (100 μg/ml) and stained with Alexa 594-conjugated phalloidin (1/500 in PBS) to visualize F-actin. Fluorescence images were captured with a Nikon Eclipse 800 fluorescence microscope equipped with Easy Image software (Tekno Optik). For each set of experiments the images were taken with identical camera settings. To ensure that the majority of the particles were fully phagocytosed under these conditions, the fluorescence quenching method (23) was used in experiments run in parallel.

To evaluate the degree of phagocytosis, the TAT-transduced cells were either kept in suspension (6) or were allowed to adhere to glass slides (22). FITC-labeled yeast, prepared as previously described (24), was added to the cells (ratio 5:1 yeast/cell) and incubated at 37°C. Phagocytosis was stopped by the addition of ice-cold PBS, after which the cells were kept at 4°C until counted. The cells were resuspended in an equal volume of trypan blue to quench the extracellular fluorescence and thereby distinguish between intracellular and extracellular yeast particles (23). Phagocytosis was quantitated microscopically in coded samples by counting ingestcd yeast particles per 100 cells for each treatment. The statistical significance was determined by Student’s t test (two-sample, equal variance).

Translocation of CD63 to the phagosomal membrane

The phagocytosis experiment was performed on the substratum as described above, but the cells were fixed in ice-cold 4% PFA before permeabilization with saponin (0.1%; v/v). Nonspecific binding was blocked with BSA (2%; w/v) and goat serum (10%; v/v). The preparations were incubated with a mouse anti-CD63 Ab (1/500), washed, and incubated with a FITC conjugated anti-mouse Ab (1/400). Phagocytosed yeast were visualized with phalloidin (1/500) to identify phagosomes. The images were captured with a Nikon Eclipse 800 fluorescence microscope equipped with Dual detectors, a Nikon microscope, and a DakoCytomation fluorescent mounting medium. Imaging was performed in a Sarastro 2000 confocal microscope (Molecular Dynamics) equipped with dual detectors, a Nikon microscope, and a ×60 oil immersion objective (numerical aperture 1.40; Nikon). Excitation of the Alexa 594 fluorophore was achieved with an argon ion laser filtered through a dichroic mirror (cutoff wavelength of 535 nm). For detection, a 600-nm long-pass emission filter was used. For all samples, blinded analysis was performed and only cells with single, well-ingested phagosomes were analyzed. At least 50 phagosomes per treatment in three independent experiments were scanned. For each set of experiments the images were taken with identical camera settings. The images were evaluated with respect to fluorescent staining at the phagosomal membrane and classified as positive or negative for CD63 staining. For the costained preparations, the Alexa 594 and Bodipy fluorophores were excited as above and detected with a 600-nm long-pass emission and a 545DF30-nm filter, respectively. This filter setup ensured no detection of the red signal in the green channel or vice versa. χ2 analysis was used to determine the significance in reduction of phagosomal maturation observed with transduced vs nontransduced cells.

Preparations for scanning electron microscopy

TAT-transduced cells were incubated with phalloidin (0.004% v/v) for 5 min on ice to stabilize the F-actin cytoskeleton. The cells were extracted by the addition of Triton X-100 (0.5%; v/v) in a microturbule stabilizing buffer

Abbreviations used in this paper: PMB, polymyxin B; HA, hemagglutinin; KRG, Krebs-Ringer phosphate buffer; N17Cdc42, dominant negative form of Cdc42; V12Cdc42, constitutively active form of Cdc42.
(1 mM EGTA, 100 mM PIPES without Ca\(^{2+}\), 4% (w/v) polyethylene glycol (pH 6.9), and phenol red) (25) for 5 min at room temperature. The concentration of phalloidin was maintained at 0.004% during the extraction procedure. Cells were washed once in PBS before fixation for 30 min at room temperature in 2.5% glutaraldehyde in 0.15 M sodium cacodylate buffer. For dehydration, the samples were washed once in PBS and then placed in 50% ethanol and the alcohol content of the solution was gradually increased to 95.5% during an ~1-h period. The samples were placed inside an E-3000 critical point drying apparatus (Polaron). Ethanol was drained from the samples and exchanged for carbon dioxide. After ~1.5 h, when the exchange was complete, the temperature was raised to 10°C to 40°C and the CO\(_2\) was slowly let out. The dried and water-free samples on glass coverslips were mounted on metal stubs and sputter-coated with 12-nm of tungsten. Scanning micrographs were taken in a JEOL 8400 scanning electron microscope. To evaluate the morphology of the cytoskeleton of the cells, images from coded samples were made without tilting the stubs with the same magnification \((\times 650)\) from the proximity of the center of the glass coverslip and, using the same images, the area of 140 (nontransduced) and 70 (TAT-V12Cdc42 and TAT-N17Cdc42-transduced, respectively) randomly chosen cells per treatment was measured with Scion Image software. The areas were compared groupwise with the nonparametric Mann-Whitney \(U\) test on ranks, which was chosen to avoid assumptions of the distribution of the data.

**Following lysosomes with LysoTracker**

To allow observation of granule movement in living cells, the granules were prelabeled with LysoTracker Red DND-99 as follows: The cells, nontransduced or transduced with TAT-V12-Cdc42 and N17-Cdc42, were allowed to adhere to the glass bottom of a Delta T dish (Biorad) for 15 min at 37°C, after which LysoTracker (50 nM) was added for an additional 15-min incubation at 37°C. The labeled cells were washed twice and the dish was mounted in a Confocal microscope (Bio-Rad Radiance 2100) equipped with a temperature control set at 37°C. IgG-opsonized yeast particles were added to the cells and the coded samples were evaluated by time-resolved microscopy using the \(\times 60\) oil immersion objective (numerical aperture 1.40; Nikon). Each sample was scanned every fourth second using the 514-nm line of the argon laser and a 600-nm long-pass filter for the emitted signal. At least 15 images were taken to create a movie.

**Quantitative image analysis**

To perform objective quantifications of granule movement in living cells, representative confocal microscope images were subjected to quantitative image analysis using the ImageJ software package (26). In the original red-green-blue color microscope image, LysoTracker-dyed granules appeared in the red channel only. The center of the phagosome and the cell boundary were manually identified in green and blue, respectively. This allowed for all further analyses to be performed automatically by using a specially developed ImageJ plugin. The red, green, and blue channels of the image were thereafter extracted into separate 8-bit grey scale images. The red image was thresholded (minimum of 50) and a binary image of LysoTracker-dyed areas depicted in black on a white background was created. Using ImageJ, the blue vector was converted into a binary mask representing the cell area. Combining the mask with the binary red images created a new image containing only the LysoTracker-dyed areas of the studied cell. To find and measure individual regions, the “Analyze Particles” function in ImageJ was applied, resulting in “area” and “center of mass” statistics of each identified region in the image. Each separate region was termed a “granule population,” and this term was applied to populations regardless of the number of granules present in the population. The green image was used to automatically obtain the spatial coordinates of the “center of mass” of the phagosome region.

For each cell, the degree of accumulation of granules around the phagosome as well as the cellular distribution of granules within the cell was established. This was done by determining the following: 1) the relative size of granule populations in each cell; 2) the number of granule populations within the cell; 3) the “closeness” to the phagosome of the granule populations within the cell; and 4) the spatial distribution of the granule populations within the cell. Statistical analysis of the data using GraphPad InStat version 3.06 (GraphPad Software) was performed with the nonparametric Mann-Whitney \(U\) test.

**The relative size of granule populations**

We estimated the degree of accumulation of granules around the phagosome and within the cell by measuring the area of the granule populations with the assumption that the area corresponds to the number of accumulated granules. The relative size \(r_i\) of granule populations in the cell was measured as the ratio between the area of the largest image region and the total area of all image regions in the cell. This measure is dimensionless and independent of individual cell sizes and is depicted in Equation 1,

\[
r_i = \frac{\max \text{area}_i}{\sum_{i=1}^{N} \text{area}_i}
\]

where \(\text{area}_i\) is the area of a region and \(N\) is the number of image regions in the cell.

**The number of granule populations**

Another way to estimate the degree of accumulation of granules is to measure the number of granule populations by assuming that a low number of granule populations corresponds to a high accumulation of granules. The number of granule populations was established by counting the number of areas that are located within a distance \(d\) to the phagosome. The average “closeness” to the phagosome \(C\) of all image regions in the cell was established as a measure where the “closeness” of an individual image region is defined as the area of the image region divided by the square of its distance to the phagosome center. This measure is dimensionless and independent of individual cell size and is shown in Equation 2,

\[
C = \frac{1}{N} \sum_{i=1}^{N} \frac{\text{area}_i}{d_i^2}
\]

where \(\text{area}_i\) is the area of a region, \(d_i\) is the Euclidean distance from one region’s “center of mass” \((x_i, y_i)\) to the phagosome “center of mass” \((c_x, c_y)\), and \(N\) is the number of regions in the cell.

**The spatial distribution of the granule populations**

To measure the spatial distribution of granules in the cell, the statistical dispersion of the distances between granule populations within each cell was established. The distribution of the intracellular spatial distribution of image regions was expressed by the relative SD (RSD) of all interregional distances \(d_i\), as shown in Equation 3,

\[
d_i = \sqrt{(x_i - x_j)^2 + (y_i - y_j)^2} \quad i \in \{1, \ldots, N - 1\} \quad j \in \{i + 1, \ldots, N\}
\]

where \(d_i\) is the Euclidean distance from one region’s “center of mass” \((x_i, y_i)\) to another region’s “center of mass” \((x_j, y_j)\) and \(N\) is the number of regions in the cell, RSD is a dimensionless measure and is independent of cell size. A high RSD value represents a more scattered granule distribution.

**Phosphorylation of p38 MAPK**

After TAT transduction, the cells were washed twice and resuspended in KRG. The cells \((2 \times 10^7)/\text{sample}) were preincubated for 1 min at 37°C and stimulated with IgG-opsonized yeast particles (ratio 1:5). Phagocytosis was stopped after 15 min by the addition of ice-cold PBS supplemented with Na\(_2\)VO\(_4\) (1 mM). The cells were pelleted \((3,500 \times g \text{ for } 30 \text{ s at 4°C})\) and resuspended in lysis buffer \((50 \text{ mM HEPES (pH 7.5), } 150 \text{ mM NaCl, } 1 \text{ mM EDTA, } 2 \text{ mM EGTA, } 10\% \text{ glycerol, } 1\% \text{ Triton X-100, } 1 \mu\text{M aprotinin, } 2 \mu\text{g/}\mu\text{L leupeptin, } 1 \mu\text{M Na}_{2}\text{VO}_{4}, \text{ and } 1 \mu\text{M Pefabloc})\). After lysis \((15 \text{ min at } 4\text{C})\), the samples were centrifuged \((10,000 \times g \text{ for } 10 \text{ min at } 4\text{C})\) and the supernatant was diluted with sample buffer. The samples were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 4% BSA overnight. Phosphorylation of p38 MAPK was detected by means of ECL using an anti-phospho-p38 MAPK Ab (1/1,000) and a secondary HRP-conjugated anti-rabbit Ab. To visualize total amounts of p38 MAPK proteins, the membrane was stripped and reblotted with a rabbit polyclonal anti-p38 MAPK Ab. The blots were scanned and the relative intensity of the bands was quantified using Scion Image Alpha version 4.0.3.2. The statistical significance was determined by the nonparametric Mann-Whitney \(U\) test.

**Results**

**TAT-Cdc42 transduction in human neutrophils and HL60 cells**

To study the role of Cdc42 during FcγR-mediated phagocytosis, human neutrophils or DMSO-differentiated promyelocytic human leukemia HL60 cells were transduced with constitutively active
The TAT transduction did not influence the viability of either human neutrophils or HL60 cells as determined by trypan blue staining (not shown). Western blot analysis revealed that only traces of TAT-V12Cdc42 or TAT-N17Cdc42 remained in human neutrophils after short transduction times (5–10 min) and that longer treatment further reduced the amount of protein (data not shown). A HA-tagged peptide of lower molecular mass could be detected after 30 min of TAT-transduction (not shown), indicating that the TAT-Cdc42 protein was subject to proteolytic degradation in these cells. Previous studies have shown that the pretreatment of cells with chloroquine, a drug that elevates lysosomal pH and modulates degranulation, protects TAT proteins from degradation (27, 28). However, treatment of neutrophils with 100 μM chloroquine before transduction with TAT-Cdc42 did not protect the protein from degradation (not shown).

In HL60 cells, by contrast, TAT-V12Cdc42 and TAT-N17Cdc42 were stable during an extended incubation after transduction (15, 30, and 60 min) (Fig. 1B). The efficacy of the TAT-transduction was found to be 65% or more as determined by critical point drying, and tungsten coating. Scanning electron micrographs were taken in a JEOL 840 scanning electron microscope.

![Figure 1. Structure, intracellular stability, and effect of TAT-Cdc42 on the morphology of HL60 cells.](http://www.jimmunol.org/)

### Table I. The effect of TAT-Cdc42 on the cell area of HL60 cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean Cell Size ± SEM</th>
<th>Maximum Cell Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontransduced</td>
<td>3,150 ± 130</td>
<td>9,000</td>
</tr>
<tr>
<td>TAT-V12Cdc42</td>
<td>4,400 ± 370</td>
<td>22,450</td>
</tr>
<tr>
<td>TAT-N17Cdc42</td>
<td>3,600 ± 260</td>
<td>10,800</td>
</tr>
</tbody>
</table>

*Adherent HL60 cells, nontransduced or transduced with 200 nM TAT-V12Cdc42 or 200 nM TAT-N17Cdc42, were extracted by Triton X-100, fixed in 2.5% glutaraldehyde, and prepared for scanning electron microscopy by dehydration, critical point drying, and tungsten coating. Scanning electron micrographs were taken in a JEOL 840 scanning electron microscope.

### Table II. Phagocytosis of IgG-opsonized yeast particles in TAT-Cdc42 transduced HL60 cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Associated Yeast Particles</th>
<th>Ingested Yeast Particles</th>
<th>Adherent Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Associated</td>
<td>Ingested</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yeast Particles</td>
<td>Yeast Particles</td>
<td>Yeast Particles</td>
</tr>
<tr>
<td>Nontransduced</td>
<td>211 ± 23</td>
<td>104 ± 18</td>
<td>68</td>
</tr>
<tr>
<td>TAT-V12Cdc42</td>
<td>212 ± 29</td>
<td>119 ± 34</td>
<td>58</td>
</tr>
<tr>
<td>TAT-N17Cdc42</td>
<td>173 ± 18</td>
<td>84 ± 11</td>
<td>61</td>
</tr>
</tbody>
</table>

*Differentiated HL60 cells were incubated with a buffer or transduced with 200 nM TAT-V12Cdc42 or TAT-N17Cdc42 for 30 minutes at 37°C. The cells were kept in suspension or allowed to adhere on glass slides. FITC-conjugated, IgG-opsonized yeast particles were added to the cells (ratio 5:1). Phagocytosis was stopped by ice-cold PBS after 15 minutes of incubation at 37°C for the adherent cells and after 30 minutes for cells in suspension. The extracellular fluorescence was quenched by trypan blue. For each experiment, bound and ingested yeast particles were counted in 100 cells per treatment.

Expressed as mean ± SEM, n = 6.

Expressed as mean, n = 2, performed in duplicate.

*p = 0.98, NS (compared with associated yeast particles in nontransduced cells in suspension).

*p = 0.38, NS (compared with ingested yeast particles in nontransduced cells in suspension).

*p = 0.18, NS (compared with associated yeast particles in nontransduced cells in suspension).

*p = 0.14, NS (compared with ingested yeast particles in nontransduced cells in suspension).
Cytoskeleton rearrangements in TAT-Cdc42-transduced HL60 cells

TAT-Cdc42-treated HL60 cells were allowed to adhere, migrate toward, and engulf IgG-opsonized yeast particles on glass slides as described previously (22). Initially, the effect of Cdc42 on F-actin reorganization was analyzed in nonphagocytosing migrating cells (Fig. 1C). It is well established that Cdc42 controls cell polarity and the remodeling of F-actin at the leading edge of moving cells (for review, see Refs. 29 and 30). In line with this, TAT-V12Cdc42-transduced HL60 cells displayed a pronounced accumulation of F-actin at the leading edge, whereas cells treated with TAT-N17Cdc42 displayed lower levels of cortical and cytoplasmic F-actin compared with nontransduced cells (i.e., control) (Fig. 1C). Scanning electron micrographs supported these findings by revealing altered cytoskeletal organization and significant differences in cell size between nontransduced and TAT-V12Cdc42-transduced HL60 cells. The TAT-V12Cdc42-transduced cells were found to be flattened, spread out, and larger in size compared with nontransduced cells ($p < 0.05$; Table I) and displayed a rich mesh of cytoskeletal filaments (Fig. 1D). Cells transduced with TAT-N17Cdc42 were of the same size as nontransduced cells ($p = 0.16$, NS; Table I), but with a less abundant peripheral cytoskeletal network (Fig. 1D).

Effects of Cdc42 on IgG-mediated phagocytosis

The reorganization of actin into F-actin-rich pseudopodia and phagocytic cups is a prerequisite for FcγR-mediated phagocytosis in human neutrophils (24, 31). Cdc42 has, in other types of cells, been suggested to play a role in this process (32, 33). The effect of TAT-V12Cdc42 and TAT-N17Cdc42 on IgG-mediated phagocytosis was assayed both in cells in suspension and in adherent cells.
using fluorescent yeast particles opsonized with IgG (Table II). No significant changes in phagocytosis were observed in TAT-V12Cdc42- or TAT-N17Cdc42-transduced cells when compared with nontransduced cells (Table II).

Accumulation of F-actin around phagosomes in TAT-Cdc42-transduced HL60 cells

To understand how Cdc42 affects cellular morphology and the distribution of F-actin around the phagosome during ingestion of IgG-opsonized particles, cells were allowed to adhere and engulf yeast particles on glass slides. In nontransduced cells, we observed phagosomes surrounded by various amounts of F-actin (Fig. 2A), reflecting the presence of newly formed phagosomes (i.e., with a broad F-actin rim) as well as older phagosomes (i.e., with less or no F-actin accumulation) (10). Transduction of the cells with TAT-V12Cdc42 resulted in a strong accumulation of F-actin around the phagosomes (Fig. 2B). In fact, no phagosomes that lacked a distinct ring of actin were observed in these cells. When evaluating 50 phagosomes in TAT-V12Cdc42-transduced cells, 75% were classified as having strong F-actin accumulation (40 and 30% inhibition, respectively; Fig. 3).

Effect of Cdc42 on phagosomal maturation

The disassembly of periphagosomal F-actin, which occurs shortly after ingestion, is crucial for phagosomal maturation (10, 11, 13, 14). Thus, the sustained activity of Cdc42, resulting in accumulated F-actin, may influence the phagosomal maturation process. We investigated the effect of Cdc42 on phagosomal maturation by studying the translocation of the azurophilic granule marker CD63 to the phagosomal membrane. Phagocytosis was performed by allowing HL60 cells to engulf IgG-opsonized yeast particles on glass slides before staining the cells with an anti-CD63 Ab. Fusion of the azurophilic granule with the phagosome was identified as CD63 staining of the phagosomal membrane in images obtained by confocal microscopy. In randomly chosen cells, both TAT-V12Cdc42 and TAT-N17Cdc42 significantly reduced the number of CD63-positive phagosomes (40 and 30% inhibition, respectively; Fig. 3).

V12Cdc42 and phagosomal maturation

Our observation that periphagosomal F-actin accumulated upon Cdc42 activation (Fig. 2) together with our previous studies showing a requirement for the disassembly of periphagosomal F-actin during phagosomal maturation (10, 11, 13, 14), prompted us to investigate whether any correlation exists between the presence of a pronounced coat of F-actin and the absence of CD63 staining at

FIGURE 5. The effect of TAT-Cdc42 on lysosome mobility in LysoTracker-loaded HL60 cells. HL60 cells were incubated for 30 min with buffer, i.e., nontransduced (I) or transduced with 200 nM TAT-V12Cdc42 (II) or 200 nM TAT-N17Cdc42 (III) while adhering to the bottoms of Delta T dishes. During the last 15 min of incubation LysoTracker Red DND-99 was present. The cells were washed and equilibrated with KRG at 37°C after mounting the dish in the temperature setting unit of a confocal microscope. After adding yeast particles, phagocytosing cells were identified and images were captured every fourth second. The position of each yeast particle was localized by phase contrast microscopy before and after scanning the series. A, Shown are representative images from 20 recorded cells per treatment obtained at two independent experiments. Phagosomes are indicated with an asterisk. Scale bar, 10 μm. Movies showing the whole sequences are available as supplementary material (Video 1–3). B, The images were subjected to quantitative image analysis (ImageJ software) to objectively determine the degree of accumulation of granules around the phagosome. For each cell, the size of granule population was established as the area of the maximum population of LysoTracker-dyed lysosomes in relation to the total area of granules. The distance between the granules and the phagosome was established as the “closeness” to the phagosome of the populations of LysoTracker-dyed lysosomes within the cell. Thus, a high value of “closeness” corresponds to small distances between granules and the phagosome. The data shown are from representative images of six recorded cells per treatment. C, The images were subjected to quantitative image analysis to objectively determine the cellular distribution of granules. For each cell, the number of populations of LysoTracker-dyed lysosomes was established. The spatial distribution of granules was established as the RSD of LysoTracker-dyed lysosome distances within the cell. Thus, a high RSD value corresponds to a more scattered granule distribution. The data shown are from representative images of six recorded cells per treatment.
the phagosomes (Fig. 3). Fig. 4 shows confocal microscopy images of cells costained with Bodipy-phalloidin and anti-CD63 Abs. Nontransduced cells with no detectable F-actin around the phagosome displayed a pronounced translocation of CD63 to the phagosome (Fig. 4). In contrast, the accumulation of F-actin around the phagosomes of TAT-V12Cdc42-transduced cells coincided with the absence of CD63 translocation to the phagosomal membrane (Fig. 4). In these cells, CD63-positive granules were found outside but in close vicinity of the F-actin ring or accumulating at the cell periphery (Fig. 4). None of the investigated TAT-V12Cdc42-transduced cells displayed costaining of F-actin and CD63, nor was any staining for CD63 observed inside the F-actin rim. In cells without prey, the cortical F-actin at the leading edge effectively excluded all CD63-positive granules (data not shown).

To further analyze the role of Cdc42 in regulating the mobility of granules in HL60 cells, TAT-V12Cdc42-cells were loaded with LysoTracker and movies observing movement of lysosomes during phagocytosis of IgG-opsonized yeast were obtained. Analysis of the movies revealed that, in nontransduced cells, lysosomes were present at the site of ingestion at an early stage and that continuous fusion of LysoTracker-positive granules with the phagosome occurred (Fig. 5A and Video 1). These data were supported by quantitative image analysis showing that lysosomes accumulated close to each other in the vicinity of the phagosome (Fig. 5, B and C). The granules in V12Cdc42-transduced cells accumulated at the cell periphery or localized close to the ingested particle; however, no fusion could be observed (Fig. 5AII and Video 2). Quantitative image analysis revealed that the LysoTracker-positive granules accumulated near each other and there was no significant difference in relative size, number of granule populations, “closeness” to a phagosome, or spatial distribution compared with granules in nontransduced cells (Fig. 5, B and C).

N17Cdc42 and phagosomal maturation

In N17Cdc42-transduced cells, confocal microscopy images of cells costained with Bodipy-phalloidin and anti-CD63 Abs revealed that the CD63-positive granules were evenly dispersed in the cytoplasm with no obvious accumulation around or translocation to the phagosomal membrane (Fig. 4). This was the case even if the phagosome was lacking its rim of F-actin (not shown). The observation of a dysregulated granule translocation in TAT-N17Cdc42-transduced cells was supported by our findings from the movies of granule movement in living cells. In contrast to nontransduced cells, where continuous fusion of LysoTracker-positive granules with the phagosome occurred (Fig. 5A and Video 1), the granules in N17Cdc42-transduced cells remained dispersed in the cytoplasm and no fusion with the phagosome was detected (Fig. 5AIII and Video 3). Quantitative image analysis supported these data by showing that in N17Cdc42-transduced cells the LysoTracker-positive granules did not accumulate around the phagosome (p < 0.01 compared with nontransduced cells; Fig. 5B). The granules were significantly more dispersed (p < 0.01, relative size; p < 0.05, number of granule populations; Fig. 5, B and C) and had a more scattered distribution (p < 0.01; Fig. 5C) compared with granules in nontransduced cells.

The finding that dominant negative Cdc42 results in reduced mobilization of granules suggests that Cdc42 is part of the signal transduction involved in the regulation of granule trafficking. p38 MAPK was recently shown to be involved in phagosomal maturation and degranulation (34). We therefore hypothesized that dominant negative Cdc42 could block the translocation of CD63 via inhibition of p38 MAPK. To test whether Cdc42 influences p38 MAPK during FcγR-mediated phagocytosis, TAT-V12Cdc42- or TAT-N17Cdc42-transduced cells were allowed to phagocytose IgG-opsonized yeast. Fig. 6 shows that phagocytosis of IgG-opsonized yeast induced a marked phosphorylation of p38 MAPK within 15 min (p < 0.05). The phosphorylation was also enhanced in cells transduced with V12Cdc42. Moreover, V12Cdc42 induced p38 MAPK phosphorylation in the absence of IgG-opsonized yeast. N17Cdc42 did not, however, prevent the IgG-induced p38 MAPK phosphorylation and had no effect on the phosphorylation of p38 MAPK per se (Fig. 6).

Discussion

The present study demonstrates that the inactivation of Cdc42 at the phagosomal membrane is a prerequisite for the depolymerization of F-actin around the phagosome. Efficient depolymerization of the periphagosomal F-actin is necessary for the subsequent phagosomal maturation (10, 11, 13, 14, 35). Consistent with this, impaired phagosomal maturation was observed in cells with permanently activated Cdc42. This inhibition was not due to impaired granule movement, because CD63-positive granules accumulated in close proximity to the periphagosomal rim of F-actin. The observation that CD63 staining was completely excluded from areas with F-actin staining (both cortical and periphagosomal F-actin)

---

3 The online version of this article contains supplemental material.
shows that the inhibition of phagosomal maturation by active Cdc42 is due to a barrier of F-actin meshwork that prevents the granules from reaching the phagosome. In fact, F-actin and lysosomal markers are never observed simultaneously on the phagosomal membrane in macrophages and fibroblasts (36, 37), suggesting that the disassembly of periphagosomal F-actin is a general requirement for granule fusion with target membranes. The importance of the depolymerization of periphagosomal F-actin is reflected by the fact that multiple signaling molecules are involved in regulating this process, including intracellular Ca$^{2+}$ (10, 11) and protein kinase Cε (13, 38). Depletion of Ca$^{2+}$ or overexpression of dominant negative protein kinase Cε results in a phenotype similar to that observed with permanently active Cdc42 in the present study.

Activation of Cdc42 and Rac is a prerequisite for the uptake of particles by macrophages (39, 40). In our study, no significant reduction of phagocytosis was observed in neutrophil-like HL60 cells transduced with dominant negative Cdc42. In line with this observation, we and others have reported that in neutrophils the impaired function of Rho GTPases has no major effect on the phagocytosis of IgG- or C3bi-opsonized particles (6, 8, 9). An alternative explanation is that the loss of Cdc42-induced function may be compensated by endogenous Rac to restore phagocytic capacity. Cdc42 and Rac are activated with the same time kinetics during the phagocytosis of IgG-opsonized particles in neutrophils (6), and both of their activities are required to coordinate F-actin accumulation at nascent phagosomes in macrophages (41).

It is well known that many pathogens are able to manipulate the functions of the actin cytoskeleton in host cells to avoid the host immune response (42–45). Internalized Salmonella reside in F-actin-coated phagosomes, thereby escaping elimination by macrophages (12). Moreover, the intracellular parasite Leishmania donovani secures its intracellular survival by forming a coat of F-actin (13, 14) in a Cdc42- and Rac1-dependent manner (46). In line with this, Cdc42 together with proteins required for F-actin assembly (i.e., Arp2/3, Wiskott-Aldrich syndrome protein (WASP), myosin, and α-actinin) is retained on phagosomes containing virulent L. donovani but not on the phagosomes of an attenuated strain that is incapable of intracellular survival (47). It is therefore tempting to speculate that the accumulation of F-actin around the phagosome by the manipulation of Cdc42 function is a common pathogenic trait shared by different intracellular pathogens.

In cells transduced with dominant negative Cdc42, CD63 granules were evenly dispersed in the cytoplasm with no obvious accumulation at or translocation to the phagosomal membrane. This observation suggests that Cdc42 is part of the signal transduction involved in regulating granule trafficking. In line with this observation, neutrophils with impaired Rac and Cdc42 function exhibited a reduced ability to perform phagolysosome fusion and oxidative activation (6, 8, 9). An attractive hypothesis for our finding is that the azurophilic granules move through the cytoplasm in a Listeria-like, F-actin-dependent, and Cdc42-dependent manner. This hypothesis is supported by two recent studies that reported actin-dependent movement of lysosomes, endosomes, and phagosomes involving neural Wiskott-Aldrich syndrome protein (N-WASP) and Arp2/3 (48, 49), which are downstream effectors of Cdc42 (50). Further support for this theory comes from a study on fibroblasts showing that actin polymerization is required for the fusion of endocytic and phagocytic pathways (36). An alternative explanation to our findings is that the microtubule organizing center, which has been shown to be regulated by Cdc42 in different cell types (51, 52), is required for the efficient translocation of a granule toward the phagosome and that impairment of Cdc42 function using N17Cdc42 results in a defective microtubule organizing center. These two notions regarding the mechanism involved in Cdc42-regulated translocation of granules will be subject to future studies.

Degranulation in neutrophils and macrophages is regulated by p38 MAPK (53, 54), and it was recently suggested that p38 MAPK enhances phagosomal maturation by increasing granule mobility via Rab (34). We therefore speculated that p38 MAPK could be part of the signaling transduction involved in the Cdc42-regulated translocation of granules. The FcγR-mediated phagocytosis per se was found to trigger p38 MAPK phosphorylation. However, the reduced mobility of granules observed after the inhibition of Cdc42 must involve an alternative signaling pathway, because the phosphorylation of p38 MAPK by IgG-opsonized yeast could still be observed in N17Cdc42-transduced cells. An alternative possibility is that Cdc42 acts downstream of p38 MAPK. The absence of N17Cdc42-mediated effects might also be explained by an insufficient amount of dominant negative Cdc42 to significantly affect the p38 MAPK activity, because the amount of modified proteins in the cell subjected to TAT transduction is considerably lower than that found after traditional cell transfection.

Under normal conditions, the periphagosomal rim of F-actin is depolymerized shortly after particle uptake (11), enabling phagolysosome fusion. For phagosomal maturation to take place, it is therefore essential that F-actin remodeling is precisely regulated. The present study shows a dual role of Cdc42 in regulating phagosomal maturation as summarized in the model proposed in Fig. 7. In the presence of active Cdc42, phagosomal maturation is blocked by the dense meshwork of F-actin around the phagosomes, which physically obstructs the granule from fusing with the phagosomal membrane. The inhibition of Cdc42 also prevents phagosomal maturation by modulating the granule translocation per se, most probably by being part in the signal transduction involved in regulating granule trafficking to the phagosomal membrane.

**Acknowledgments**

We thank Pia Druid, Kristina Orselius, and Maria Gustavsson for excellent technical assistance, Dr. Margaretha Lindroth for advice regarding preparations for scanning electron microscopy, and Patrik Lindblom, M.Sc. Engineering, for setting up and performing the quantitative image analysis. Dr. Steve Dowdy is thanked for providing the pTAT-HA constructs and Dr. Dirk Roos for the anti-CD63 Ab.
Disclosures

The authors have no financial conflict of interest.

References