The EMBO Journal Vol.17 No.21 pp.6219–6229, 1998

Fc receptor-mediated phagocytosis requires CDC42 and Rac1

Philippe Massol, Philippe Montcourrier[1], Jean-Claude Guillemot and Philippe Chavrier[2]

Centre d'Immunologie INSERM-CNRS de Marseille-Luminy, Case 906, 13288 Marseille Cedex 9 and [1]CNRS UMR 5539, Université Montpellier II, 34095 Montpellier Cedex 5, France

[2]Corresponding author
e-mail: chavrier@ciml.univ-mrs.fr

At the surface of phagocytes, antibody-opsonized particles are recognized by surface receptors for the Fc portion of immunoglobulins (FcRs) that mediate their capture by an actin-driven process called phagocytosis which is poorly defined. We have analyzed the function of the Rho proteins Rac1 and CDC42 in the high affinity receptor for IgE (FcεRI)-mediated phagocytosis using transfected rat basophil leukemia (RBL-2H3) mast cells expressing dominant inhibitory forms of CDC42 and Rac1. Binding of opsonized particles to untransfected RBL-2H3 cells led to the accumulation of F-actin at the site of contact with the particles and further, to particle internalization. This process was inhibited by *Clostridium difficile* toxin B, a general inhibitor of Rho GTP-binding proteins. Dominant inhibition of Rac1 or CDC42 function severely inhibited particle internalization but not F-actin accumulation. Inhibition of CDC42 function resulted in the appearance of pedestal-like structures with particles at their tips, while particles bound at the surface of the Rac1 mutant cell line were enclosed within thin membrane protrusions that did not fuse. These phenotypic differences indicate that Rac1 and CDC42 have distinct functions and may act cooperatively in the assembly of the phagocytic cup. Inhibition of phagocytosis in the mutant cell lines was accompanied by the persistence of tyrosine-phosphorylated proteins around bound particles. Phagocytic cup closure and particle internalization were also blocked when phosphotyrosine dephosphorylation was inhibited by treatment of RBL-2H3 cells with phenylarsine oxide, an inhibitor of protein phosphotyrosine phosphatases. Altogether, our data show that Rac1 and CDC42 are required to coordinate actin filament organization and membrane extension to form phagocytic cups and to allow particle internalization during FcR-mediated phagocytosis. Our data also suggest that Rac1 and CDC42 are involved in phosphotyrosine dephosphorylation required for particle internalization.

Keywords: *Clostridium difficile* toxin B/Fc receptor/phagocytosis/Rho-GTP binding protein/tyrosine protein phosphatase

Introduction

Phagocytosis of antibody-opsonized particles by macrophages is a sequential process that starts by the interaction of the particles with surface receptors for the Fc portion of immunoglobulins (FcRs) and culminates with the appearance of the particles into membrane-sealed actin-free phagosomes. Following progressive zipper-like engagements between FcRs and opsonic ligands, membrane protrusions extend over the particle surface to form a phagocytic cup which engulfs the particle (reviewed by Swanson and Baer, 1995). Actin polymerization and actin filament accumulation underneath the plasma membrane, at the site of contact with the particle, are thought to provide the driving force for protrusion extension. This assumption is consistent with the inhibitory effect of cytochalasins on particle engulfment (Zigmond and Hirsch, 1972; Greenberg *et al.*, 1991).

FcR-mediated phagocytosis requires the activation of protein tyrosine kinases (PTKs), as demonstrated by the effects of PTK inhibitors that block the formation of the phagocytic cups and the ingestion of particles (Greenberg *et al.*, 1993). Upon FcR aggregation, Src-family PTKs become activated and phosphorylate tyrosine residues which are present in cytoplasmic motifs of the receptor-associated subunits and are referred to as ITAMs (immunoreceptor tyrosine-based activation motif) (Ghazizadeh *et al.*, 1994; Sarmay *et al.*, 1994; Wang *et al.*, 1994). Phosphorylated ITAMs serve as docking sites for the SH2 domains of the cytosolic PTK $p72^{Syk}$ (Kiener *et al.*, 1993; Darby *et al.*, 1994; Durden and Liu, 1994; Greenberg *et al.*, 1994). The crucial role of $p72^{Syk}$ in coordinating early signaling events to actin cytoskeleton reorganization in FcR-mediated phagocytosis has been illustrated recently by different approaches (Indik *et al.*, 1995; Cox *et al.*, 1996; Greenberg *et al.*, 1996), including the use of $p72^{Syk}$-deficient mouse macrophages that bind opsonized erythrocytes and form actin-rich phagocytic cups but are incapable of subsequent internalization of the opsonized particles (Crowley *et al.*, 1997). The absence of detectable tyrosine phosphorylation of the phosphoinositide 3-kinase (PI 3-kinase) p85 subunit in $p72^{Syk}$-deficient macrophages is particularly remarkable (Crowley *et al.*, 1997), because chemical inactivation of PI 3-kinase activity by wortmannin (Wtn) also blocks phagocytosis before formation of a sealed phagosome (Araki *et al.*, 1996; Crowley *et al.*, 1997). Altogether, these findings suggest that $p72^{Syk}$ is required for proper activation of PI 3-kinase, this latter event eliciting closure of protrusions and completion of particle engulfment by an unknown mechanism.

The formation of phagocytic cups around particles during FcR-mediated phagocytosis closely resembles the current model for growth factor-induced membrane ruffling. Stimulation of membrane ruffling by platelet-

derived growth factor (PDGF) and insulin requires the activity of PI 3-kinase (Wennström *et al.*, 1994). PDGF activation can be mimicked and PI 3-kinase requirement can be bypassed by microinjection of an activated form of the Rho GTP-binding protein Rac1 (Ridley *et al.*, 1992; Nobes *et al.*, 1995). This suggests that Rac1 is one of the effector proteins for PI 3-kinase signaling (Hawkins *et al.*, 1995) and that Rac1 may be involved in actin reorganization during FcR-mediated phagocytosis. RhoA, Rac1 and CDC42 are involved in the regulation of actin cytoskeleton organization (for review see Tapon and Hall, 1997). In macrophages these three GTP-binding proteins control actin cytoskeleton organization and cell adhesion (Allen *et al.*, 1997; Cox *et al.*, 1997). There is direct evidence that RhoA is essential for FcγR-mediated phagocytosis (Hackam *et al.*, 1997) and a recent study has also implicated Rac1 and CDC42 in FcγR-mediated phagocytosis in a macrophagic cell line although the stage of inhibition was not precisely defined (Cox *et al.*, 1997). Therefore, Rho GTP-proteins may control the cytoskeletal rearrangements that operate during FcR-mediated phagocytosis.

To test the importance of CDC42 and Rac1 in FcR-mediated phagocytosis, we used stably transfected rat basophil leukemia (RBL-2H3) cells, a rat-tumor mast cell line, that express trans-dominant inhibitory mutant forms of Rac1 or CDC42 (Guillemot *et al.*, 1997). RBL-2H3 cells express the high affinity receptor for IgE (FcεRI) which, upon aggregation, triggers the secretion of inflammatory mediators that are responsible for anaphylactic responses. In addition, FcεRI can trigger endocytosis and phagocytosis of soluble and particulate ligands, respectively (Pfeiffer *et al.*, 1985; Daëron *et al.*, 1993; Daëron *et al.*, 1994). As for FcγRs, aggregation of FcεRI activates the Src-family PTK Lyn, which phosphorylates the ITAMs of the β and γ subunits allowing the recruitment of $p72^{Syk}$ (for review see Jouvin *et al.*, 1995). We have recently established that CDC42 and Rac1 control specific pathways that lead to actin cytoskeleton reorganization in response to FcεRI activation (Guillemot *et al.*, 1997).

We have analyzed FcεRI-mediated phagocytosis of dinitrophenyl (DNP)-conjugated zymosan by the RBL-2H3 transfectants expressing the inhibitory Rac1 and CDC42 mutants. Using scanning and transmission electron microscopy, and fluorescence confocal microscopy we observed that dominant inhibition of Rac1 and CDC42 function did not prevent F-actin and phosphotyrosine accumulation at the sites adjacent to the particles. The phagocytic cups that assembled at the surface of the transfected cells did not close and the internalization of the particles was severely compromised. These effects were correlated with the persistence of tyrosine-phosphorylated proteins in the phagocytic cups of transfected cells even after prolonged exposure to the particles.

Results

FcεRI-mediated phagocytosis in RBL-2H3 rat-tumor mast cells

We have set up an assay to investigate the function of the Rho GTP-binding proteins in FcR-mediated phagocytosis in RBL-2H3 rat-tumor mast cells. RBL cells were sensitized with anti-DNP IgE and incubated with fluoresceinated DNP-conjugated zymosan (Z-DNP). After 15 min in the presence of Z-DNP, F-actin accumulated around the particles to form actin-rich phagocytic cups (Figure 1A, arrows). After 45 min at 37°C, nearly all bound particles were internalized and accumulated in the perinuclear region of the cells and were free of F-actin (Figure 1B). In contrast, unconjugated zymosan particles or RBL cells that were not pre-sensitized with the anti-DNP IgE did not allow phagocytic cup formation nor particle internalization (data not shown). These results demonstrate that FcεRI-mediated phagocytosis in RBL cells is restricted to FcεRI-bound, IgE-opsonized particles.

Rho GTP-binding proteins are essential for FcεRI-mediated phagocytosis

The *Clostridium difficile* toxin B was used to inhibit the function of endogenous Rho proteins within RBL cells (Just *et al.*, 1995). Cells were incubated in the presence of Toxin B for 16 h and Z-DNP phagocytosis was analyzed as described above. Toxin B did not prevent binding of Z-DNP at the surface of RBL cells, but Z-DNP particles were not internalized and did not induce the accumulation of F-actin in toxin B-treated cells (Figure 1C). The phagocytic process was inhibited and not simply delayed in Toxin B-treated cells since actin-free Z-DNP particles were still observed at the cell surface after 45 min (Figure 1D). These data indicate that Rho GTP-binding proteins are essential for FcεRI-mediated phagocytosis and that inhibition of their function by *C.difficile* toxin B prevents F-actin accumulation beneath the bound particles.

Rac1N17 and CDC42N17 inhibit FcεRI-mediated phagocytosis

We investigated the function of Rac1 and CDC42 in FcεRI-mediated phagocytosis using the phagocytic assay on stably transformed RBL cell lines that constitutively express dominant-negative, N-terminal myc-tagged forms of these two Rho GTP-binding proteins (Guillemot *et al.*, 1997). High resolution scanning electron micoscopy (SEM) revealed that binding of Z-DNP-induced membrane ruffling over the surface of control RBL cells and Z-DNP particles were observed in phagocytic cups that were formed by membrane protrusions in close contact with the particles (Figure 2A). After 45 min, RBL cells had returned to their quiescent, flat morphology and the phagocytozed particles were no longer visible (Figure 2B).

After 15 min in the presence of Z-DNP, Rac1N17-expressing cells also formed membrane protrusions around the bound particles (Figure 2C), but in contrast to control cells, there were no ruffles forming at the particle-free surface of the cells (compare Figure 2A and C). The difference with control cells became even more apparent after 45 min at 37°C since Z-DNP particles were still present in cup-like structures at the surface of Rac1N17-expressing cells (Figure 2D).

This analysis was repeated on CDC42N17-expressing cells and revealed that after 15 min at 37°C many Z-DNP particles had triggered the formation of elongated pedestal-like structures with particles at their tips (Figure 2E). Such structures were never observed at the surface of control or Rac1N17-expressing cells. After 45 min, non-internalized particles were still observed in small cup-like structures that accumulated on the dorsal surface of the cells (Figure 2F).

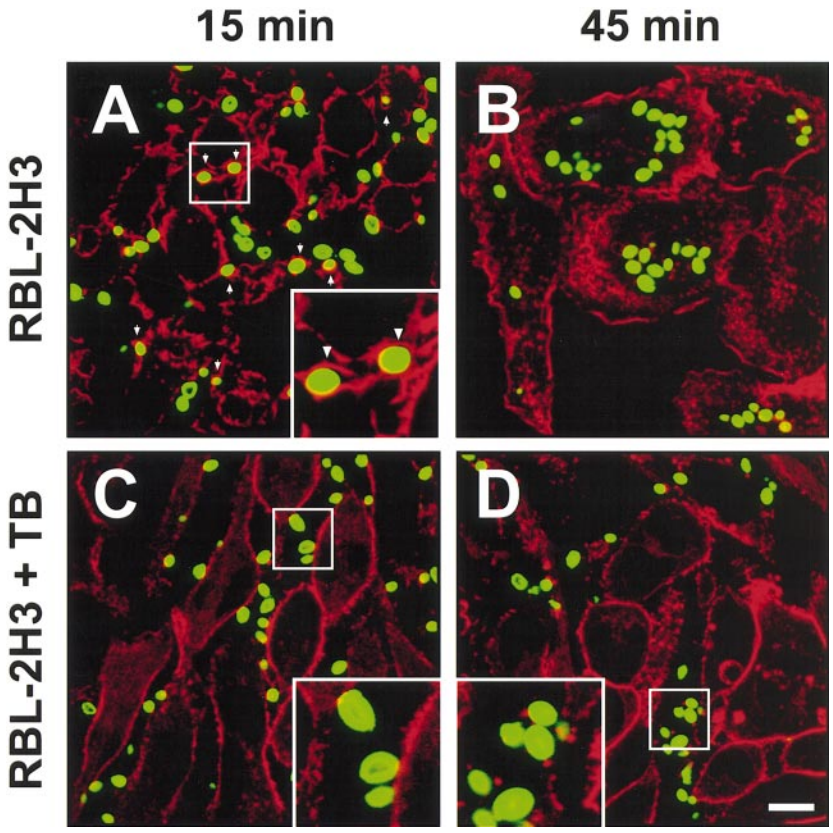

Fig. 1. Inhibition of Rho proteins by *C.difficile* toxin B blocks FcεRI-mediated phagocytosis. RBL-2H3 cells treated or not treated for 16 h with *C.difficile* toxin B (100 ng/ml) were sensitized with anti-DNP IgE. After washing, Z-DNP was sedimented onto the cells by centrifugation at 4°C. Phagocytosis was started by warming at 37°C. After 15 or 45 min at 37°C, cells were fixed and labeled with TR-phalloidin to visualize F-actin distribution by confocal microscopy. (**A**, **C** and **D**) show optical sections corresponding to the dorsal plane of the cells, while in (**B**) the section was recorded close to the cell–substratum interface. F-actin is shown in red and fluoresceinated Z-DNP particles, visualized in the FITC channel, are shown in green. (A and B), untreated RBL-2H3 cells. After 15 min, TR-phalloidin staining is evident around the particles and reveals the formation of phagocytic cups that enclose the particles (arrowheads and see higher magnification inset). After 45 min, F-actin around particles has disassembled and actin-free particles are distributed in the perinuclear region of the cells. (C and D) RBL-2H3 cells treated with *C.difficile* toxin B do not internalize Z-DNP particles. Particles are blocked at the cell surface and do not induce the accumulation of F-actin (see higher magnification insets). Bar, 10 μm.

Electron microscopy on ultra-thin sections in Rac1N17-expressing cells confirmed that Z-DNP particles were still observed at the cell periphery surrounded by unfused membrane protrusions after 45 min (Figure 3B), whilst in control cells Z-DNP accumulated in the perinuclear region (Figure 3A). In CDC42N17-expressing cells, Z-DNP particles were also observed at the cell periphery within phagocytic cups showing very short membrane protrusions over the particle edges (Figure 3C). In both mutant cell lines, the membrane protrusions surrounding the particles appeared to contain a homogeneous cytoplasm devoid of organelles (Figure 3, arrows), suggesting that polymerized actin filaments are present in these structures (Greenberg, 1995).

Altogether, these observations indicate that expression of dominant inhibitory mutant forms of Rac1 and CDC42 interferes with FcR-mediated phagocytosis in RBL-2H3 cells by inhibiting the extension and/or fusion of membrane protrusions over the particles. As a consequence, phagocytic cups cannot close and particles cannot be internalized. In addition, dominant inhibition of Rac1 or CDC42 function resulted in distinct effects on phagocytic cup morphology: inhibition of CDC42 was accompanied by the formation of aberrant phagocytic cups that had a pedestal-like structure with very short membrane extensions and impaired particle internalization. The membrane protrusions that form at the surface of Rac1N17-expressing cells were longer but still impaired in their ability to fuse and to mediate particle internalization.

Inhibition of actin phagocytic cup disassembly by Rac1N17 and CDC42N17

We have recently shown that expression of Rac1N17 and CDC42N17 in RBL-2H3 cells interferes with FcεRI-induced actin cytoskeleton reorganization (Guillemot *et al.*, 1997). Therefore, we examined whether the phagocytic defects we observed in the stably transformed cell lines could be the consequence of a defective F-actin assembly at the sites of contact with Z-DNP.

Rac1N17 expression did not prevent the accumulation of F-actin around particles at the onset of phagocytosis (Figure 4A) but in contrast to the wild-type situation, particles were still present in phagocytic cups that were heavily stained with TR-phalloidin after 45 min at 37°C (compare Figures 1B and 4B). CDC42N17-expression led to the formation of elongated actin-rich pedestal-like phagocytic cups with Z-DNP at their tip, confirming the electron microscopy (Figure 4C). After 15 min, the tips

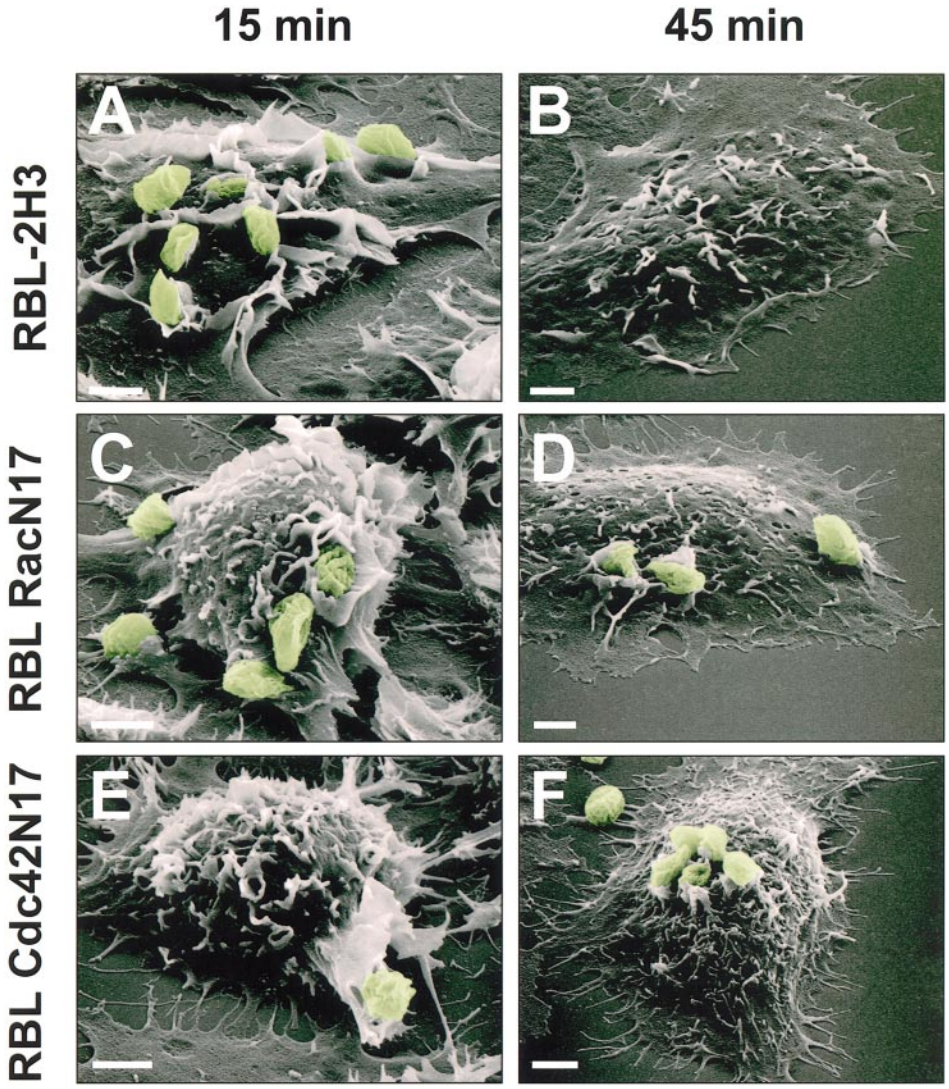

Fig. 2. Constitutive expression of Rac1N17 and CDC42N17 inhibits FcεRI-mediated phagocytosis in RBL-2H3 cells at the internalization step. The phagocytosis assay is as described in the legend of Figure 1. After 15 or 45 min of phagocytosis of Z-DNP, cells were fixed and processed for SEM analysis. (**A** and **B**) Control RBL-2H3 cells. (**C** and **D**) Rac1N17-expressing cells. (**E** and **F**) CDC42N17-expressing cells. Note Z-DNP at the tip of pedestal-like structure after 15 min of phagocytosis in CDC42N17-expressing cells (E). Z-DNP particles are artificially colored. Bars, 2 μm.

of these pedestals were flat, extending only shortly around the edges of the particles (arrows). After 45 min, most of the particles had not been internalized and were surrounded by polymerized actin (Figure 4D, arrowheads), but only few pedestal-like structures were still visible. For both mutant cell lines, non-internalized Z-DNP particles surrounded by F-actin were still visible after 2 h at 37°C, indicating that phagocytosis was severely inhibited and that at least some particles were irreversibly blocked at the surface (data not shown). Inhibition of phagocytosis in the mutant cell lines was estimated by counting the number of external particles present in actin-rich phagocytic cups and the number of internalized actin-free particles after 45 min (Table I). Only 1% of Z-DNP particles were still present at the surface of control cells, while in Rac1N17- and CDC42N17-expressing cells, 25 and 42% of the particles were trapped in phagocytic cups, respectively. In conclusion, Rac1 and CDC42 are not essential for F-actin recruitment around the particles, but they are both required for particle internalization.

Rac1 translocates to the cytoskeleton during FcεRI-mediated phagocytosis

The association of Rac1 with the cytoskeleton of RBL-2H3 cells during the phagocytic process was studied by comparing the amount of Rac1 in Triton X-100 detergent-soluble and -insoluble fractions. In control RBL-2H3 cells, in the absence of Z-DNP stimulation, Rac1 was detected in the Triton X-100 soluble fraction (Figure 5A, compare lanes 1 and 5). A significant fraction of Rac1 became resistant to detergent extraction in cells activated with Z-DNP for 15 min at 37°C (Figure 5A, lane 6). In Rac1N17-expressing cells, most of the myc-tagged Rac1 mutant protein was constitutively associated with the cytoskeleton even in absence of particles (Figure 5A, lane 7, arrowhead). This fraction increased upon engagement of FcεRI by Z-DNP concomitantly with endogenous Rac1 protein translocation (Figure 5A, lane 8). These observations indicate that expression of Rac1N17 did not significantly interfere with the translocation of endogenous Rac1 to the cytoskeleton and suggest that inhibition of

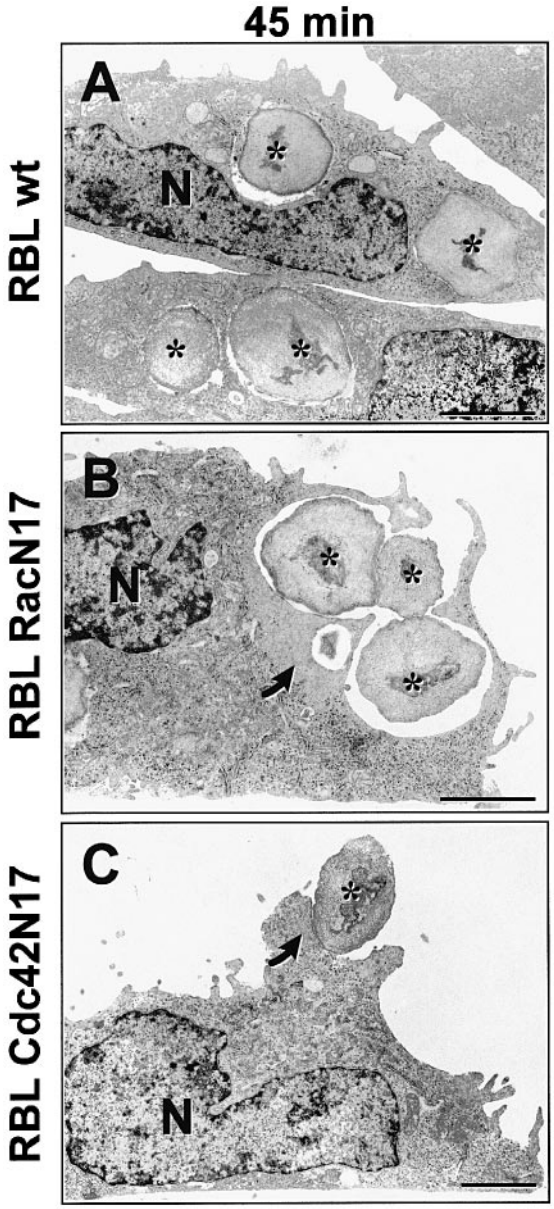

Fig. 3. Electron micrographs of Rac1N17 and CDC42N17 RBL transfectants blocked at the internalization step of phagocytosis. Cells were incubated for 45 min with DNP-conjugated zymosan and then processed for electron microscopy. (**A**) Control cells with internalized Z-DNP particles in phagosomes. (**B**) Rac1N17-expressing cells. (**C**) CDC42N17-expressing cells. In RBL-2H3 transfectants, most Z-DNP particles remain in continuity with the extracellular milieu and are not enclosed in a sealed phagosome. Arrows indicate the organelle-free region of cytoplasm surrounding the particles and corresponding probably to polymerized actin (see text). N, nucleus. Asterisk, Z-DNP particle. Bars, 2 μm.

endogenous Rac1 activation by the inhibitory mutant occurred after Rac1 translocation to the cytoskeleton. Expression of the inhibitory form of CDC42 did not prevent Rac1 translocation to the Triton X-100 insoluble fraction (data not shown). In parallel experiments, using specific antibody reagents, CDC42 and RhoA were detected in the detergent-soluble fractions but we failed to detect these proteins in the detergent-insoluble fractions (data not shown). Therefore, we conclude that engagement of FcεRI during phagocytosis leads to the translocation of Rac1 to the actin cytoskeleton, suggesting that Rac1 is recruited and activated within the newly formed phagocytic cups, allowing completion of the phagocytic process.

Persistence of tyrosine-phosphorylated proteins in the phagocytic cups correlates with an inhibition of particle internalization

To characterize further the defect in FcεRI-mediated phagocytosis in the mutant cell lines, we analyzed the content of the phagocytic cups in phosphotyrosine containing proteins. Protein tyrosine phosphorylation is required for the onset of phagocytosis (Greenberg *et al.*, 1993) and the PTK $p72^{Syk}$ plays a crucial role in both FcγR-mediated phagocytosis (Greenberg *et al.*, 1996; Crowley *et al.*, 1997) and FcεRI-signaling in mast cells (Jouvin *et al.*, 1995). As already reported for FcγR-mediated phagocytosis in macrophages (Greenberg *et al.*, 1993), immunofluorescence staining of phagocytozing RBL-2H3 cells with anti-phosphotyrosine antibody revealed the accumulation of tyrosine-phosphorylated proteins in the nascent phagocytic cups (Figure 6A), whereas tyrosine phosphorylation had returned to the basal level by 45 min (Figure 6B). RBL cells treated with PP1, a selective inhibitor of Src-family PTKs (Hanke *et al.*, 1996) which inhibits FcεRI-mediated phosphorylation of Lyn and $p72^{Syk}$ (Amoui *et al.*, 1997), did not form phagocytic cups upon binding of Z-DNP (data not shown). Phosphotyrosine accumulation in newly formed phagocytic cups was also clearly observed in Rac1N17-expressing cells (Figure 6C) as well as in CDC42N17-expressing cells (Figure 6E). In the latter cells, anti-phosphotyrosine antibody decorated elongated structures corresponding to the previously described pedestal-like phagocytic cups (arrowheads). In contrast to wild-type cells, tyrosine phosphorylation was still present in the phagocytic cups after 45 min incubation in both mutant cell lines (Figure 6D and F).

The levels of tyrosine-phosphorylated proteins during phagocytosis were compared in control and mutant cell lines by Western blot analysis. Comparative analysis of Triton X-100-soluble and -insoluble fractions revealed that the level of tyrosine phosphorylation increased upon stimulation with Z-DNP (Figure 7). The basal level in the absence of Z-DNP was similar in the different cell lines. After 15 min of stimulation with Z-DNP, a marked difference was observed in the Triton X-100-insoluble material where Z-DNP induced the tyrosine phosphorylation of several species in the 30–40, 46 and 90 kDa molecular weight ranges that were stronger in both Rac1N17- and CDC42N17-expressing cells compared with control cells (Figure 7, right panel, arrowheads). The difference between control and mutant cells was still apparent after 45 min of activation with Z-DNP.

Inhibition of PTPase activity prevents phagocytic cup disassembly

High phosphotyrosine levels in the phagocytic cups of the mutant cell lines were correlated with an inhibition of particle internalization suggesting that protein tyrosine phosphatases may be implicated in phagocytosis. To verify this assumption, we treated RBL-2H3 cells with phenylarsine oxide (PAO), a general inhibitor of protein phosphotyrosine phosphatases (PTPases). Five minutes after the initiation of phagocytosis by Z-DNP, PAO was

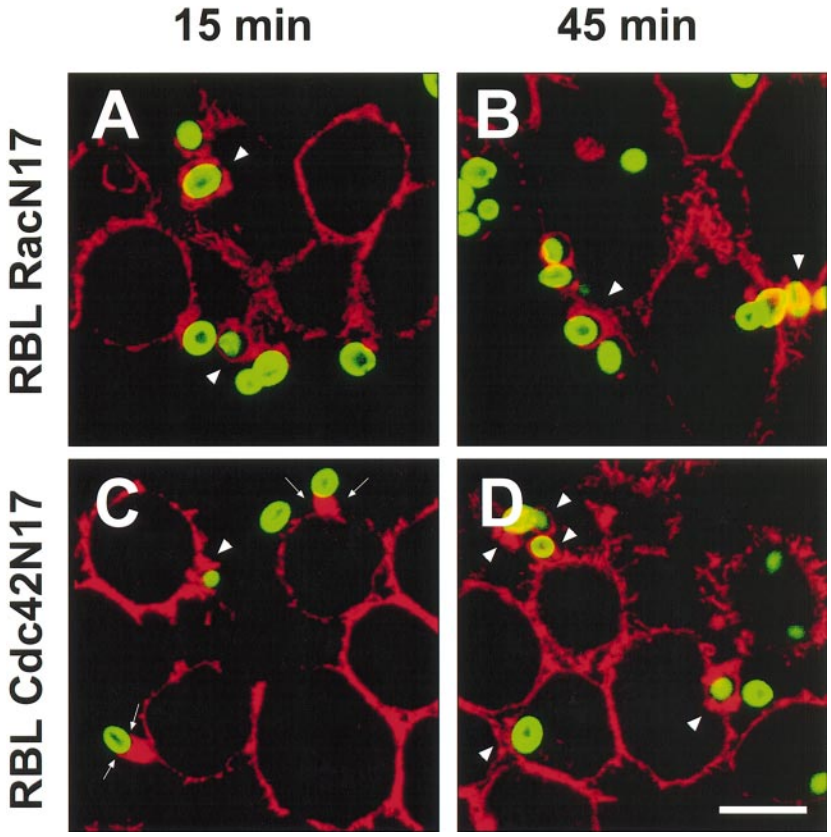

Fig. 4. Phagocytic cup architecture in the mutant cell lines. (**A** and **B**) F-actin distribution in Rac1N17-expressing cells. Note that after 45 min, particles are still present in phagocytic cups (arrowheads). (**C** and **D**) CDC42N17-expressing cells. About half of the particles are observed at the tip of F-actin rich pedestal-like structures with very short extensions over the particle edges (arrows). The remaining half are embedded in shorter and thicker F-actin-rich cups (arrowheads). By 45 min, particles are external, surrounded by thick F-actin rings (arrowheads). Optical sections were recorded through the dorsal plane of the cells. Bar, 10 μm.

Table I. Phagocytic activity of the different RBL-2H3 cell lines

	Distribution of Z-DNP particles External particles[a] and number of particles/cell[b]		External particles[a] (% of total number)
Cell line	External[a]	Internal[b]	
RBL-2H3	0.13 ± 0.04	10.57 ± 0.33	1.2
Rac1N17	1.93 ± 0.15	5.6 ± 0.42	25.6
CDC42N17	2.6 ± 0.19	3.58 ± 0.28	42.0
RBL-2H3 + Wtn	2.78 ± 0.2	0.75 ± 0.2	78.7

RBL-2H3 control cells or stably transformed cell lines were sensitized with anti-DNP IgE. After washing, cells were incubated with Z-DNP for 45 min at 37°C in the absence or presence of 100 nM Wtn. Cells were then fixed and F-actin was stained with TR-phalloidin. One hundred cells were examined per condition by indirect immunofluorescence microscopy. These data are representative of three experiments.
[a]External Z-DNP particles are defined as particles at the cell periphery surrounded by F-actin.
[b]Internal particles are defined as particles inside the cells and free of actin.

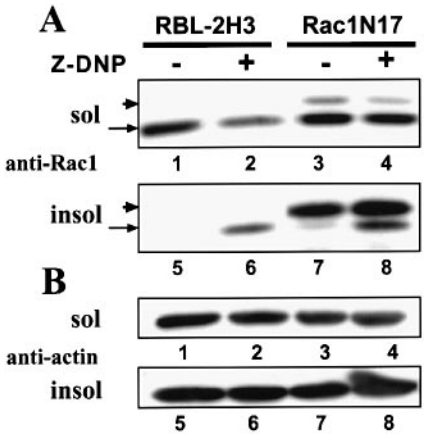

Fig. 5. Immunoblotting and distribution of Rac1 in Triton X-100-extracted control or mycRac1N17-expressing RBL-2H3 cells. Anti-DNP IgE sensitized cells were incubated in the absence (–) or in the presence (+) of Z-DNP for 15 min at 37°C. Cells were solubilized and proteins were separated into detergent-soluble (sol) and -insoluble (insol) fractions. (**A**) After separation by SDS–PAGE and electrotransfer, Rac1 was detected with anti-Rac1 mAb. Arrowheads indicate the position of the myc-tagged mutant Rac1 protein expressed in Rac1N17 transfectant which migrates more slowly on SDS–polyacrylamide gel than does the endogenous protein (arrows). (**B**) Immunoblotting analysis with anti-actin antibodies.

added and strongly enhanced overall tyrosine phosphorylation (Figure 8A, compare lanes 3 and 5; 4 and 6). The effect of PAO on phagocytosis was analyzed after 45 min of activation with Z-DNP. PAO addition led to the inhibition of particle internalization (Figure 8B) and to the persistence of actin-rich phagocytic cups (Figure 8C, arrows). Under the same experimental conditions, okadaic acid (up to 150 nM), a serine/threonine phosphatase inhibitor, had no effect (not shown).

The correlation between the defect in particle internaliz-

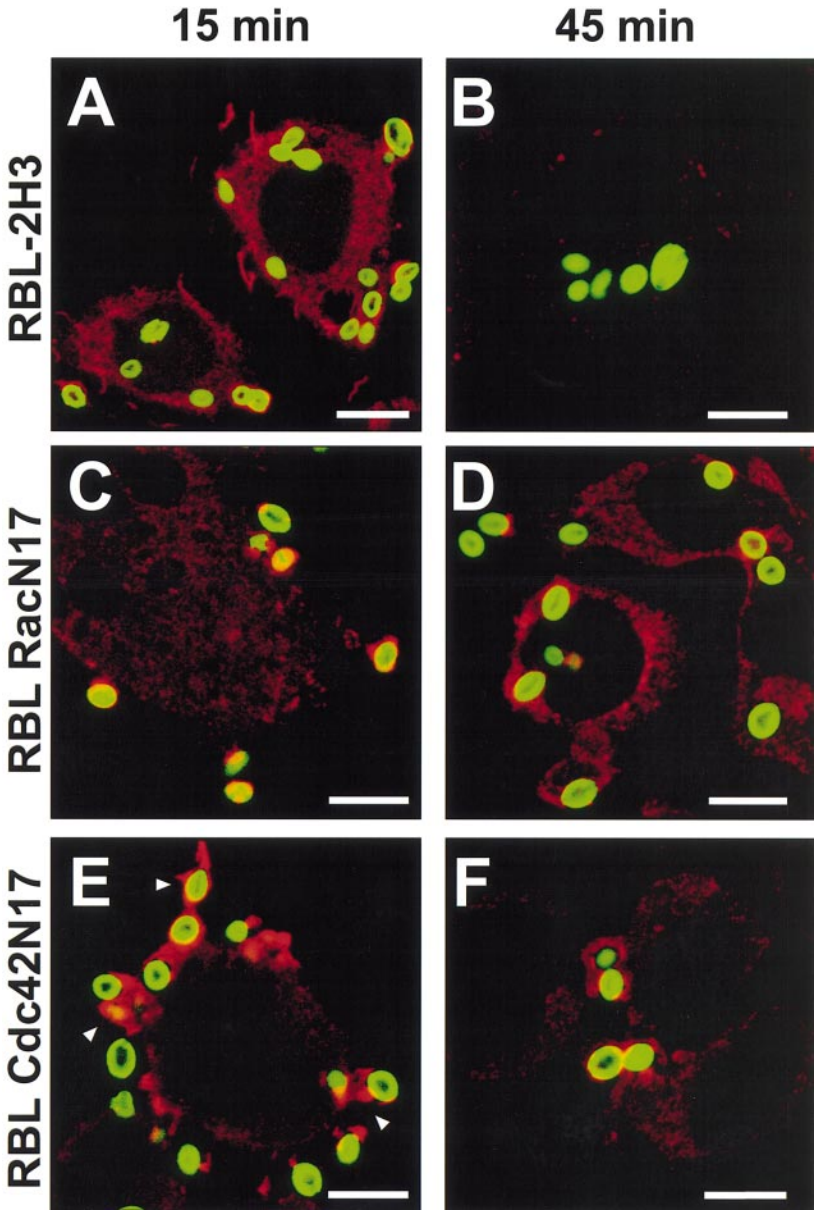

Fig. 6. Accumulation of tyrosine-phosphorylated proteins in the phagocytic cups. Tyrosine-phosphorylated proteins were detected by labeling with 4G10 mAb, revealed with TR-conjugated anti-mouse IgG secondary antibodies (red) and Z-DNP is shown in green. (**A** and **B**) RBL-2H3 cells. (**C** and **D**) Rac1N17-expressing cells. (**E** and **F**) CDC42N17-expressing cells. Arrowheads point at pedestal-like structures in CDC42N17 transfectant. Bars, 10 μm.

ation, persistence of unclosed phagocytic cups and sustained tyrosine phosphorylation of some phagocytic cup components suggests that a balance between tyrosine phosphorylation/dephosphorylation events regulates the assembly/disassembly of phagocytic cups and that this balance is perturbed upon dominant inhibition of Rac1 and CDC42 function. Our findings also demonstrate that PTPase activity is required for phagocytic cup closure and particle internalization during FcεRI-mediated phagocytosis.

Discussion

The data presented here demonstrate that Rho GTP-binding proteins are required for phagocytosis by FcRs and indicate that Rac1 and CDC42 act at different stages of the phagocytic process. First, we demonstrated the role of Rho proteins in FcεRI-mediated phagocytosis in RBL cells by treating them with *C.difficile* toxin B that inhibits RhoA, Rac1 and CDC42 (Just *et al.*, 1995). Under those conditions, Z-DNP particles could bind to the surface of RBL cells, but F-actin recruitment and phagocytic cup formation were abolished. Interestingly, *Clostridium botulinum* C3 exotoxin, a RhoA-specific inhibitor, also causes an inhibition of F-actin and phosphotyrosine-containing protein recruitment at the sites of contact with opsonized particles in microinjected J774 macrophages (Hackam *et al.*, 1997). These effects correlate with an inhibition of Fcγ receptor clustering that is likely to explain the inhibition of phagocytosis by C3 (Hackam *et al.*, 1997). In contrast, expression of CDC42N17 or Rac1N17 in RBL cells did not inhibit the accumulation of F-actin nor

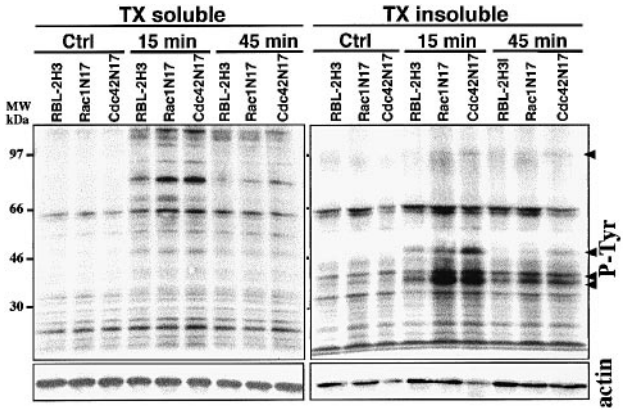

Fig. 7. Persistence of high tyrosine phosphorylation levels in Rac1 and CDC42 mutant cells in response to Z-DNP binding. (**A**) Tyrosine-phosphorylated proteins were analyzed by immunoblotting of Triton X-100-soluble and -insoluble extracts prepared from cells cultured in the absence (ctrl) or in the presence of Z-DNP particles for 15 or 45 min at 37°C. Molecular weight standards are indicated (kDa). Arrowheads indicate tyrosine-phosphorylated species present in Triton X-100-insoluble extracts that are stronger in Rac1N17- and CDC42N17-expressing cells compared with control cells. (**B**) The blot was stripped and reprobed with anti-actin antibodies to verify that the amount of protein in the different fractions was equal.

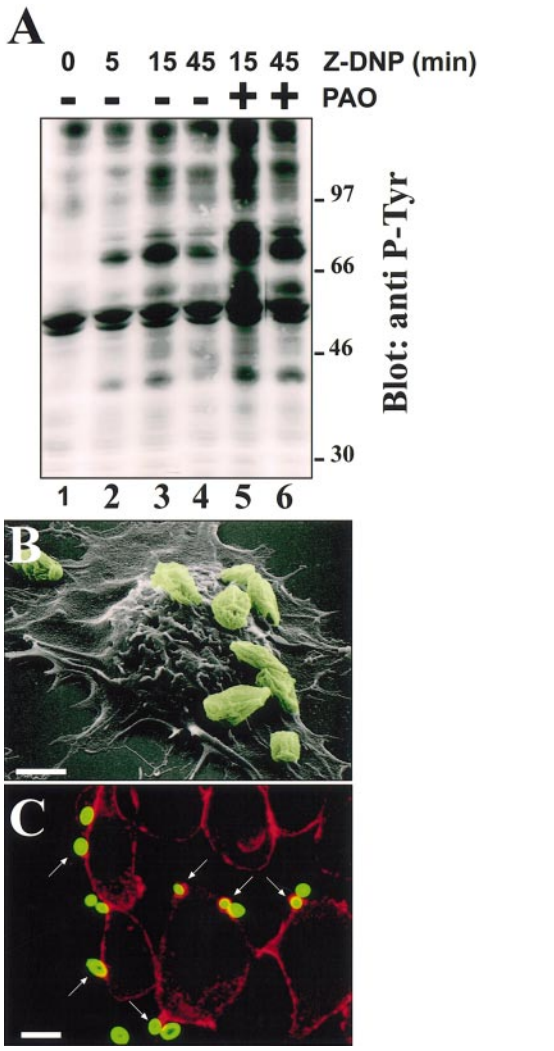

Fig. 8. PAO inhibits internalization of Z-DNP. (**A**) RBL-2H3 cells were lysed after 0, 5, 15 or 45 min of Z-DNP phagocytosis (lanes 1 to 4), or were cultured for 5 min in the presence of Z-DNP, then phenylarsine oxide (PAO) at a concentration of 5 μM was added and cells were lysed after a total incubation time of 15 or 45 min (lanes 5 and 6, respectively). Total cellular proteins were immunoblotted with the anti-phosphotyrosine mAb 4G10. Molecular weight markers (kDa) are indicated on the right. (**B**) Scanning electron micrographs of FcεRI-mediated phagocytosis in RBL-2H3 cells cultured for 5 min with Z-DNP and then treated with PAO (5 μM). Cells were fixed after a total incubation time of 45 min and processed for SEM. Images are artificially colored to visualize Z-DNP particles. Bar, 2 μm. (**C**) As (B), except that cells were fixed and labeled with TR-phalloidin to visualize F-actin distribution by confocal microscopy. Bar, 10 μm.

did it prevent the recruitment of tyrosine-phosphorylated proteins in the nascent phagocytic cups (we have observed that Rac2 is also expressed in RBL cells, but it is likely that Rac1N17 inhibits Rac2 as well as Rac1 activity). Similarly, expression of Rac1N17 or CDC42N17 in murine macrophages was also unable to inhibit the accumulation of F-actin beneath IgG-opsonized erythrocytes (Cox *et al.*, 1997). Altogether, these observations suggest that RhoA acts on the early events of the phagocytic process while Rac1 and CDC42 act on more distal steps by regulating the dynamic organization of polymerized actin filaments to form membrane protrusions enclosing the particles and allowing particle internalization. Our data also suggest that Rac1 and CDC42 have distinct functions during FcR-mediated phagocytosis. CDC42, which controls filopodial extension in fibroblast cells (Tapon and Hall, 1997), and is required for bud formation by restricting membrane growth to the bud in *Saccharomyces cerevisiae* (Adams *et al.*, 1990), may regulate the extension of membrane over the particle edges. Inhibition of CDC42 by preventing the extension of protrusions would result in the formation of the observed actin-rich pedestal-like structures because of unproper coupling of the actin polymerization response with membrane growth over the particle edges. This assumption is supported by the absence of membranes to enclose particles at the tip of pedestals. On the other hand, the phenotype of Rac1N17-expressing cells that consists of thin membrane extensions surrounding the particles which are incapable of fusing, appears more reminiscent of the situation recently described in Wtn-treated macrophages (Araki *et al.*, 1996) and in Wtn-treated RBL cells (Table I). Rac1, together with PI3-K, may be involved in completion of phagocytosis by allowing membrane fusion and closure of the phagocytic cup that are required prior to the formation of a membrane-sealed phagosome. Altogether, these findings suggest that RhoA, Rac1 and CDC42 act in a coordinated fashion to organize actin filaments, to regulate membrane extension in order to build a functional phagocytic cup and to allow particle internalization.

We are proposing that Rac1 and CDC42 are involved in a pathway that couples the recognition of opsonized particles by FcRs with particle internalization. First, upon FcR clustering, Src-family PTKs become activated and phosphorylate the ITAMs of the receptor-associated subunit allowing recruitment and activation of $p72^{Syk}$ (Jouvin *et al.*, 1995). Secondly, $p72^{Syk}$ triggers various pathways that lead to phagocytic cup formation (Indik *et al.*, 1995; Cox *et al.*, 1996; Greenberg *et al.*, 1996; Crowley *et al.*, 1997) and is probably directly involved in PI 3-kinase activation during phagocytosis. This is supported by the similarity between the phagocytic defects in Syk-deficient

cells (Crowley *et al.*, 1997) and cells treated with the PI 3-kinase inhibitor Wtn (Araki *et al.*, 1996; and our data). The inhibitory effect of Wtn and Rac1N17 on phagocytosis appear very similar and we postulate that PI 3-kinase uses Rac1 as an effector in FcR-mediated phagocytosis. Studies on PDGF-R signaling have documented a pathway connecting PI 3-kinase with the activation of Rac1 function in membrane ruffling in fibroblasts (Wennström *et al.*, 1994; Hawkins *et al.*, 1995; Nobes *et al.*, 1995). PI 3-kinase, producing PtdIns(3,4,5)P_3, may act at the level of guanine exchange factors (GEFs) that promote Rac1 activation. These GEFs share a conserved catalytic domain (Dbl domain) and a pleckstrin homology (PH) domain. In some cases, PH domains bind to PtdIns(3,4,5)P_3 with high affinity (Shaw, 1996). Local activation of PI 3-kinase at the site of FcεRI-clustering could allow PH domain-dependent recruitment of the GEF to the PtdIns(3,4,5)P_3-enriched membrane and the subsequent activation of Rac1. This assumption is supported by the observation that Rac1 translocates from the cytosol (detergent-soluble fraction) to the cytoskeleton (detergent-insoluble fraction) where it may become accessible to specific GEFs during the phagocytic process. $p95^{vav}$, which belongs to the Dbl-family of GEFs, is an attractive candidate as a GEF in this system since it becomes rapidly tyrosine phosphorylated in response to FcεRI aggregation (Margolis *et al.*, 1992) and interacts directly with, and is a substrate for $p72^{Syk}$ (Deckert *et al.*, 1996). Moreover, $p95^{vav}$ is a Rac1-specific GEF whose activity is induced by tyrosine phosphorylation (Crespo *et al.*, 1997).

In the present study we found a relationship between inhibition of particle internalization in the trans-dominant negative transfectants and persistence of high level of tyrosine-phosphorylated proteins in the defective phagocytic cups of the mutant cell lines. This led us to investigate whether PTPases might be required for completion of phagocytosis. Loss of PTPase activity by PAO treatment of phagocytozing RBL-2H3 cells inhibited particle internalization. This observation shows the requirement for tyrosine phosphatase activity for phagocytic cup closure and subsequent particle internalization during FcR-mediated phagocytosis. There are also recent observations that support a role for PTPase activity in regulating focal adhesion and stress fiber formation in Swiss 3T3 fibroblasts (Retta *et al.*, 1996). Dephosphorylation of critical substrates in the phagocytic cup by as yet uncharacterized PTPases may promote actin filament reorganization and/or depolymerization that is required for the complete internalization of the particle in a membrane-sealed actin-free phagosome. There are several PTPases expressed in basophils or mast cells including CD45, HePTP, SHP-1 and SHP-2 (Hook *et al.*, 1991; Swieter *et al.*, 1995; Kimura *et al.*, 1997) that may participate in that process. We do not know how CDC42 and/or Rac1 actually control PTPase activity during phagocytosis. One possibility is that PTPase recruitment to the phagocytic cups depends on the organization of the actin cytoskeleton in the phagocytic cups and may be affected in the mutant cell lines due to the abnormal phagocytic cup architecture. Another possibility is that CDC42 and/or Rac1 directly recruit PTPAses within the phagocytic cups. In this respect, as the CDC42 effector protein WASp interacts with the cytoskeletal protein PSTPIP (Wu *et al.*, 1998) that associates with PEST family PTPases (Spencer *et al.*, 1997; Dowbenko *et al.*, 1998), it appears possible that activated CDC42 can activate/recruite PTPases during phagocytosis.

Materials and methods

Antibodies and reagents

Rat IgE anti-DNP (clone LO-DNP30) was purchased from LO-IMEX (Brussels, Belgium). Texas red (TR)-conjugated phalloidin was purchased from Molecular Probes. Anti-Rac1 (clone 23A8; Witke *et al.*, 1995) and anti-RhoA mouse mAbs were kindly provided by Dr D.Kwiatkowski (Harvard Medical School, Boston, USA). Anti-CDC42 polyclonal antibodies have been described (Lang *et al.*, 1993). Cell culture reagents were from Gibco-Life Sciences. Unless indicated, chemicals were purchased from Sigma.

Fluoresceinated DNP-conjugated zymosan preparation

Fluoresceinated DNP-conjugated zymosan **(**Z-DNP) was prepared as follows. Zymosan was first boiled in distilled H_2O for 1 h, washed three times in 150 mM NaCl and resuspended in 150 mM NaCl at a final concentration of 25 mg/ml. Zymosan was modified with 2,4-dinitrobenzene-sulfonic acid (ACROS) by mixing 1 vol. of boiled zymosan with an identical volume of 0.1 M K_2CO_3 pH 9.5 and then by adding the same volume of 25 mg/ml 2,4-DNBS drop-wise. The mixture was incubated for 18 h at room temperature (rt) under constant agitation and then washed several times in 50 mM K_2CO_3 pH 9.5. Finally, 0.5 vol. of 1 mg/ml 6-[(4,6-dichlorotriazin-2 YL)amino]-fluorescein in 50 mM K_2CO_3 pH 9.5 was added and coupling was performed at 37°C for 15 min. Then Z-DNP was washed extensively and resuspended at 25 mg/ml in 150 mM NaCl and stored in aliquots at –20°C.

Phagocytosis assay

RBL-2H3 cells, obtained from Dr M.Daëron (Institut Curie, Paris, France), were cultured in DMEM supplemented with 10% fetal calf serum (FCS) at 37°C in a 6% CO_2 incubator. Cells (10^5) were plated in a 24-well plate containing a glass coverslips 24 h before analysis. Cells were preincubated with IgE anti-DNP (1 μg/ml) for 1 h at 37°C and washed twice. Then 300 μl of Z-DNP (0.2 mg/ml) in culture medium at 4°C was added to the well. Particles were sedimented by centrifugation at 450 *g* for 2 min at 4°C and cells were kept on ice for 15 min. Phagocytosis was started by warming at 37°C. After different periods of incubation, cells were washed five times with ice-cold phosphate-buffered saline (PBS) to stop phagocytosis and remove unbound particles, and then fixed and processed for immunofluorescence or electron microscopy analyses.

Drug treatments

The effect of *C.difficile* toxin B (100 ng/ml) was assessed after 16 h of treatment followed by sensitization with anti-DNP IgE and incubation Z-DNP for the indicated time. 4-amino-5-(4-methylphenyl)-7-(*t*-butyl) pyrazolo[3,4-*d*]pyrimidine (PP1) (Calbiochem), a selective inhibitor of Src-family PTKs (Hanke *et al.*, 1996), was added to the cells at 10 μM at the same time as Z-DNP. PAO at 5 μM (10 mM stock solution in dimethyl sulfoxide) was added after 5 min of incubation of the cells at 37°C in the presence of Z-DNP and kept for the indicated periods of time. Control cells were treated with the same final concentration of dimethyl sulfoxide.

Immunofluorescence and SEM analyses

Immunofluorescence and SEM analyses were performed as described previously (Guillemot *et al.*, 1997). SEM images were artificially colored to visualize Z-DNP using Photoshop 4.0 (Adobe). SEM and confocal images were assembled and labeled using Freehand 7.0.1 software (Macromedia Inc.).

Transmission electron microscopy

Cells were fixed in suspension with 2% glutaraldehyde in 0.1 M cacodylate buffer containing 0.1 M sucrose pH 7.3, for 4 h at rt. After washing in the same buffer, the cell pellet was post-fixed with 2% osmium tetroxide and stained *en bloc* with 1% aqueous uranyl acetate, dehydrated and embedded in Epon 812. Lead citrate contrasted ultra-thin sections were observed with a Hitachi 7100 electron microscope.

Detergent extraction and immunoblotting analysis

Cells (3×10^6) were stimulated as described above in phagocytosis assay, rinsed in PBS and incubated for 3 min in 0.25 ml of extraction buffer (0.5% Triton X-100, 75 mM KCl, 0.1 mM $MgCl_2$, 1 mM EGTA, 10 mM imidazole pH 7.4, 10 mM Tris–HCl pH 8.0, 1 mM PMSF, 0.5 M iodoacetamide, 0.5 M NaF, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 0.4 mM orthovanadate) at 4°C. Then cells were scraped off the dishes and the detergent-soluble supernatant was recovered after centrifugation at 15 800 *g* for 1 h at 4°C and mixed with 5× Laemmli buffer. For anti-phosphotyrosine detection, all of the detergent-insoluble fraction and one tenth of the detergent-soluble supernatant were loaded onto a 7–13% SDS–polyacrylamide gradient gel and transferred onto nitrocellulose. After incubation with specific antibodies diluted in 5% milk powder, 0.1% Tween 20 in PBS, detection of bound antibodies was performed using goat anti-rabbit or anti-mouse peroxidase-conjugated antisera with the enhanced chemiluminescence system (ECL, Amersham).

Acknowledgements

This work was supported by INSERM and CNRS institutional fundings and a grant from the CNRS (Appel d'offres Biologie Cellulaire). J.C.G. was a recipient of the Association Recherche et Partage. We are indebted to Drs P.Cossart, I.deMendez and P.Boquet for their critical reading of the manuscript. The *C.difficile* Toxin B was a kind gift of Dr P.Boquet. We also thank the Centre Régional d'Imagerie Cellulaire de Montpellier for electron microscopy facilities.

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Received March 23, 1998; revised August 14, 1998; accepted September 10, 1998