Rho and Rac exert antagonistic functions on spreading of macrophage-derived multinucleated cells and are not required for actin fiber formation

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Accepted 21 January; published on WWW 7 March 2000

SUMMARY

Multinucleated giant cells (MNGC) derived from avian blood monocytes present, like osteoclasts, an unusual cytoskeletal organization characterized by (1) cortical rings of actin filaments, (2) unique adhesion structures called podosomes and (3) vinculin containing focal complexes which are not visibly connected to F-actin structures. The Rho family of small GTPases plays an essential role in the regulation and organization of cellular cytoskeletal structures including F-actin and vinculin associated structures. Using bacterial toxins such as modified exoenzyme C3 (C3B) and toxin B or overexpression of constitutively active Rac and Rho proteins fused to the green fluorescent protein (GFP), we show that Rac and Rho play antagonistic roles in regulating the morphology of osteoclast-like cells. Inhibition of Rho by C3B triggered MNGC spreading whereas activated Rho promoted cell retraction. However, inhibition or activation of Rho led to complete disorganization of fibrillar actin structures, including podosomes. Toxin B inhibition of Rho, Rac and Cdc42 induced a time dependent F-actin and vinculin reorganization. Initially, actin fibers with associated adhesion plaques formed and disappeared subsequently. Finally, only small focal complexes remained at the MNGC periphery before retracting. At the time when actin fibers formed, we observed that Rac was already inhibited by toxin B. By combining C3B treatment and overexpression of a dominant negative form of Rac (N17Rac), we show that the formation of these focal adhesion and actin fiber structures required neither Rho nor Rac activity. Moreover, our results show that podosomes are extremely unstable structures since any modifications of Rho or Rac activity resulted in their dissociation.

Key words: F-actin, Osteoclast, Podosome, GTP-Rac, Actin fiber, Focal complex, GFP, Toxin B, Exoenzyme C3

INTRODUCTION

Small GTP-binding proteins of the Rho subfamily have been implicated in diverse cellular events such as membrane trafficking, transcriptional regulation, growth control, and cytoskeletal organization. Rho subfamily GTPases belong to the superfamily of Ras GTPases and include RhoA, B, C, D, E and G, Rac1 and 2, Cdc42 and TC10. Like all members of the Ras superfamily, GTPases of the Rho subfamily are molecular switches which are active in a GTP-bound state and inactive in a GDP-bound state. The switch between the active and inactive form of GTPase is closely regulated by exchange factors (for reviews see Bourne et al., 1991; Van Aelst and D’Souza-Schorey, 1997).

Rho GTPases are dynamic regulators of the actin cytoskeleton. Overexpression of inactive or active variants of Rho GTPases has demonstrated their involvement in the regulation of F-actin organization in various cell types, e.g. in fibroblasts (Nobes et al., 1995; Paterson et al., 1990; Ridley et al., 1992, 1995; Ridley and Hall, 1994), in macrophages (Allen et al., 1997), in epithelial cells (Braga et al., 1997) or in neuronal cells (Leeuwen et al., 1997). In fibroblasts, Cdc42 regulates filopodia formation in response to bradykinin (Kozma et al., 1995). Rac1 is responsible for the formation of lamellipodia and ruffles in response to PDGF or insulin, and RhoA induces the formation of stress fibers in response to LPA (Ridley and Hall, 1994). Moreover, focal adhesion complex formation has been also shown to be regulated by RhoA, Rac1 and Cdc42 (Clark et al., 1998; D’Souza-Schorey et al., 1998; Hotchin and Hall, 1995; Mackay et al., 1997; Nobes and Hall, 1995; Ridley et al., 1992). However, individual functions of Rho GTPases remain uncharacterized in additional cell types and some data on their general function are contradictory. Indeed, it has been shown that activated RhoA is a negative regulator of human monocyte spreading because it maintained the cells in a rounded morphology (Aepfelbacher et al., 1996). In addition, Rho inhibition enhanced adhesion of monocytes to fibronectin via the $\alpha_5\beta_1$ integrin receptor (Aepfelbacher, 1995). However, in fibroblasts, the interaction of integrins with the extracellular matrix was impaired by blocking Rho activity...

...focal complexes which are not visibly connected to F-actin structures. The Rho family of small GTPases plays an essential role in the regulation and organization of cellular cytoskeletal structures including F-actin and vinculin associated structures. Using bacterial toxins such as modified exoenzyme C3 (C3B) and toxin B or overexpression of constitutively active Rac and Rho proteins fused to the green fluorescent protein (GFP), we show that Rac and Rho play antagonistic roles in regulating the morphology of osteoclast-like cells. Inhibition of Rho by C3B triggered MNGC spreading whereas activated Rho promoted cell retraction. However, inhibition or activation of Rho led to complete disorganization of fibrillar actin structures, including podosomes. Toxin B inhibition of Rho, Rac and Cdc42 induced a time dependent F-actin and vinculin reorganization. Initially, actin fibers with associated adhesion plaques formed and disappeared subsequently. Finally, only small focal complexes remained at the MNGC periphery before retracting. At the time when actin fibers formed, we observed that Rac was already inhibited by toxin B. By combining C3B treatment and overexpression of a dominant negative form of Rac (N17Rac), we show that the formation of these focal adhesion and actin fiber structures required neither Rho nor Rac activity. Moreover, our results show that podosomes are extremely unstable structures since any modifications of Rho or Rac activity resulted in their dissociation.
protein from and Rac inhibition resulted in the decrease of the resorption role of Rho GTPases in osteoclasts and showed that both Rho Marchisio et al., 1984; Teti et al., 1991). How F-actin (Lakkakorpi al., 1993; Lakkakorpi and Väänänen, 1991; found in osteoclasts in between phases of bone resorption vinculin and talin. It has been suggested that the appearance of osteoclasts is characterized by the presence of a sealing zone, which is composed of a ring of F-actin surrounded by talin and vinculin. In non resorbing osteoclasts, F-actin is essentially organized into podosomes, small dots surrounded by a ring of vinculin and talin. It has been suggested that the appearance of podosomes correlates with a migratory cell phenotype found in osteoclasts in between phases of bone resorption (Lakkakorpi et al., 1993; Lakkakorpi and Väänänen, 1991; Marchisio et al., 1984; Teti et al., 1991). How F-actin organization and adhesion structures in osteoclasts are regulated is not known. To date only two studies analyzed the role of Rho GTPases in osteoclasts and showed that both Rho and Rac inhibition resulted in the decrease of the resorption activity of osteoclasts. Exoenzyme C3, an ADP-ribosylating protein from Clostridium botulinum known to selectively inhibit Rho (A, B and C) activity (Kikuchi et al., 1988; Rubin et al., 1998; Sehr et al., 1998; Sekine et al., 1989), induced actin reorganization by disrupting the ring of F-actin (Zhang et al., 1995) and, inhibition of Rac1 by introducing anti-Rac antibody into permeabilized osteoclasts triggered their rounding up (Razzouk et al., 1999).

We have established an in vitro differentiation model in which monocytes from chicken peripheral blood differentiate into adherent macrophages. After a phase of proliferation, these macrophages first aggregate, then spread and finally fuse to multinucleated giant cells (MNGC) which express the major osteoclast marker proteins, but do not resorb bone (Woods et al., 1995). These multinucleated cells are in turn able to generate mononucleated cells by a unique budding mechanism (Solari et al., 1995).

In the present study, we looked at the function of Rho and Rac proteins in the MNGC. We used two bacterial cytopathic toxins whose targets are known to be small Rho GTP-binding proteins; the cytotoxin B (ToxB) from Clostridium difficile spontaneously penetrates cell membranes and inactivates Rho, Rac and Cdc42 (Just et al., 1994, 1995), and C3B, a fusion protein between the exoenzyme C3 and the non-catalytic B fragment of diptheria toxin which allows the internalization of active C3 into intact cells expressing the diptheria toxin receptor. C3B inhibits RhoA, B and C (Aullo et al., 1993). In addition, by microinjection, we transiently overexpressed GFP-tagged constitutively active RhoA and Rac1 variants in MNGC lysates by a recently presented affinity precipitation technique. Our results show that both RhoA and Rac1 are necessary to maintain the F-actin organization in MNGC and that they have opposing effects on MNGC spreading. Moreover, we show that the cellular retraction induced by expression of dominant negative N17Rac1 needs active Rho protein.

**MATERIALS AND METHODS**

**Cells**

Cells were isolated from heparinized adult chicken (SPAFAS) whole blood as previously described (Solari et al., 1995). After separation on lymphocyte separation medium (LSM, Organon-technica, Durham, USA), leukocytes were collected from the gradient interface, washed and seeded at a density of $7.8 \times 10^6$ cells/cm² in tissue culture-treated Petri dishes in the presence of BT88 (DMEM Stabilix, DMEMNGS 10822470, BioMedia, Boussens, France) complete medium containing 5% chicken serum (Sigma, Saint-Quentin Falavier, France), 5% fetal calf serum (Roche, Mannheim, Germany), 10% tryptose phosphate broth (Difco, Michigan, USA), 1% penicillin streptomycin (BioMedia) and 0.2% amphotericin B (BioMedia). Non-adherent cells were removed with washes with PBS (BioMedia) and remaining adherent cells were primary macrophages. After 2 days in culture, macrophages were trypsinized (trypsin (0.5%)/EDTA (0.2%) (Roche) and reseeded at a density of $2.7 \times 10^5$ cells/cm². Cells were maintained at 37°C and 5% CO2. MNGC formation was monitored by phase contrast microscopy. Cell morphology was observed with an inverted microscope (Olympus IMT-2) using Hoffman contrast. For morphological analysis, cells were first fixed in methanol for 10 minutes and incubated in Wright-Giemsa (1% Wright, 10% Giemsa stain in H2O) for 15 minutes to stain nuclei and cytoplasm. For C3B experiment, diameter of at least one hundred cells were measured with Pegase software for each condition.

Avian macrophages from the HD11 cell line or fibroblasts from the NIH3T3 cell line were maintained in the same medium. For the transfection experiment, cells were transfected with expression vectors coding for either GFP alone, or fused to V14RhoA, V12Rac1 or N17Rac1 products using Fugene 6 (Roche, Mannheim, Germany) following the manufacturer instructions.

**Reagents**

Monoglucoisyltransferase toxin B extracted from Clostridium difficile (Just et al., 1994), as well as the modified ADP-ribosylating exoenzyme C3 from Clostridium botulinum fused to the B fragment of diptheria toxin (Aullo et al., 1993) were generous gifts from Dr P. Boquet (Inserm U452, Nice, France). The toxin B solution was stored at 1 mg/ml in 3% BSA/PBS (w/v) at −20°C.

Chimeras between enhanced GFP and GTPases were obtained by insertion of the mutated GTPases ORFs into the pEGFP-C1 expression vector (Clontech, Palo Alto, CA; Gauthier-Rouvière et al., 1998; Roux et al., 1997). DNA constructs (V14RhoAgfp, V12Rac1gfp and N17Rac1gfp) were amplified by a standard protocol and adjusted to a final concentration of 1 mg/ml in H2O.

The monoclonal anti-chick vinculin antibody VN3-24 was obtained from Developmental Studies Hybridoma Bank developed by Dr Shinuske Saga, maintained by the department of Pharmacology and Molecular Sciences, John Hopkins University School of Medicine (Baltimore, MD 21205) and the Department of Biological Sciences, University of Iowa (Iowa City, IA 52242) under contract NO1-HD-2-3144 from the NICHID, Goat anti-mouse IgG (H+L), TRITC or Cyalin 5 conjugated, were obtained from Jackson ImmunoResearch Laboratories (USA), FITC and TRITC labelled phalloidin were from Molecular Probes (Eugene, USA).

The GST-CRIB construct used for measuring GTP-Rac in cell
lysates was kindly provided by Dr J. Collard (Netherlands Cancer Institute, Amsterdam, Netherlands).

Microinjection
MNGC were trypsinized, seeded on 14 mm diameter glass coverslips and maintained for 24 hours in complete medium. Then, complete medium was buffered with 10 mM Hepes to prevent pH variation during the microinjection step. Intracellular microinjection of GFP constructs (0.25 mg/ml in H2O) was performed on an Axiovert 35M inverted microscope (Zeiss, Oberkochen, Germany) using a micromanipulator 5170 and a microinjector 5242 from Eppendorf (Hamburg, Germany). Microinjection was performed at 37°C. After injection, cells were further maintained at 37°C and 5% CO2 for 6 hours in the same medium or, when indicated, in the medium containing 10⁻⁶ M C3B.

GTP-Rac affinity precipitation assay
The Rac activity assays were performed as described by Sander et al. (1998). Briefly, fusion protein containing the Rac- and Cdc42-binding domain (CRIB) from human PAK1B (amino acids 56-272) fused to GST were produced in Escherichia coli BL21 cells. After isopropylthiogalactoside (IPTG) induction, pellets of bacteria were resuspended in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 2 mM MgCl₂, 0.2 mM Na₂SO₄, 10% glycerol, 20% sucrose, 2 mM DTT, protein inhibitor cocktail (Roche, Mannheim, Germany) and then sonicated. Cell lysates were centrifuged for 20 minutes at 4°C at 45000 g and the supernatants were incubated with glutathione-coupled Sepharose 4B beads (Pharmacia Biotech) for 30 minutes at 4°C. After 3 washes with lysis buffer, the amount of GST-CRIB fusion protein bound to the beads was estimated using Coomassie stained SDS gels.

HD11 macrophages transfected with recombinant GFP-GTPases, or MNGC treated or not with 1 mg/ml toxin B for 3 or 6 hours, were rapidly washed in ice-cold PBS and lysed on ice with 50 mM Tris-HCl (pH 7.4), 2 mM MgCl₂, 1% Triton X-100, 10% glycerol, 100 mM NaCl and protein inhibitor cocktail. Lysates were centrifuged for 5 minutes at 17000 g at 4°C and aliquots were taken from the supernatant to determine the protein concentration in the total cell lysate. 20 μg of bacterially produced GST-CRIB fusion protein bound to glutathione-coupled Sepharose beads were added to cell lysate and maintained for 30 minutes at 4°C. Beads and proteins bound to the fusion protein were washed 4 times in lysis buffer, eluted in Laemml sample buffer and then analysed for bound Rac molecules by western blotting using the monoclonal antibody 23A8 against human Rac (Upstate Biotechnology, NY, USA). In experiments with HD11 macrophages, recombinant GFP-GTPases were revealed by western blotting using a monoclonal antibody directed against GFP (Roche).

Immunofluorescence and confocal laser scanning microscopy
Trypsinized MNGC were seeded on glass coverslips that had been previously coated for 1 hour with complete medium and maintained for 48 hours in culture before drug addition. After the indicated times, cells were fixed for 10 minutes in 3.7% (v/v) formaldehyde in PBS, and permeabilized with 0.1% (v/v) Triton X-100 in PBS for 5 minutes. Permeabilized cells were then incubated for 45 minutes at room temperature with the monoclonal anti-chick vinculin antibody diluted in 3% BSA/PBS (w/v), washed in PBS and then revealed with either TRITC or cyanin-5-conjugated goat anti-mouse antibody in PBS/3% normal goat serum (v/v) for 45 minutes at room temperature. F-actin distribution was revealed after incubation with either TRITC- or FITC-conjugated phalloidin for 20 minutes at room temperature, according to the manufacturer’s procedure. Cells were extensively washed in PBS and coverslips were mounted in glycerol/PBS (9:1) supplemented with 0.1% p-phenylenediamine (Sigma) as antibleaching agent. Cells were observed under an LSM510 laser scanning confocal microscope (Zeiss) using a ×63 (NA 1.4) Zeiss Plan Neo Fluar objective. Available illumination sources were at 488 nm, 543 nm and 633 nm. To prevent cross-contamination between fluorochromes, the multitrack recording module was used which allows a sequential acquisition of each channel before merging. Images were processed in Adobe Photoshop and printed on a Kodak XLS 8600 PS thermal sublimation printer.

RESULTS

F-actin and vinculin organization in MNGC
MNGC appear as large, strongly adherent multinucleated cells, typically presenting several bright mononucleated cells attached to their surface (Fig. 1A). Time lapse microscopy observations have shown that MNGC are constantly changing shape and rapidly oscillating between a spread and more retracted morphology (not shown). This dynamic implies tightly regulated changes in the cytoskeletal organization. Since the small GTP binding proteins of the Rho subfamily are involved in F-actin rearrangement in most cell types, we decided to investigate their function in these cells, reminiscent of osteoclasts. At first, we characterized their F-actin and vinculin organization.

Secondary macrophage cell cultures containing MNGC were maintained for 2 days on glass coverslips before being fixed and labelled for F-actin and vinculin. Confocal microscopy analysis showed that MNGC, like osteoclasts, possessed at their periphery and at the level of the substratum, specific adhesion structures called podosomes (Fig. 1B-D), characterized by F-actin dots (Fig. 1E,G) surrounded by a ring of vinculin (Fig. 1F,G). Moreover, at the substratum level, F-actin was also organized into cortical rings of filaments (Fig. 1B, arrowheads), whereas vinculin accumulated in occasional adhesion plaques (Fig. 1C, arrows) or in focal complexes (Allen et al., 1997) not visibly associated with fibrilar actin structures (Fig. 1F,G, arrowheads). At the surface of the cells, F-actin was found in membrane ruffles (not shown).

Activated RhoA and Rac1 fused to GFP promote stress fibers and membrane ruffles, respectively, in NIH3T3 cells
In order to analyze functions of RhoA and Rac1, we either used mutated forms of RhoA (a constitutively active form, V14RhoA) and Rac1 (a constitutively active or a dominant negative form, V12Rac1 or N17Rac1, respectively) fused to the GFP protein. We constructed an expression vector derived from the pEGFP-C1 plasmid in which mutated GTPase-ORFs were inserted downstream to the enhanced green fluorescent protein (GFP) coding sequence. The resulting cDNAs encoded chimeric GTPaseGFP proteins. Before introducing the cDNAs by microinjection into MNGC, we tested the ability of V14RhoA-GFP and V12Rac1GFP to induce the formation of stress fibers and ruffles, respectively, after transient NIH3T3 fibroblasts transfection (Nobes and Hall, 1995). Whereas the expression of GFP protein alone did not change NIH3T3 cell morphology or their F-actin organization (Fig. 2A,B), V14RhoAGFP transfected NIH3T3 fibroblasts exhibited an increased number of actin stress fibers (Fig. 2C,D) and, as expected, the V12Rac1GFP construct was able to induce ruffles (Fig. 2E,F).
Activated RhoA triggers rounding up of MNGC, reorganization of actin filaments and vinculin clustering

In order to analyze the role of the Rho proteins in MNGC, we either used the membrane permeable C3B chimera toxin to inhibit Rho (A, B, C) proteins (Aullo et al., 1993), or the V14 mutated form of RhoA fused to the GFP protein (V14RhoA<sub>GFP</sub>).

Microinjection of V14RhoA<sub>GFP</sub> expression vector into the cytoplasm did not result in expression of recombinant RhoA protein. We therefore decided to microinject cDNA directly into one of the nuclei of multinucleated cells. We have previously checked that in MNGC, all nuclei are transcriptionally active (P. Boissy et al., unpublished). After microinjection into the nucleus of any of the GFP constructs, GFP fluorescence could be detected as soon as 4 hours later. We analyzed the microinjected cells 6 hours after injection. When vector expressing only the GFP protein was microinjected, GFP was diffusely distributed in the cytoplasm without significantly disturbing F-actin or vinculin structures (Fig. 3A-C). Occasionally, GFP accumulated in nuclei.

We then checked the ability of the V14RhoA<sub>GFP</sub> protein product to induce actin and vinculin rearrangements. At a low expression level of recombinant RhoA protein, as judged from GFP fluorescence intensities (Fig. 3D-F), vinculin formed bright complexes at the extreme periphery of the cells (Fig. 3E) and F-actin staining revealed dissolution of podosomes and bright peripheral fiber bundles (Fig. 3F). At higher

Fig. 1. Confocal microscopy analysis of F-actin and vinculin organization in MNGC. (A) Phase contrast image of a spread multinucleated giant cell (MNGC) with centrally localized nuclei (arrows) and round refringent mononuclear cells on top of the MNGC. (B-G) The same cell was analyzed at the substrate level for its F-actin (B,E) or vinculin (C,F) content by confocal microscopy at either low (B-D) or high magnification (E-G). Colocalization of both F-actin and vinculin is shown in D and G after merging the two channels. The actin cytoskeleton of MNGC is composed of cortical actin fibers (B, arrowheads) and podosomes (B, arrow; E-G). Arrows in C and D point to vinculin-labelled focal adhesion plaques and arrowheads in F and G point to vinculin-labelled focal complexes not associated with F-actin structures. Bars: 25 μm (A-D); 3 μm (E-G).
magnification, no more F-actin could be detected in association with vinculin complexes (Fig. 3H,I, arrows). Higher expression levels of V14RhoA
 led to strong retraction of MNGC (Fig. 3J-L). Vinculin accumulated in clusters and formed numerous peripheral focal complexes associated with fine and small F-actin filaments (Fig. 3K,L, arrowhead) whereas F-actin was located in small retraction fibers (Fig. 3L, arrow) as well as in cytoplasmic aggregates resembling to abnormal podosomes. Vinculin formed aggregates instead of a ring around actin dots (Fig. 3K,L, empty arrow). Although MNGC were completely retracted when V14RhoA
 was highly expressed, they were still adherent, even 18 hours after microinjection (not shown).

**Rho inhibition in MNGC triggers their spreading as well as the disassembly of actin filaments and redistribution of vinculin**

Since Rho activation induced cytoskeletal rearrangements and MNGC retraction, we investigated the effect of Rho inactivation by means of the C3B chimeric toxin. Compared to untreated MNGC (Fig. 4A), when MNGC were incubated with $10^{-8}$ M C3B for 3 (Fig. 4B), or 6 hours (Fig. 4C), they increasingly spread out. When we compared the diameter of MNGC normalized to the number of nuclei per cell, after 6 hours treatment, we observed an increase of about 40% in the diameter of treated versus untreated MNGC (Fig. 4D).

Confocal immunofluorescence studies showed that the spreading of MNGC correlated with a complete disorganization of their actin cytoskeleton as soon as 3 hours after treatment (Fig. 4E-G). Cortical actin fibers, as well as podosomes, completely disappeared. Actin could still be found in small upper located membrane ruffles (data not shown) and in F-actin microspikes at the periphery of the cells (Fig. 4E). On the other hand, vinculin was also redistributed from podosomes and small adhesion plaques to small peripheral focal complexes sometimes at the base of F-actin microspikes (Fig. 4E-G, arrow). After 6 hours treatment, vinculin accumulated at the cell periphery forming continuous and discontinuous lines along or near the cell border, partially colocalized with F-actin (Fig. 4G-H).

These results show that Rho activity is necessary to maintain the typical F-actin organization in MNGC and that Rho inactivation triggers flattening and spreading of MNGC concomitantly with F-actin disassembly and vinculin redistribution. Therefore, in MNGC, Rho activation promotes cell retraction and bundling of cortical actin without forming typical stress fibers whereas Rho inhibition allows additional cell spreading. However, contrary to fibroblasts in which stress fiber formation depends on Rho activity, no characteristic F-actin structures in MNGC appear to be directly controlled by Rho since activation as well as inhibition of Rho lead to disorganization of podosomes and cortical fibers. We then decided to investigate the involvement of the other Rho GTPase proteins.

**Inhibition of Rho family GTPases induces time-dependent actin and vinculin reorganization and MNGC retraction**

To analyze the involvement of the other Rho GTPase proteins, including Rho, Rac and Cdc42, in F-actin and vinculin organization of MNGC, we used cytotoxin B (ToxB) which inactivates Rho, Rac and Cdc42. MNGC were maintained on
glass coverslips for 48 hours and treated with 1 ng/ml toxin B for 3, 6 or 18 hours. After 3 hours of treatment, the MNGC remained spread with apparent normal morphology (Fig. 5A,B). 6 hours after drug addition, 40% of MNGC were retracted and presented only few cytoplasmic extensions (Fig. 5C, arrows). At that time, spread cells appeared wrinkled and some of them exhibited small cytoplasmic extensions indicating that they were in the retraction process (Fig. 5C, arrowheads). As shown in Fig. 5D, MNGC treated for 18 hours had dramatically retracted and maintained cytoplasmic bridges connecting the cellular bodies, suggesting complete disruption of the cytoskeleton. We further analyzed the distribution of F-actin and vinculin in spread MNGC at the early stages (3 and 6 hours treatment) of ToxB-induced Rho GTPases inhibition before complete retraction.

In contrast to untreated cells, MNGC treated for 3 hours with ToxB had lost their podosomes as well as their cortical actin fibers (Fig. 5E-G). Their apical plasma membranes appeared smooth and devoid of ruffles (not shown). Surprisingly, most of the cells had developed actin fiber structures (Fig. 5E, arrow) which ended in vinculin containing focal adhesion plaques (Fig. 5F-G, arrows). After 6 hours treatment, the actin fibers had completely disappeared from the still spread MNGC and F-actin appeared diffuse and completely disorganized within the cells (Fig. 5H). However, vinculin still aggregated at the periphery of the cells into small focal complexes (Fig. 5I, arrow). This complete disruption of cytoskeleton 6 hours after ToxB treatment was followed by the complete retraction of the MNGC as observed 18 hours after drug addition (see above). In conclusion, ToxB treatment induced sequential changes
in cytoskeletal organization of MNGC which could reflect a
time-dependent differential inhibition of Rho GTPases by
ToxB. As Rac activity is closely linked to Rho in the control
of cell contractility and spreading (Sanders et al., 1999), we
analyzed more precisely the role of the Rac GTPases in
MNGC.

**Activated Rac promotes MNGC spreading, actin filament assembly and focal complex formation, whereas Rac inactivation leads to a dramatic retraction**

MNGC were microinjected either with plasmids coding for
V12Rac1gfp, the constitutively active form, or N17Rac1gfp, the
dominant negative form of Rac GTPase fused to GFP, and
analysed 6 hours later. Confocal microscopy observations after
V12Rac1 microinjection indicated that F-actin and vinculin
were dramatically reorganized as shown in Fig. 6A-C. At the
substratum level, the active Racgfp product was found
colocalized with actin ruffles (Fig. 6A,C, bold arrow) as well
as at the extreme periphery of the cell (Fig. 6A,C); vinculin
formed lots of small focal adhesions at the end of numerous,
radially oriented thin F-actin fibers (Fig. 6B,C, arrow).
Podosomes were absent. This cytoskeletal reorganisation was
accompanied by flattening and increased spreading of the
MNGC. Indeed, microinjected MNGC containing only 3 or 4
nuclei usually reached at least 140 μm in diameter whereas
corresponding control MNGC never spread over 100 μm, as
indicated in Fig. 4D.

On the other hand, microinjection of the transdominant-
negative form of Rac, N17Rac1gfp (Fig. 6D-F) induced MNGC
retraction concomitantly with vinculin aggregation in small
spots throughout the cell (Fig. 6F). F-actin disorganized and
short filaments aggregated in a part of the cell (Fig. 6F, arrow).
Some filopodia-like structures were also formed (Fig. 6F,
arrowhead). It has to be noted that N17Rac1gfp grossly
colocalized with F-actin (Fig. 6D,F) and did not induce actin
fibers polymerization.

Since in V12Rac1gfp-microinjected MNGC, small actin
fibers were formed, we asked whether actin fibers formation in
ToxB treated MNGC could be the consequence of a differential
inhibition of either Rho or Rac and specially if Rac is well
inhibited by ToxB.

**Rac is rapidly inhibited in toxin B treated MNGC**

To evaluate Rac activity in MNGC, we used an affinity
precipitation assay based on GST-CRIB, the GTPase binding
domain (CRIB) of the p21 activated kinase (PAK) fused to the
glutathione S-transferase (GST) as described by Sander et al.
(1998). The CRIB domain of PAK is known to interact with
GTP-bound Rac and Cdc42 (Manser et al., 1994). We first
checked that GST-CRIB was able to precipitate V12Rac1gfp
expressed in the avian macrophage HD11 cell line. HD11 cells
were transfected either with GFP alone, V14RhoAexpressed
V12Rac1gfp, or N17Rac1gfp expression vectors. We performed
affinity precipitation assays and revealed bound GFP-GTPase

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Fig. 4. C3B inhibition of Rho increases MNGC
spreading and disrupts organization of F-actin and
vinculin. MNGC were treated or not with 10^{-8} M
C3B for 3 or 6 hours, fixed and stained either with
Wright-Giemsa solution (A-C) or for F-actin (E,G) or
vinculin (FH). In D, the diameter of spread MNGC
is represented as a function of the number of nuclei
per cell. Error bars represent standard deviations for
each conditions. After C3B treatment, MNGC
appeared increasingly spread and flattened, and cell
limits became hardly visible after 6 hours treatment
(C, arrows indicate cell margins).

Immunofluorescence labelling shows that cortical
actin, podosomes and small actin containing-focal
adhesion plaques were no longer visible after 3 hours
C3B treatment (E). At the same time, vinculin
accumulated at the periphery in fine focal complexes
(F) and can be found associated with actin-containing
microspikes (E,F, arrow). After 6 hours treatment,
vinculin as well as F-actin were found partially
colocalized in a discrete peripheral labelling at the
cell margin (G,H). Bars: 40 μm (A-C), 25 μm (E-H).
by western blotting with an anti-GFP antibody. Whereas GFP, V14RhoA<sup>GFP</sup>, V12Rac1<sup>GFP</sup>, or N17Rac1<sup>GFP</sup> were expressed and detected in total cell lysates (Fig. 7A, lanes 1-4), GFP, V14RhoA<sup>GFP</sup> and N17Rac1<sup>GFP</sup> were never detected in precipitates (lanes 5, 6, 8). As expected, only the constitutively activated form of Rac was precipitated together with GST-CRIB (lane 7). We then performed assays on ToxB-treated MNGC. Cell lysates derived from untreated or 3 or 6 hours ToxB-treated cells were incubated with the GST-CRIB fusion protein and analysed by western blotting with anti-Rac antibody for the presence of CRIB-bound GTP-Rac. As shown in Fig. 7B, the total amount of Rac protein was identical in each condition (Fig. 7B, lanes 3-5) whereas the level of GTP-Rac decreased very rapidly in ToxB-treated cells (compare lane 6 to lanes 7, 8). After 3 hours of treatment, levels of GTP-bound Rac were diminished 50 fold and after 6 hours, Rac was completely inhibited. These results show that Rac is rapidly inhibited by ToxB and, as a consequence, actin fibers formation at 3 hours of ToxB treatment did not require Rac activity. Similarly, we raised the possibility that actin fibers formation in ToxB-treated MNGC could be due to residual Rho activity.

**Actin fiber formation in ToxB-treated MNGC is Rho independent**

To make sure that Rho activity in ToxB-treated MNGC was completely inhibited, they were maintained in the presence of 10<sup>-8</sup> M C3B for 6 hours before addition of ToxB for 3 (Fig. 7C) or 6 hours (Fig. 7D). Concomitantly, as a control, a subset of MNGC was treated with C3B alone for 9 (Fig. 7E) or 12 hours (Fig. 7F). F-actin (green) and vinculin (red) contents were analysed by confocal microscopy. Merged channels are shown in G and J. The analysis of the F-actin and vinculin organization showed that, in contrast to untreated cells exhibiting cortical actin fibers and podosomes, MNGC maintained for 3 hours in the presence of ToxB developed thick F-actin fibers (E, arrow) associated with focal adhesion plaques (F,G, arrows). After 6 hours of treatment, actin was diffusely distributed all over the MNGC (H) and vinculin accumulated at the cell periphery in fine focal complexes (I, arrow). Bars: 40 µm (A-D); 25 µm (E-J).
Role of Rho and Rac on osteoclast cytoskeleton distribution than those found in MNGC treated for 6 hours (Fig. 4G-H): focal complexes are formed and found at the cell periphery (arrowheads). These data indicate that actin fibers and focal adhesion plaques formation in ToxB treated MNGC are independent of Rho and Rac activities.

Finally, we checked whether simultaneous inhibition of Rac and Rho activities was sufficient to induce actin fibers and focal adhesion plaques formation.

Fig. 7. Actin fibers formation in MNGC does not depend on Rac and Rho activities. (A) Avian macrophages from the HD11 cell line were transfected with expression vectors expressing either GFP alone (lanes 1 and 5), V14RhoGFP (lanes 2 and 6), V12Rac1GFP (lanes 3 and 7) or N17Rac1GFP (lanes 4 and 8). Cell lysates of transfected cells were incubated with GST-CRIB fusion protein bound to the glutathione-Sepharose beads (lanes 5-8). The amount of bound recombinant GFP-GTPases was determined by western blotting using a monoclonal anti-GFP antibody and compared to the expression level of transfected protein in total cell lysates (lanes 1-4). (B) MNGC were treated with 1 ng/ml ToxB for 0 hours (lanes 3 and 6), 3 hours (lanes 4 and 7) or 6 hours (lanes 5 and 8). Rac level in total cell lysates (lanes 3-5) or in GST-CRIB precipitates (lanes 6-8) were determined by western blotting with monoclonal anti-Rac antibody. As a control, MNGC lysate was incubated with GST bound to the glutathione Sepharose beads (lanes 1 and 2). To analyse the consequence of Rho inhibition on actin fibers formation in ToxB-treated MNGC, cells were treated with a combination of $10^{-8}$ M C3B and 1 ng/ml ToxB. MNGC were previously treated with C3B for 6 hours and ToxB was added into the medium for an additional 3 (C) or 6 hours (D). Cells treated for 9 or 12 hours with C3B alone are shown in E and F. F-actin and vinculin were labelled and analysed by confocal microscopy. Merged channels are shown. Arrowheads point to focal complexes and arrow to actin fibers associated with focal adhesion plaques. Bar, 25 µm (C-F).
Rho is required for the retraction of MNGC induced by Rac inhibition. MNGC were microinjected with either GFP- (A-C) or N17Rac1gfp-expression vectors (D-F). Immediately after microinjection, MNGC were treated with 10⁻⁸ M C3B for 6 hours. Cells were then fixed and monitored under a confocal microscope for GFP fluorescence (A,D). Vinculin distribution was revealed by indirect immunofluorescence (B,E) whereas F-actin organization was probed with phallolidin rhodamine (C,F). As shown in Fig. 4, C3B treated MNGC microinjected with GFP control vector (A) spread out and accumulated vinculin at their periphery (B, arrows), whereas podosomes and fibrillar actin disappeared (C). On the other hand, N17Rac1gfp expressing and C3B-treated MNGC remained spread (D-F) and developed focal adhesions (E, arrow) at least partially associated with actin fibers (F, arrow). Moreover, vinculin accumulated at the periphery of the MNGC (compare E to B), Bar, 25 μm.

**DISCUSSION**

Macrophage-derived multinucleated giant cells (MNGC), like osteoclasts lying on bone, have a unique cytoskeletal organization most obviously represented by highly specific adhesion structures, called podosomes. These are dot-like F-actin structures containing an F-actin core, which are in tight contact with the substratum (Teti et al., 1991). In addition, F-actin is essentially organized into rings of cortical filaments and abundant membrane ruffles covering the surface of the cells. Vinculin is found in small rings surrounding the actin core of podosomes as well as in small focal complexes at the cell periphery or very few typical adhesion plaques. In this study, we have shown that this unique cytoskeletal architecture is tightly regulated by the balanced activities of Rho-GTPases. Indeed, activation as well as inhibition of Rho proteins resulted in the disassembly of podosomes and cortical actin filaments. Thus, whereas Rho activity governs the stress fiber cytoskeleton of fibroblasts, Rho is not directly involved in the formation of a typical MNGC actin structure but is necessary to maintain these structures once they are formed. Moreover, Rho activity counterbalanced MNGC spreading. Rho activation triggered cellular retraction whereas, as already reported for monocytes (Aepfelbacher et al., 1996), Rho inhibition increased MNGC spreading. Interestingly, even though F-actin was completely disorganized in C3B treated MNGC, vinculin still accumulated at the cell margin in focal complexes. Such a vinculin distribution in peripheral focal complexes has already been described in C3-treated fibroblasts adherent to fibronectin which do not develop focal adhesion plaques. These focal complexes have been considered as precursors of focal adhesion plaques (Clark et al., 1998; Rottner et al., 1999). In the MNGC, accumulation of focal complexes at the cell margin correlated with increased cell spreading in response to Rho inhibition. This suggests that the spreading process is dependent upon Rho inhibition and characterized by focal complex formation; podosomes and cortical actin filaments would then be characteristic of MNGC in a stabilization process. The switch between spreading cells and immobilized cells could need only transient Rho activation since constitutively activated Rho leads to cellular retraction.
To evaluate the involvement of other Rho GTPases in the control of F-actin and vinculin organization in MNGC, we used toxin B which inhibits Rho GTPases members. Toxin B treatment of MNGC resulted in a rapid dissociation of actin from cortical filaments and podosomes, and subsequently, in a transient assembly of actin fibers terminating in vinculin-containing focal adhesion plaques. After 6 hours treatment, some MNGC retracted whereas the still spread cells exhibited vinculin-containing focal complexes at their periphery. After 18 hours of treatment, MNGC were completely retracted. These experiments suggested that ToxB sequentially inhibited Rho members and then ToxB treatment unmasked a delicate equilibrium in the activities of the various Rho GTPases. Thus, to define the relative function of Rho and Rac GTPases, it was necessary to know the effect of Rac activity modification. Microinjection of constitutively active V12Rac promoted spreading, F-actin polymerisation into fine peripheral fibers and formation of small focal contacts at the end of fine actin fiber structures. Nevertheless, cortical filaments and vinculin complexes formed under these conditions were different from those found in untreated MNGC and, then, actin fibers were thinner than those found in ToxB-treated MNGC. As expected for the V12Rac-induced MNGC spreading, overexpression of dominant-negative N17Rac triggered cell retraction and dramatic F-actin and vinculin disorganization.

Taken together, these data suggest that at least Rho and Rac play opposing roles on MNGC spreading. Moreover, specific inhibition of either Rac or Rho did never result in actin fiber formation or in inducing typical F-actin or vinculin structure found in normal MNGC. It suggests that a complex regulatory network maintains their normal morphology and cytoskeletal architecture. The F-actin and vinculin structures found in MNGC are not formed by the dominant activity of a single Rho GTPase and in these cells, Rho and Rac activity seem to be antagonistically linked; our data are in agreement with recent reports showing that, in Swiss3T3, Rho and Rac influence the development of focal contacts and focal complexes through mutually antagonistic pathways. Rac is required for the initiation of new contact sites whereas Rho serves in the maturation of preformed contacts into focal contacts (Rottner et al., 1999). This relationship between Rho and Rac could explain the phenotype inducible in ToxB-treated MNGC. GTP-Rac precipitation assays indicated that Rac was rapidly inhibited in the first hours of treatment. If we considered a differential inhibition of Rac and Rho by ToxB, Rho activity could be sufficient to form actin fibers from existing focal complexes in the first 3 hours of treatment. After 6 hours of treatment, Rho was finally inhibited by ToxB and Rho could not trigger actin fibers formation from the remaining peripheral focal complexes. However, a combination of C3B and ToxB treatment shows that, in MNGC, ToxB-induced actin fibers were reduced in thickness and number, but were not inhibited. This suggested that either a C3B-insensitive pool of Rho GTPases was involved in these actin fibers formation or that these actin fibers did not depend on Rho protein. This hypothesis was reinforced by experiment using concomitant C3B treatment and N17RacGFP microinjection leading to large focal adhesion plaque assembly and localized actin fiber polymerization. Thus, it could be possible that an upper regulatory level exists between Rho GTPases. Indeed, a new family of Rho members, the Rdn proteins, have been recently identified. They are constitutively bound to GTP and overexpression of Rdn1 and Rdn3/RhoE were able to disassemble stress fibers and focal adhesion plaques in response to LPA or membrane ruffles in response to PDGF in fibroblasts, suggesting that Rdn proteins can antagonize the Rho and Rac pathways. Moreover, Rdn proteins are not substrates for ADP-ribosylation by exoenzyme C3 (Nobes et al., 1998). Thus, actin fibers and focal adhesion plaques formed when Rho and Rac were inhibited in MNGC could be the consequence of an unidentified Rho GTPase which, in turn, should be a regulator of Rho and Rac in untreated MNGC.

As ToxB inhibits all Rho GTPase members, it will be of interest to investigate the function of Cdc42 in MNGC. It has been shown that, in macrophages, high levels of Cdc42 activity antagonize the formation of lamellipodia (Allen et al., 1997) and that spreading of monocytes is associated with an increased membrane association of Cdc42 (Aepfelbacher et al., 1994). Moreover, it has been already reported, in BHK cells, that downregulation of either Rho, Rac or Cdc42 leads to the activation of the two non inhibited Rho GTPases and that treatment with Clostridium difficile toxin A leads to the transient appearance of a variety of activation phenotypes (Moorman et al., 1999). Thus, it seems plausible that the relative amounts of Rho, Rac and Cdc42 activities control the cytoskeletal organization and the cell morphology in MNGC and a change in the activity of each one modifies the activities of the others.

Podosomes were characterized as adhesion structures composed of a fine F-actin core surrounded by a ring of vinculin (Teti et al., 1991). We show that modification of either Rho or Rac activity disassembles these structures in MNGC, indicating that podosomes are highly unstable structures that require finely tuned activities of several Rho GTPases for their function. Podosomes were first identified in v-src-transformed fibroblasts (Tarone et al., 1985). It is striking to note that osteoclasts are among the cells expressing the highest levels of c-src (Horne et al., 1992) and c-src –/– mice have osteoclasts unable to resorb bone due to bone matrix adhesion defects (Lowe et al., 1993). It is thus tempting to speculate that, at least in macrophage-derived MNGC as well as in osteoclast cells, c-src is an upstream regulator of Rho GTPases.

S. Ory was supported by a fellowship from the Ministère de l’Éducation et de la Recherche (France). We are grateful to Dr J. Collard for providing us with the GST-CRIB construct and to Dr S. Van Delft for technical comments. We specially thank Dr M. Pfaff for helpful discussions and critical reading of the manuscript. This work was supported by grants from the C.N.R.S., the Ligue Nationale Française contre le Cancer, the Association pour la Recherche contre le Cancer et an A.I.P. from I.N.R.A.

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