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The RhoA Effector mDia Is Induced During T Cell Activation and Regulates Actin Polymerization and Cell Migration in T Lymphocytes

Miguel Vicente-Manzanares,* Mercedes Rey,* Manuel Pérez-Martínez,* María Yáñez-Mó,* David Sancho,* José Román Cabrero,* Olga Barreiro,* Hortensia de la Fuente,* Kazuyuki Itoh,† and Francisco Sánchez-Madrid2*  

Regulation of actin polymerization is critical for many different functions of T lymphocytes, including cell migration. Here we show that the RhoA effector mDia is induced in vitro in activated PBL and is highly expressed in vivo in diseased tissue-infiltrating activated lymphocytes. mDia localizes at the leading edge of polarized T lymphoblasts in an area immediately posterior to the leading lamella, in which its effector protein profilin is also concentrated. Overexpression of an activated mutant of mDia results in an inhibition of both spontaneous and chemokine-directed T cell motility. mDia does not regulate the shape of the cell, which involves another RhoA effector, p160 Rho-coiled coil kinase, and is not involved in integrin-mediated cell adhesion. However, mDia activation blocked CD3- and PMA-mediated cell spreading. mDia activation increased polymerized actin levels, which resulted in the blockade of chemokine-induced actin polymerization by depletion of monomeric actin. Moreover, mDia was shown to regulate the function of the small GTPase Rac1 through the control of actin availability. Together, our data demonstrate that RhoA is involved in the control of the filamentous actin/monomeric actin balance through mDia, and that this balance is critical for T cell responses. The Journal of Immunology, 2003, 171: 1023–1034.  

Cell migration can be considered a multistep cycle that includes the formation of filopodia to explore the external milieu (1), followed by the extension of a lamellipodium in the direction of cell movement, consolidation of cell adhesion in the vicinity of the leading edge, translocation of the cell body forward, and, finally, retraction of the rear part of the cell (2). Filopodia and lamellipodium formation at the leading edge requires active actin polymerization. Therefore, at the leading edge of migrating cells intracellular signals are translated into actin polymerization, which probably requires chemoattractant receptors connected to actin polymerization machinery (3). In this regard, molecules involved in actin polymerization and branching, such as Wiskott-Aldrich syndrome protein (WASP),3 the Arp2/3 complex, vasodilator-activated phosphoprotein (VASP), or profilin, have been located at the leading edge of motile cells (4–7).  

The small GTPase, RhoA, exhibits a dual role in the formation of focal adhesions and the regulation of actin stress fibers (8). These effects are mediated through different downstream effectors. The serine/threonine kinase p160 Rho-coiled coil kinase (ROCK) is involved in myosin phosphorylation and actomyosin assembly (9). The 140-kDa protein mDia, the mammalian homologue of Drosophila melanogaster diaphanous (10), belongs to the family of formins, proteins that possess two or three formin homology (FH) domains, one of them interacting with the actin-polymerizing protein profilin (11). mDia seems to regulate actin polymerization through its interaction with profilin (10). The recent finding that the yeast homologue of mDia, Bni1p, is able to nucleate de novo actin polymerization through its FH2 domain has shed new light on the roles of these molecules in actin remodeling (12, 13).  

Data obtained in yeast models have demonstrated that the regulation of actin polymerization by WASP (Bee1)-Arp2/3 and that by mDia (Bni1p)-profilin are different. Each route regulates the formation of different actin-based structures, namely patches and cables, respectively (14, 15), which relate to the different behaviors of the Arp2/3 complex and formins in the nucleation of actin polymerization, originating branched or linear actin cables, respectively (16). Furthermore, comparison with mammal fibroblasts indicates that whereas the Cdc42/Rac1-WASP/Scar-Arp2/3 cascade is involved in the generation of new structures through interaction of Arp2/3 with the pointed ends of actin filaments (17), the RhoA-mDia-profilin pathway regulates actin polymerization in preformed structures such as stress fibers through the association of mDia with the barbed end of growing actin filaments (16). However, some degree of cross-talk exists between the two mechanisms, as mDia may act upstream of Rac1 in the regulation of actin-based membrane ruffles (18).  

RhoA is involved in lymphoid polarization and chemotaxis (19). Interaction of chemokines such as stromal cell-derived factor 1α (SDF-1α) with its receptor CXCR4 activates RhoA and its effector p160ROCK, leading to myosin L chain phosphorylation and actomyosin contraction (20), as well as to tail retraction of lymphocytes (20) and monocytes (21), which are absolutely required for

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3 Abbreviations used in this paper: WASP, Wiskott-Aldrich syndrome protein; EGFP, enhanced green fluorescence protein; F-actin, filamentous actin; FH, formin homology; PMN, polymorphonuclear leukocytes; SDF-1α, stromal cell-derived factor 1α; TIL, tumor-infiltrating lymphocytes; VASP, vasodilator-activated phosphoprotein; ROCK, Rho-coiled coil kinase; G-actin, monomeric actin.
chemokine-induced lymphoid migration (20). The contribution of mDia to cytoskeletal reorganization has been assessed in adherent cells such as fibroblasts (22, 23) and epithelial cells (24), but no information is available on mDia expression or function in hematopoietic cells. In this report we show that mDia is induced in human T lymphocytes in an activation-dependent manner. In addition, we demonstrate that mDia is critically involved in T cell motility through regulation of the filamentous actin (F-actin)/monomeric actin (G-actin) ratio and the availability of actin for other effectors, such as the small GTPase, Rac1. Thus, mDia regulates actin in T cells, suggesting its critical involvement in T cell migration.

Materials and Methods

Cells

Human HeLa cells and the T cell lines Jurkat, HSB-2, and Peer were cultured in RPMI 1640 medium with 10% FCS. Human PBL and polymorphonuclear leukocytes (PMN) were obtained as previously described (20, 25). T lymphoblasts were obtained from human PBL by treatment with 1 µg/ml PHA-L (Sigma-Aldrich, St. Louis, MO) for 48 h and were maintained with 50 U/ml IL-2. Tumor-infiltrating lymphocytes (TIL) and T lymphocytes from rheumatoid synovium of volunteer donors were isolated as previously described (26).

Abs and reagents

The following Abs were employed: T3b (anti-CD3) and the irrelevant control P3/H11003 (26), anti-mDia (Transduction Laboratories, Lexington, KY); anti-RhoA (Santa Cruz Biotechnology, Santa Cruz, CA), anti-α-tubulin and vimentin mAbs (Sigma-Aldrich), and 2H11 Ab against profilin (gift from Dr. B. M. Jockusch, Zoological Institute, Braunschweig, Germany; previously described (27)). Phalloidin-Alexa 568 and -Alexa 647 and DNase I-Alexa 594 were obtained from Molecular Probes (Eugene, OR). Human rIL-2 was obtained through the National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, from M. Gately. Lectin from Phaseolus vulgaris (PHA-L), human fibronectin, poly-1-lysine, manganese chloride, and PMA were purchased from Sigma-Aldrich.

cDNA and transfection

The pEGFP-C1 and pEGFP-F control plasmids were purchased from Clontech Laboratories (Palo Alto, CA). The activated mutant of p160ROCK (Δ4-ROCK) and the kinase-dead mutant (KD-ROCK) have been previously described (33). The wild-type enhanced green fluorescence protein (EGFP)-mDia, activated mutant EGFP-mDia ΔN3, EGFP-mDia F2, and EGFP-mDia ΔN3 (HindIII) have been described previously (18, 28). EGFP-mDia F1 (aa 570–735) was generated by PCR (primers 5′-GGAGGATCTGCTGCTGTTCCCCTG-3′ and 3′-AGGGGGCCCAAATCCAAAAGGGGAGGT-5′), cloned in-frame in BglII/SmaI of the multiple cloning site of pEGFP-C1, and confirmed by sequencing. V12Rac1 and N17Rac1 constructs (originally made available by Dr. A. Hall, Medical Research Council, University College, London, U.K.) were generated by subcloning into the EcoRI site of pCDNA3, and orientation was confirmed by restriction. Jurkat, Peer, or HSB-2 T cells were transfected as described previously (19), and transfection efficiency was estimated by flow cytometry.

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** Up-regulation of mDia expression during T cell activation. A, Expression of mDia by different cell lineages. Lysates from the indicated T cell lines, HeLa cells, PMN, and PBL, were blotted for mDia. One representative experiment of three performed is shown. B, Induction of mDia upon activation of PBL. Fresh isolated PBL were cultured in the presence of PHA and later IL-2, and samples of cells were harvested at the indicated times. Cells were then lysed and blotted for mDia and RhoA. A representative experiment of four performed is shown. C, Up-regulation of mDia expression by CD3 stimulation. Fresh isolated PBL were seeded at the indicated concentration of immobilized anti-CD3 in the presence or the absence of SDF-1α for the indicated times. Cells were lysed and blotted for mDia, RhoA, and vimentin. A representative experiment of four performed is shown. D, In vivo up-regulation of mDia upon T cell activation. Samples of fresh isolated PBL, TIL, and rheumatoid arthritis synovial T cells were lysed and blotted for mDia and vimentin.
Western blot

Samples of PBL (5 × 10^6), PMN (2 × 10^6), Jurkat, Peer, and HSB-2 (2 × 10^6 each); and HeLa (1 × 10^6) cells were lysed in RIPA buffer (1% Triton X-100, 0.1% SDS, and 0.5% deoxycholate in ice-chilled TBS with protease inhibitors). Then, cell lysates were assayed for protein concentration and analyzed by Western blot (20).

Flow cytometry

Expression of mDia in HSB-2 cells was performed as follows: cells (2 × 10^6) were fixed in 2% formaldehyde for 10 min, rinsed in TBS, and permeabilized with 0.5% Triton X-100 in TBS for 5 min. The cells were rinsed again, stained with 100 μl of anti-mDia Ab (2.5 μg/ml) for 30 min at 4°C, rinsed, and incubated for another 30 min with an anti-mouse Ab coupled to allophycocyanin (BD Pharmingen, San Diego, CA). Fluorescence intensity was determined in a FACSCalibur flow cytometer (BD Biosciences). Three cell subsets were defined according to GFP expression (NULL, LOW-MED, and HIGH; for an example, see Fig. 5A), and the level of mDia expression in each one was calculated.

For expression of the integrin activation epitope HUTS-21, cells were incubated for 30 min with 1 mM Mn^{2+} at 37°C, followed by incubation with the HUTS-21 Ab (10 μg/ml) for another 30 min and an allophycocyanin-coupled anti-mouse Ab, after which HUTS-21 expression in each GFP-expressing population was determined as described above.

Immunofluorescence and confocal microscopy

Indirect immunofluorescence assays were performed as previously described (19). Staining for G-actin was performed as reported previously (29). Briefly, cells were fixed with 4% paraformaldehyde in PBS containing 2 M sucrose and 2 mM MgCl2, for 20 min at room temperature, permeabilized in 0.5% Triton X-100 in PBS for 10 min, and stained with a 1/10,000 dilution of Alexa 594-labeled DNase I (Molecular Probes, Eugene, OR). Cells were observed using a DMR photomicroscope equipped with QFISH software (Leica, Mannheim, Germany). For quantification of cell polarization, at least 300 transfected cells were counted in three independent experiments. For spreading experiments, the cells were seeded on either anti-CD3 Ab (25 μg/ml) or fibronectin in the presence of 25 ng/ml PMA, allowed to settle for 30 min, and stained for F-actin. Spreading was scored as the percentage of cells exhibiting ectopic lamellae formation.

Confocal images (series of optical sections) were obtained with a TCS-SP confocal laser scanning equipment (Leica Microsystems, Heidelberg, Germany).

Migration assay

Migration assays were performed in Boyden-modified chambers (Transwell, Costar, Cambridge, MA) as previously described (19). Quantification of cell migration was performed, counting transfected and nontransfected cells by flow cytometry for 60 s. Transfected and nontransfected cells were individually evaluated as indicated.

Actin polymerization assay

Determination of the levels of polymerized actin was performed as follows. Transfected T cells at 2 × 10^5/ml were resuspended in RPMI 1640 medium supplemented with 0.1% human serum albumin, and 100 μl of cells were incubated, or not, with 10 nM SDF-1α at 37°C for 15 s. Then cells were fixed and permeabilized with 200 μl of CELLwash buffer (BD Biosciences, Mountain View, CA) for 10 min at 37°C and incubated for 30 min at 4°C with 5 μg/ml of Alexa 647-conjugated phalloidin (Molecular Probes). The levels of polymerized actin were determined for each GFP-expressing population as described above.

Adhesion assay

Adhesion was estimated by flow cytometry as follows. Jurkat or HSB-2 T cells were transfected with the different constructs and seeded on flat-bottom, tissue culture-coated, 96-well plates coated with 10 μg/ml fibronectin at 10^5 cells/well and allowed to settle for 15 min. When indicated, the cells were treated with 1 mM Mn^{2+}. After adhesion, unbound cells were removed by gently washing (three times), and adhered cells were detached by treatment with trypsin. Adhered, transfected, and nontransfected cells were individually evaluated as indicated.

Results

Expression of mDia is induced upon activation of T lymphocytes

To explore the expression of mDia in immune cells, lysates from different lymphoid lines and primary PBL and PMN were subjected to Western blot. PMN, Jurkat, and Peer T cells showed a strong band of the expected molecular mass, whereas HSB-2 T cells and PBL had low and very low levels of the molecule, respectively (Fig. 1A). On the other hand, HeLa cells showed strong expression of mDia (10).

We then investigated whether the expression of endogenous mDia was associated with the activation of T cells. PBL were stimulated with PHA and IL-2, and the expression of mDia was determined at different times during activation. mDia was up-regulated during cell activation with PHA (48 h) and persisted when the cells were maintained with IL-2 (Fig. 1B). CD3-mediated T cell activation also increased mDia expression at 48 and 72 h (Fig. 1C), but the induction at 24 h was only observed in some experiments (data not shown). No additive or inhibitory effect on mDia expression was observed when T cells were coincubated with anti-CD3 and 20 nM SDF-1α, a chemokine that exerts a costimulatory effect on CD4+ T cells (30) (Fig. 1C). Conversely, the expression of the mDia upstream regulatory GTPase RhoA was not increased

FIGURE 2. mDia expression does not depend on RhoA activity. A, PBL were pretreated for 14 h with 50 μg/ml C3 exoenzyme or were left untreated, and were incubated for another 24 h with 1 μg/ml anti-CD3 or 1 μg/ml PHA. Cells were lysed and blotted for mDia, p160ROCK, and vimentin. A representative experiment of six performed is shown. B, HSB-2 T cells were transfected with GFP, V14, RhaO, or wild-type mDia coupled to GFP, and mDia expression was assessed by flow cytometry. The background level is set with the irrelevant Ab P3 X 63. Results represent the mean ± SD of two independent experiments performed in duplicate.
under the same conditions (Fig. 1, B and C). The expression of mDia was also up-regulated in in vivo activated T cells, such as TIL and T cells from synovial fluid of rheumatoid arthritis patients (Fig. 1D), indicating that in vivo activation is associated with enhanced expression of mDia.

We then investigated whether mDia up-regulation is dependent on RhoA activity. PBL were pretreated overnight with the exoenzyme C3 from Clostridium botulinum, which has been described to inhibit RhoA activity (31). C3 was shown to inhibit RhoA, as it induced previously described morphological changes (spike formation) in PBL (20) (data not shown). C3-treated PBL were further stimulated with either anti-CD3 or PHA, and mDia expression was assessed 24 h later. We found that C3 did not block anti-CD3- or PHA-induced mDia up-regulation (Fig. 2 A, upper panel); thus, we conclude that RhoA activity is dispensable for mDia protein expression. Another RhoA effector, the kinase p160ROCK, remained unaffected by any of the treatments (Fig. 2A, middle panel). On the other hand, HSB-2 T cells, which bear low levels of mDia (Fig. 1), were transfected with the activated mutant of RhoA, V14 RhoA, and the levels of mDia protein were studied by flow cytometry on permeabilized cells. Cells expressing the V14 RhoA mutant exhibited similar levels of mDia compared with untransfected cells or GFP-transfected cells (Fig. 2B), thus confirming that mDia expression is independent of RhoA activity.

**Localization of mDia and profilin in polarized T cells**

To determine the localization of mDia upon T cell activation, T lymphoblasts showing up-regulated expression of mDia (Fig. 1B) were allowed to adhere to fibronectin and were stained for mDia, F-actin, and α-tubulin (Fig. 3, A and B). mDia was polarized at the leading edge of the polarized lymphocyte (Fig. 3A). More precisely, mDia localized in an area immediately posterior to the leading lamella, clustered along the height of the cell body at the leading edge (Fig. 3, A and B, side view), colocalizing partially with F-actin in this structure. On the other hand, profilin, a well-characterized effector of mDia, also localized to the leading edge, co-localizing with F-actin (Fig. 3C).

**Activation of mDia inhibits lymphoid motility**

To address the role of mDia in T lymphocyte migration, Peer T cells, which express high levels of mDia (Fig. 1), were transiently transfected with different constructs of mDia fused to EGFP, namely mDia wild type, a constitutively activated form of mDia (mDia ΔN3); mDia F2, which lacks both the Rho-binding domain
and the FH1 domain (28); mDia F1, containing only the profilin-binding domain; and a dominant negative form, mDia ΔN3 (HindIII), which has been reported to inhibit Y-27632-induced, Rac-mediated actin ruffling (18) (Fig. 4). All constructs were expressed at comparable levels (Fig. 5, A and B, and data not shown). Migration assays showed that cells expressing the activated mutant mDia ΔN3 had a dose-dependent reduction in spontaneous migration (Fig. 5C) and chemotaxis in response to 10 nM SDF-1α (Fig. 5, B and D). In contrast, cells expressing the wild-type molecule as well as mDia F2, mDia F1, or mDia ΔN3 (HindIII) behaved normally compared with the untransfected control (Fig. 5, C and D). Similar results were obtained with mDia ΔN3-transfected Jurkat and HSB-2 T cells in spontaneous motility and chemotaxis to SDF-1α (data not shown).

FIGURE 4. EGFP-mDia constructs employed through this study. The constructs employed are represented, including the representative functional domains involved in the regulation of the molecule. RBD, Rho-binding domain.

FIGURE 5. An activated mutant of mDia impairs lymphocyte motility and chemotaxis. A, Expression profile of Peer T cells transfected with GFP-ΔN3 mDia. All constructs were expressed at the same level. A representative profile is shown. B, Expression profiles of Peer T cells transfected with the activated mutant of mDia, ΔN3 mDia (a) and F2 mDia (b) after migration in Transwell filters. Compare the levels of transmigrated transfected cells expressing the activated mutant ΔN3 mDia (B) with the profile of cells transfected with the F2 mDia mutant. C, Quantification of the effects of various mutants of EGFP-mDia on spontaneous motility of Peer T cells. The cells were allowed to migrate for 4 h. Results represent the mean ± SD of five independent experiments performed in triplicate. D, Quantification of the effects of various mutants of EGFP-mDia on chemotaxis of Peer T cells. The cells were allowed to migrate in the presence of 10 nM SDF-1α for 90 min. Results represent the mean ± SD of five independent experiments performed in triplicate.
mDia is not involved in the regulation of integrin-mediated cell adhesion, but controls T cell motility through postadhesion events

mDia has been postulated to control cell adhesion through its involvement in cell substrate contractile forces in adherent cells. To investigate the possible role of mDia in integrin-mediated lymphocyte adhesion, Jurkat cells were transfected with the mDia mutants mentioned above and allowed to adhere to fibronectin, and adhesion was quantified by flow cytometry. None of the constructs affected T cell adhesion to fibronectin (Fig. 6A), even in the presence of manganese (Fig. 6A, ■), which suggests that β1 integrins from transfected cells are fully functional and are unaffected by mDia expression. Furthermore, the appearance of activation epitopes in β1 integrins induced by Mn2+, such as those recognized by the HUTS-21 Ab (32), remained unaffected by overexpression of the mDia fusion proteins (Fig. 6B). Remarkably, cellular spreading induced by either PMA or anti-CD3 was severely impaired in cells expressing the mDia ΔN3 mutant (Fig. 6, C and D), which suggested that the activation of mDia was interfering with postadhesion events related to the actin cytoskeleton rather than having a direct effect on integrin-mediated cell adhesion.

The RhoA effectors p160ROCK and mDia coordinately control T cell migration by regulating T cell shape and actin balance

To ascertain the possible mechanism by which mDia controls T cell motility, HSB-2 T cells, which are constitutively polarized (19), were transfected with the different GFP fusion proteins of mDia (see above). As a control, we employed EGFP-F, which is specifically targeted to the plasma membrane and which showed

FIGURE 6. mDia does not regulate integrin-dependent adhesion, but is involved in postadhesion events related to the actin cytoskeleton. A, Jurkat T cells were transfected with GFP, an activated mutant of mDia (mDia ΔN3), or mDia F2 and were allowed to adhere to human fibronectin (10 μg/ml). Adhesion was quantified by flow cytometry. When indicated, the cells were allowed to adhere in the presence of 1 mM Mn2+. Results represent the mean ± SD of three independent experiments performed in triplicate. B, Jurkat T cells were transfected with GFP, an activated mutant of mDia (mDia ΔN3), or mDia F2 and were incubated in the presence or the absence of 1 mM Mn2+. The exposure of activation-related epitopes of β1 integrins was assessed by staining with the HUTS-21 mAb and was analyzed by flow cytometry. Results represent the mean ± SD of three independent experiments performed in duplicate. C, Jurkat T cells were transfected with GFP, an activated mutant of mDia (mDia ΔN3), or mDia F2; allowed to adhere to human fibronectin (10 μg/ml) in the presence of 25 ng/ml PMA or anti-CD3 (25 μg/ml); and stained for F-actin (red panels). Representative fields are shown. D, Quantification of cell spreading on both fibronectin with PMA and anti-CD3. Results represent the mean ± SD of three independent experiments.
no specific accumulation at the leading edge (Fig. 7Aa). All constructs exhibited a patchy localization very similar to that of the endogenous molecule, accumulating at the leading edge of the cell (Fig. 7, Ad and Af, and data not shown), although localization of the F2 mutant was not as confined to the leading edge as the rest of the constructs (Fig. 7A, s and i). On the other hand, the activated mutant EGFP-mDia ΔN3 induced a dramatic accumulation of F-actin specifically at the leading edge of the polarized T cell (Fig. 7Ae), which was not induced by any of the other constructs (Fig. 7Ah and data not shown). However, none of the aforementioned cDNA altered the shape of the cells (Table I); this alteration is the cause of the inhibition of motility observed in T cells (19).

To further characterize the mechanism by which RhoA controls the morphology of T cells, polarized HSB-2 cells were transfected with cDNA encoding FLAG-fused forms of p160ROCK (33), namely, an activated mutant of the kinase (Δ4 ROCK) and a kinase-dead form (KD ROCK). Δ4 ROCK profoundly affected the morphology of HSB-2 cells, inducing cell rounding and loss of defined cell poles (Fig. 7B, upper panels, and Table I). On the other hand, KD ROCK did not induce dramatic changes in the morphology of polarized lymphocytes (Fig. 7B, lower panels, and Table I), although occasional defects in tail retraction could be observed in 5–10% of transfected cells (data not shown). Together our data demonstrate that RhoA regulates lymphoid cell shape.

FIGURE 7. RhoA regulates T cell shape through p160ROCK, but not mDia. A, Polarized HSB-2 T cells transfected with GFP-mDia ΔN3 (d), GFP-mDia F2 (g), or GFP-F (a) were stained for F-actin (b, e, and h) and α-tubulin (blue in c, f, and i, which represent merged panels). Cyan shows blue-green colocalization, Yellow means green-red colocalization, and magenta is blue-red costaining. B, HSB-2 cells were transfected with FLAG-Δ4 ROCK or FLAG-KD ROCK, and stained for F-actin (red). Flag staining is shown in green. Representative fields are shown.
through p160ROCK, but also that normal mDia activity is required for motility.

To investigate the mechanism through which mDia is actually involved in lymphocyte motility, Peer T cells were transfected with the different mDia constructs, and F-actin was visualized by immunofluorescence. The activated mutant of mDia (mDiaΔN3) increased the levels of polymerized F-actin, whereas mDia F2 and the other constructs did not have a significant effect (Fig. 8A and data not shown). The increase in F-actin levels correlated with a decrease in the amount of G-actin, as determined by binding of fluorescence-labeled DNase I (Fig. 8B). A significant decrease in the level of G-actin was observed in 95 ± 12% (n = 140) of cells transfected with mDiaΔN3, whereas mDia F2-transfected cells showed no significant decrease (5 ± 2; n = 156). Flow cytometry experiments confirmed that mDiaΔN3 induced a clear cut dose-dependent increase in F-actin, whereas mDia, mDia

<table>
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<th>Expressed DNA</th>
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* HSB-2 cells were transfected and their polarity quantified as described under Materials and Methods. Criteria for considering a cell polarized relied on 1) the retracted position of the MTOC or 2) the existence of a well-defined F-actin-rich leading edge in the absence of secondary lamellae. Absolute numbers are the result of at least three independent experiments and percentage is shown as the mean ± SD of these experiments.

**FIGURE 8.** Activated mDia disturbs the F-actin/G-actin ratio, increasing the cellular content of polymerized F-actin. A, Peer T cells were transfected with the activated mutant of mDia (mDiaΔN3) or mDia F2, allowed to adhere to human fibronectin, and stained for F-actin (red panels). Arrowheads point to transfected cells. B, Peer T cells were transfected with the activated mutant of mDia (mDiaΔN3) or mDia F2, allowed to adhere to human fibronectin, and stained for G-actin (red panels). Arrowheads point to transfected cells. C, Measurement of F-actin content in EGFP-mDia fusion proteins by flow cytometry. Transfected cells were stained with Alexa 647-phalloidin, and the levels of F-actin were measured by flow cytometry. Cell subsets were arbitrarily established, comprising undetectable (NULL), low to medium (LOW-MED), and high (HIGH) expressions of each GFP construct (see Fig. 5A for a flow cytometry profile). Results represent the mean ± SD of five independent experiments performed in triplicate.
F2, and mDia F1 mutants showed no significant effect (Fig. 8C). These results were also observed when transfections were performed in HSB-2 and Jurkat T cells (data not shown).

To confirm the involvement of mDia in regulation of the F-actin/G-actin balance, Peer T cells were transfected with different mDia constructs. As shown above, the activated mutant of mDia induced a dramatic increase in the basal level of polymerized actin (Fig. 9A), which resulted in a blockade of the increase in F-actin elicited by chemokines. In contrast, stimulation of mDia- and mDia F2-transfected cells with SDF-1α resulted in an increment in F-actin similar to that observed in untransfected and EGFP-transfected cells (Fig. 9A). To verify that mDia overexpression was not affecting CXCR4 expression or receptor function, we studied CXCR4 internalization, observing that none of the transfected EGFP fusion proteins affected internalization of CXCR4 induced by SDF-1α or PMA (Fig. 9B).

mDia regulates the downstream effects of the small GTPase Rac1 in T cells

mDia has been previously suggested to regulate Rac1 activation induced by p160ROCK inhibition (18). On the other hand, Rac1 directly regulates actin polymerization through control of the Scar/WAVE/Arp2/3 pathway (34). To investigate the possible relationship between Rac- and mDia-regulated actin polymerization processes, cotransfection experiments were performed in Jurkat T cells. Overexpression of the activated mutant of mDia, mDia ΔN3, together with the dominant negative form, N17Rac1, resulted in a phenotype indistinguishable from that of cells expressing mDia ΔN3 alone (Fig. 10A), whereas overexpression of N17Rac1 alone resulted in cell polarization, as previously described (19). Quantitative analysis demonstrated that N17Rac1 was unable to block mDia ΔN3-induced actin polymerization (Fig. 10B), demonstrating that Rac1 was not downstream of mDia in actin polymerization processes of T cells. Conversely, overexpression of the activated mutant V12Rac1 resulted in ectopic lamellae expression in >85% of the transfected cells (Fig. 10, C and D), but coexpression of V12Rac1 with mDia ΔN3 significantly blocked Rac-induced spreading (Fig. 10, C and D), which suggests that mDia-induced actin polymerization inhibits Rac1 activity, probably by limitation of actin substrate to induce de novo actin polymerization involved in lamellae extension.

Discussion

Regulation of actin cytoskeleton is a multimolecular process in which RhoA plays a fundamental role. Downstream effectors of RhoA involved in actin reorganization and cell migration include the kinase p160ROCK and mDia, but the roles of these effectors in lymphoid motility have been assessed only in the former (20, 21). Here, it is demonstrated that mDia was present in very small amounts in freshly isolated PBL, but was induced both in vivo and in vitro during T cell activation. Previous reports suggested that small GTPases, such as Rac, are up-regulated in metastatic cells (35, 36), but this is the first report that demonstrates activation-induced expression of a Rho-binding protein. The difference in mDia expression may be related to the fact that T lymphoblasts proliferate actively, which does not occur in freshly isolated PBL. In this regard, the low expressing HSB-2 cell line proliferates at slower rate than other T cell lines employed in this study, such as Jurkat or Peer (data not shown). Since the Drosophila homologue of mDia, diaphanous, is required for efficient completion of cytokinesis (11), it is possible that mDia is induced to allow cell division. It is also feasible that the induction of mDia in immune cells is related to the cell size increment in activated T cells and the corresponding growth of actin-based structures. In this regard, Rho has been involved in the regulation of cell size during embryonic development (37). Furthermore, activated T cells exhibit larger numbers of spontaneous lamellae and actin-rich tails than PBL (M. Vicente-Manzanares, unpublished observations). Finally, mDia could be involved in the regulation of adhesion; our data clearly indicate that mDia is regulating postadhesion events rather than direct modulation of integrins; thus, it is likely to be controlling the migratory behavior of activated T cells within target tissues (for instance, TIL embedded in the tumor stroma), which are subjected to cortical tension during three-dimensional contacts with their environment. In this regard, mDia has been shown to be required for formation of adhesive contacts during cell adhesion under externally applied mechanical force (38). It is tempting to speculate that an increase in mDia activity in lymphocytes expressing high levels of mDia within inflammatory foci may reduce the motility of activated T cells, as demonstrated for overexpression of the activated mutant, and this may function as a regulatory step involved in the retention of activated T lymphocytes in target tissues.

Lymphocyte polarization is an essential feature of migration (3). RhoA has been demonstrated to regulate lymphoid polarity, as overexpression of the activated mutant V14RhoA induced cell
rounding (19). Although mDia is involved in epithelial cell polarity (39), its role in lymphocyte polarity had not been addressed. Employment of cellular systems in which we had previously studied the role of RhoA demonstrated that mDia does not regulate lymphocyte polarity, which was regulated by another RhoA effector, p160ROCK, since overexpression of an activated mutant of this molecule induced a rounded phenotype similar to that induced by V14RhoA (19).

Previous studies in HeLa cells demonstrated that exogenously introduced mDia localized to the perinuclear zone and to podosome-like regions in the periphery of the cell (28). Our data show that both endogenous and overexpressed mDia localize to the leading edge, but only scarcely at the leading lamella. Activated PAK1 as well as WASP, Arp2/3, and VASP are localized within the leading lamella of migrating fibroblasts (4, 5, 7, 40), suggesting that leading lamella formation is under the control of Rac and Cdc42. However, RhoA has been implicated in the maturation of early, Rac-mediated focal contacts (41), suggesting that downstream effectors of RhoA are located behind the Rac-regulated area to allow focal contacts to move backward during cell migration. Since mDia also regulates Rac1-mediated induction of membrane ruffles in fibroblasts (18), the localization of mDia in the vicinity of the Rac-regulated area may contribute to the spatial regulation of lamellae and ruffling. In this regard, it is worth noting that mDia seems to control Rac activity at a level different from GTP loading, since mDia activation inhibits spreading induced by the activated

FIGURE 10. mDia regulates Rac downstream activation. A, Jurkat T cells were transfected with Myc-tagged N17Rac1 (blue) alone or together with mDia ΔN3 (green), allowed to adhere to human fibronectin, and stained for F-actin (red). Representative panels are shown. B, Quantification of the experiments shown in A. Results represent the mean ± SD of two independent experiments, with counting of at least 74 cotransfected cells/condition. C, Jurkat T cells were transfected with Myc-tagged V12Rac1 (blue) alone or together with mDia ΔN3 (green), allowed to adhere to human fibronectin, and stained for F-actin (red). Representative panels are shown. D, Quantification of the experiments shown in C. Results represent the mean ± SD of two independent experiments, with counting of at least 74 cotransfected cells/condition.
mutant V12 Rac1, which cannot be down-regulated by GDP exchange. It is tempting to speculate that mDia controls the availability of G-actin to be employed in the formation of Rac-controlled structures such as lamellae, and thus the spatial localization of endogenous mDia may serve this purpose.

Formins have been demonstrated to regulate actin polymerization through their interaction with profilin (11) and more recently by their own actin nucleation capability (12, 13). However, the importance of this mechanism in the overall levels of cellular F-actin had not been previously addressed or measured. In addition, we offer definitive proof of the simultaneous requirement of both downstream mechanisms (profilin binding and actin nucleation) for the induction of actin polymerization, since neither the construct encoding only the FH1 (profilin-binding) nor the fusion protein expressing the FH2 domain alone was sufficient to induce F-actin accumulation. Moreover, overexpression of GFP-tagged profilin did not change the level of F-actin (data not shown), suggesting that mDia is key in the regulation of actin polymerization through this pathway.

Translation of a chemoattractant signal into cell migration sparks the formation of actin structures such as lamellae. In fact, exposure of lymphoid cells to chemokines induces a robust, but transient, increase in the cellular F-actin pool (42). Chemokines such as SDF-1 activate Cdc42 (43) and RhoA (20), and a detailed kinetics study of these two GTPases revealed that Cdc42 activation occurs earlier than RhoA. Furthermore, inhibition of RhoA by the ADP-ribosyltransferase C3 did not impair the increase in F-actin elicited by chemokines (20). Together these data suggest that Cdc42 or Rac, but not RhoA, is involved in the transient increment in F-actin induced by chemotaxotransfactors. However, the activated mutant of mDia abolishes the SDF-1-induced transient increment in F-actin, which is probably caused by depletion of G-actin, demonstrating the importance to chemotaxis of maintaining the F-actin/G-actin balance. Thus, regulation of the F-actin and G-actin pools by mDia may provide an additional mechanism to Rho-p160ROCK-mediated LIM kinase activation, which results in actin-depolymerizing factor/colillin inactivation (44). In this regard, mDia has been recently suggested to play a complementary role in RhoA-regulated SRF activation, which is mainly achieved through LIMK (45).

In summary, the results presented here demonstrate a role for the RhoA effectors ROCK and mDia in lymphoid motility. ROCK controls lymphocyte shape and cell contractility, whereas mDia regulates the F-actin/G-actin balance, thus being required for all actin-dependent processes.

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