The Requirement for Polyamines for Intestinal Epithelial Cell Migration Is Mediated through Rac1*

Ramesh M. Ray, Shirley A. McCormack, Claire Covington, Mary Jane Viar, Yi Zheng‡, and Leonard R. Johnson§

From the Department of Physiology and §Department of Molecular Sciences, University of Tennessee Health Science Center, Memphis, Tennessee 38163

The rapid migration of intestinal epithelial cells is important to the healing of mucosal ulcers and wounds. This cell migration requires the presence of polyamines and the activation of RhoA. RhoA activity, however, is not sufficient for migration because polyamine depletion inhibited the migration of IEC-6 cells expressing constitutively active RhoA. The current study examines the role of Rac1 and Cdc42 in cell migration and whether their activities are polyamine-dependent. Polyamine depletion with α-difluoromethylornithine inhibited the activities of RhoA, Rac1, and Cdc42. This inhibition was prevented by supplying exogenous putrescine in the presence of α-difluoromethylornithine. IEC-6 cells transfected with constitutively active Rac1 and Cdc42 migrated more rapidly than vector-transfected cells, whereas cells expressing dominant negative Rac1 and Cdc42 migrated more slowly. Polyamine depletion had no effect on the migration of cells expressing Rac1 and only partially inhibited the migration of those expressing Cdc42. Although polyamine depletion caused the disappearance of actin stress fibers in cells transfected with empty vector, it had no effect on cells expressing Rac1. Constitutively active Rac1 increased RhoA and Cdc42 activity in both normal and polyamine-depleted cells. These results demonstrate that Rac1, RhoA, and Cdc42 are required for optimal epithelial cell migration and that Rac1 activity is sufficient for cell migration in the absence of polyamines due to its ability to activate RhoA and Cdc42 as well as its own effects on the process of cell migration. These data imply that the involvement of polyamines in cell migration occurs either at Rac1 itself or upstream from Rac1.

Cell migration maintains the organization and integrity of the mucosa of the gastrointestinal tract (1). New cells migrate from the crypts of the small intestine onto the villi and differentiate as they move toward the tips. Mature cells are sloughed into the lumen and replaced from below. The damaged mucosa rapidly repairs itself through two basic processes (2). The early phase of mucosal repair is the replacement of lost cells by mitosis and does not take effect until 24 h or so after injury. We have examined the early phase and the process of cell migration using IEC-6 cells, a non-transformed, putative crypt cell line derived from adult rat intestine and originally described by Quaroni et al. (4). This model resembles the early phase of mucosal healing in the gastrointestinal tract in that cell migration is independent from DNA synthesis, has a complete dependence on actin polymerization (5), and depends on polyamines (6).

Cell migration requires an intact and functioning cytoskeleton, which for the most part consists of filamentous F-actin, tubulin, and intermediate fibers. F-actin is formed by the polymerization of 420-kDa monomers termed G-actin. These filaments of actin with their associated binding proteins make up the actin cortex, a dense network just inside the inner surface of the plasma membrane (7). Long filaments of actin traverse the cell as stress fibers, and short filaments extend into the lamellipodia that are prominent during migration (8). Focal adhesions provide the necessary attachments to the substrate and via the stress fibers allow the cytoskeleton to exert force on the extracellular matrix (9). Cells initiate migration in response to receptor signaling via integrins and the extracellular matrix (10) or in response to soluble factors (11).

Soluble factor and integrin signaling is relayed to the cytoskeleton by signal transduction pathways involving a sub-group of the Ras superfamily of small GTP-binding proteins (12). The Rho (for Ras homology) GTPases consist of three major types of small (21 kDa) proteins that bind and hydrolyze GTP. Their intrinsic GTPase activity is controlled by guanine nucleotide exchange factors and GTPase-activating proteins, known as GAPs. Rho guanine nucleotide dissociation inhibitor is an inhibitory guanine nucleotide exchange factor that prevents the dissociation of guanosine diphosphate from Rho as well as from Rac and Cdc42, the two other members of this family (13, 14). Rac and Rho regulate the polymerization of actin to produce stress fibers and lamellipodia, respectively (15, 16). Cdc42 has been shown to be responsible for the formation of filopodia (12). In NIH 3T3 fibroblasts, the Rho GTPases can be activated sequentially in that activation of Cdc42 activates Rac, which in turn activates Rho (12). Thus, there appears to be a mechanism for the coordinated regulation of the cytoskeleton through activation of the Rho GTPases.

Our laboratory has been interested in the cellular actions of polyamines, which are involved in many aspects of membrane function including stability, Ca²⁺ homeostasis, and ion transport (17). Polyamines are also involved in the organization of the cytoskeleton and cell migration. Polyamine-deficient Chinese hamster ovary cells lack actin filaments and microtubules unless polyamines are supplied exogenously (18). Inhibitors of

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‡ To whom correspondence should be addressed: Dept. of Physiology, The University of Tennessee Health Science Center, 894 Union Ave., Memphis, TN 38163. Tel.: 901-448-7088; Fax: 901-448-7752; E-mail: ljohn@physio1.utmem.edu.

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Polyamines and Rho GTPases in Cell Migration

Polypeptide synthesis prevent concanavalin A-induced expres-
sion of α-tubulin and β-actin mRNAs in mouse splenocytes (19).
In vitro, polypeptides stimulate the rapid polymerization of G- 
and formation of bundles from F-actin, indicating a possi-
bile direct effect of polypeptides on cytoskeletal organization 
(20). We have shown that the early phase of mucosal healing, 
which is due to cell migration, requires polypeptides (21, 22) and 
that polypeptide depletion inhibits migration in the IEC-6 cell 
model and leads to numerous alterations in the cytoskeleton. 
When cells were depleted of polypeptides with α-difluoromethyl-
ornithine (DFMO), 1 which inhibits ornithine decarboxylase, 
the first rate-limiting enzyme in polypeptide synthesis, there 
was a significant decrease in actin stress fibers and a corre-
sponding increase in the density of the actin cortex (23). There 
was also a redistribution of tropomyosin from stress fibers to 
the actin cortex. Additional changes in response to polypeptide 
depletion included a marked reduction in the formation of 
lamellipodia and a dissociation of actin from nonmuscle myosin 
II (24). Although no changes occurred in the absolute amounts 
of G- and F-actin, the association of actin with the sequestering 
protein thymosin β4 was inhibited (25). All of the alterations in 
the cytoskeleton as well as the inhibition of migration caused 
by DFMO were prevented if exogenous polypeptides were 
supplied in the presence of DFMO.

Santos et al. (26) demonstrate that Rho was required for the 
actual migration of IEC-6 cells after wounding or in response to 
growth factor stimulation. Migration was inhibited after micro-
jection of Rho guanine nucleotide dissociation inhibitor Clostridium botulinum C3 ADP-ribosyltransferase toxin or Rho 
T19N, a dominant negative form of RhoA. Inactivation of Rho 
by these means not only inhibited migration but also altered 
the cytoskeleton in ways that were identical to those produced 
by polypeptide depletion. With this in mind we recently exam-
ined the effects of polypeptide depletion on RhoA (27). We found 
that DFMO caused a significant decrease in RhoA levels in the 
cytosplasm and membranes of IEC-6 cells. This decrease was 
due to an approximate 50% inhibition in RhoA synthesis. Ne-
ither the half-life of RhoA nor the level of RhoA mRNA was 
affected. Constitutively active HA-V14 RhoA cells migrated 
much more rapidly than vector-transfected cells, and dominant 
negative HA-N19-RhoA cells exhibited almost no motility. Sur-
prisingly, the depletion of polypeptides almost totally inhibited 
the migration of the cells expressing constitutively active Rho.
Polyamine depletion did not affect the activity of RhoA in the 
HA-V14-RhoA cells but inhibited it dramatically in the vector-
transfected cells (27). After the loss of polypeptides, constitu-
tively active HA-V14-RhoA cells had fewer stress fibers and 
took on the appearance of the HA-N19-RhoA cells and the 
polypeptide-depleted wild type cells. Thus, although RhoA ac-
tivity is essential for the migration of intestinal epithelial cells, 
it is not sufficient. And although polypeptides are necessary 
for RhoA synthesis and activity, they are also required for an 
additional step either upstream or downstream from RhoA that 
results in cell migration (27).

Both Rac1 and Cdc42 are excellent candidates for additional 
steps in the process of cell motility to be regulated by pol-
ypeptides. There is considerable signaling cross-talk between 
Rho, Rac, and Cdc42, and each of these molecules regulates 
some aspect of cytoskeletal transformation (12, 16). In fact 
Rac1 has also been shown to be involved in stress fiber forma-
tion (16). In the current paper, we show that the activities of 
Rac and Cdc42, like that of RhoA, are polypeptide-dependent.
We demonstrate that the rates of migration of IEC-6 cells 
transfected with constitutively active Rac or Cdc42 are sig-
nificantly more rapid than those of the corresponding wild type 
cells or those transfected with empty vectors. We also demon-
strate that Rac1 activation leads to RhoA and Cdc42 activation 
independent of polypeptides, which explains the restoration of 
the cytoskeleton and migration rate in constitutively active 
Rac1 cells depleted of polypeptides.

EXPERIMENTAL PROCEDURES

Materials—Disposable culture ware was purchased from Corning 
Glass Works (Corning, NY). Media and other cell culture reagents 
were obtained from Invitrogen. Dialyzed fetal bovine serum (FBS) 
and other biochemicals were purchased from Sigma. Plasmids, pMX-IRES-GFP-
V12-Rac1, pMX-IRES-GFP-N17-Rac1, pMX-IRES-GFP-F28L-Cdc42, 
pMX-IRES-GFP-N17-Cdc42, and pMX-IRES-GFP (vector) were used 
for the transfection experiments. FuGENE™ 6 transfection reagent 
was a gift from Roche Diagnostics. The primary antibodies, affinity-
purified mouse monoclonal antibodies against RhoA and affinity-puri-
ified rabbit polyclonal antibodies against Rac1 and Cdc42, were pur-
chased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Matrigel 
was obtained from Collaborative Research Inc. (Bedford, MA). DFMO 
was the kind gift of Ilex Oncology Inc. (San Antonio, TX).

Cell Culture—The IEC-6 cell line was purchased from the American 
Type Culture Collection at passage 13. IEC-6 cells originated from 
intestinal crypt cells as judged by morphological and immunological 
criteria. They are nontumorigenic and retain the undifferentiated 
character of epithelial stem cells.

Stock cell cultures were maintained in T-150 flasks in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% FBS, 10 
μg insulin, and 0.05 mg of gentamicin sulfate/ml. The flasks were incubated at 37 °C in a humidified atmosphere of 90% air, 10% CO2. 

Stock cells were passaged once a week, 2 days after feeding at 6, 24, 
and 1-day treatments, respectively. Another group of cells was grown in DFMO plus 
4 was inhibited (25). All of the alterations in 
mDia, formin-related diaphanous protein.

1 The abbreviations used are: DFMO, α-difluoromethylornithine; 
FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; 
GST, glutathione S-transferase; GFF, green fluorescent protein; 
GTPyS, guanosine 5′-O(thio)triphosphate; IRES, internal ribosome 
entry sequence; PAK, p21-activated kinase; PKN, protein kinase N; 
mDia, formin-related diaphanous protein.

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and resuspended before the addition of the cell lysates (200 μg of total protein). The beads were washed three times in cell lysis buffer supplemented with protease inhibitors. The lysates were then sonicated and the proteins were recovered by the addition of glutathione beads to the supernatants. The beads were washed three times in cell lysis buffer and resuspended before the addition of the cell lysates (200 μg of protein for RhoA and Cdc42 and 100 μg of protein for Rac1). After 1 h of incubating at 4°C, beads were washed with lysis buffer, and the amounts of RhoA, Rac1, and Cdc42 proteins bound to GST fusion proteins were analyzed by SDS-PAGE and Western blot using RhoA-, Rac1-, and Cdc42-specific antibodies. Protein (20 μg) from each sample was resolved by SDS-PAGE to determine the levels of RhoA, Rac1, and Cdc42 proteins.

Western Blot Analysis—Protein was separated on 15% SDS-PAGE and transferred to polyvinylidene difluoride membranes by electroboothing. The membranes were then probed with an antibody directed against one of the proteins (RhoA, Rac1, Cdc42, or actin). Immunocomplexes were visualized by the enhanced chemiluminescence detection system and quantitated by densitometric scanning.

Transfection—IEC-6 cells were transfected with constitutively active and dominant negative Rac1 and Cdc42 as well as vector. DNA was prepared by using a Qiagen (endotoxin-free) plasmid preparation kit. Cells were grown as mentioned earlier to 70–80% confluency in 60-mm culture dishes. FuGENE 6 reagent was mixed with constitutively active or dominant negative (pMX-IRES-GFP-V12-Rac1), dominant negative (pMX-IRES-GFP-N17-Rac1), constitutively active (pMX-IRES-GFP-F28L-Cdc42), or vector (pMX-IRES-GFP) DNA (3 μl; 2 μg) in serum-free medium to a total volume of 100 μl and incubated 15 min at room temperature. The reaction mixture was added dropwise onto monolayers of cells containing 4.0 ml of serum-free medium and further incubated for 12 h at 37°C. Cells were fed after 12 h with fresh medium. The limiting dilution technique was used to select stable clones. Selected clones were grown to confluence, and the characterization of clones was carried out by measuring cell migration and the direct visualization of green fluorescence protein (GFP).

Immunohistochemical Localization of Endogenous and Recombinant Rac1—Transfected cells were grown for 4 days, trypsinized, replated in 35-mm dishes (each containing a matrigel-coated glass coverslip), and allowed to attach and spread. Cells were fixed with 4.0% formaldehyde, washed with DPBS, permeabilized with 0.2% Triton X-100 for 5 min, and blocked with 3% BSA for 20 min. Cell monolayers were stained with Texas-red conjugated phalloidin for 1 h. Images were captured by digital confocal microscopy and processed with NIH image.

Statistics—All data are expressed as means ± S.E. from six dishes. Autoradiographic results were repeated three times. Densitometry of Western blots was done by NIH image analysis. The significance of the differences was determined using Dunnett’s multiple-range test (D), and values of p < 0.05 were considered significant.

RESULTS

Polyamine Depletion Inhibits the Activities of RhoA, Rac1, and Cdc42—To study the effect of polyamine depletion on activities of Rho family GTPases, it is important to determine the relative amounts of activated RhoA, Rac1, and Cdc42 in normal IEC-6 cells. Whole cell extracts equivalent to 25–200 μg of protein were subjected to pull-down assay using either GST-PKN and GST-mDia (for RhoA) or GST-PAK (for Rac1 and Cdc42) protein. Pull-down samples along with 25 μg of whole cell extract was resolved by SDS-PAGE and analyzed by Western blot analysis. Confluent IEC-6 cells grown in control medium showed high levels of active Rac1 protein in as low as 25 μg of total protein (Fig. 1A). In contrast, a significant amount of active RhoA protein was detected only in 200 μg of total protein.
Although active Cdc42 protein was evident in as low as 50 μg/H9262 g of total protein, the total amount of activated Cdc42 was lower than amounts of active Rac1 and RhoA (Fig. 1C). Total protein levels of RhoA and Rac1 were comparable and significantly higher than that of Cdc42. Thus, in subsequent experiments we used 100 μg/H9262 g of total protein for Rac1 and 200 μg/H9262 g of total protein for the determination of RhoA and Cdc42 activity. Polyamine depletion by treatment of cells with DFMO for 4 days significantly decreased the activities of all three Rho GTPases (Fig. 2). Active RhoA decreased to 35 or 20% of control levels depending on whether binding to mDia or to PKN was used in the pull-down assay, respectively. There was also a 40% decrease in the level of RhoA protein when cells were grown for 4 days in the presence of DFMO. PAK1 was used to pull down both active Rac1 and active Cdc42. Rac1 activity was reduced to 40% and Cdc42 activity was reduced to 23% of that of control levels. There was no change in the level of Rac1 protein, but Cdc42 levels decreased to 45% of normal. In each case supplying putrescine along with DFMO during growth prevented the decrease (Fig. 2).

As shown in Fig. 3A the activities of RhoA, Rac1, and Cdc42 decreased little when cells were treated with the DFMO for 1, 2, and 3 days. There were dramatic decreases in the activities of all 3 to below 50% that of control on the 4th day of DFMO treatment. The levels of RhoA and Cdc42 proteins followed a similar pattern, and there was no significant decrease in Rac1 protein (Fig. 3A). Fig. 3B illustrates the means of the quantified data of the activities of all three Rho GTPases. As in the studies depicted in Fig. 2, the addition of putrescine to the incubation medium containing DFMO prevented all of the effects of ornithine decarboxylase inhibition.

Rac1 and Cdc42 Protein Expression Alters IEC-6 Cell Migration—Clones of IEC-6 cells transfected with vector and with dominant negative and constitutively active genes of Rac1 and Cdc42 were characterized by the direct visualization of green fluorescent protein and by determining rates of cell migration. Digital confocal micrographs of one clone of each of the five types are shown in Fig. 4. In each case, all cells expressed GFP, indicating successful transfection and clonal selection. Transfection with dominant negative constructs of both Rac1 and Cdc42 inhibited cell migration compared with vector-transfected cells, whereas transfection with constitutively active constructs significantly increased the rates of migration (Fig. 5A). As shown in Fig. 5B, by 8 h vector-transfected cells had migrated to cover ~40% of the original wound. This number was reduced to 25% in cells carrying dominant negative Rac1 and to 10% in cells with dominant negative Cdc42. On the other hand, cells transfected with constitutively active Rac1 covered more than 85% of the
wound width and those with constitutively active Cdc42 had migrated over 60% of the original wound.

**Expression of Activated Recombinant Rac1 and Cdc42 Influences the Migration of Polyamine-depleted IEC-6 Cells**—Polyamine depletion inhibited the migration rate of vector-transfected cells by ~50% (Fig. 6, A and B). Cells transfected with constitutively active Rac1 had migrated to close ~85% of the initial wound width by 8 h, and polyamine depletion had no significant effect in these cells (Fig. 6, A and B). Polyamine depletion inhibited migration of cells expressing constitutively active Cdc42 by ~30%. Supplying putrescine to cells incubated with DFMO prevented its effects on the migration of cells expressing only vector and those expressing active Cdc42. In summary, polyamine depletion had no effect in these cells expressing Rac1. It did inhibit the migration of cells expressing Cdc42 but to a lesser extent compared with cells transfected with vector.

**Polyamine Depletion Does Not Alter Rac1 or Cdc42 Activity in Cells Expressing Those Proteins**—The expression of active Rac1 or active Cdc42 prevented the inhibition of the activities of those respective proteins that occurred in normal wild type IEC-6 cells after polyamine depletion (Fig. 7). As one would expect, incubation with DFMO inhibited Rac1 activity in vector-transfected cells to the same extent as it did in wild type cells. Fig. 7 also shows that polyamine depletion had no effect on Rac1 activity once vector-transfected cell extracts had been activated with GTPγS. These data suggest that, irrespective of total protein levels, activated Rac1 and Cdc42 protein levels were significantly higher in DFMO-treated cells expressing constitutively active proteins, and Rac1 activation in the absence of polyamines is not limited by guanine nucleotide exchange factors.

**Expression of Constitutively Active Rac1 Prevents Cytoskeletal Changes Associated with Polyamine Depletion**—As pointed out in the Introduction, after polyamine depletion wild type IEC-6 cells lose lamellipodia and stress fibers and develop a heavy actin cortex. That same pattern is shown in vector-transfected cells (Fig. 8). In cells, expressing constitutively active constitutively active Rac1 and Cdc42 protein expression influences IEC-6 cell migration. A, cells transfected with vector (pMX-IRES-GFP), constitutively active (CA) Rac1 and Cdc42, and dominant negative (DN) Rac1 and Cdc42 were grown in DMEM, 5% FBS for 4 days. Confluent monolayers were wounded with a gel-loading tip in the center of plates marked to localize the wound site. Plates were photographed immediately to record the wound width (0 h), washed, and incubated with fresh serum free medium. Plates were photographed at the marked wound location after 10 h of incubation. A plate containing vector cells at 0 and 10 h is shown as a representative control. Representatives of three experiments are shown. B, quantitative analysis of migration showing wound width covered as compared with initial scratch size (0 h) using NIH image analysis. Values are the mean ± S.E. of six observations. *, Significantly different from vector (p < 0.05). DN, dominant negative; CA, constitutively active.
active Rac1, however, the cytoskeleton appeared immune to polyamine depletion. There were no visible differences in stress fibers, lamellipodia, or the actin cortex in polyamine-depleted V12-Rac1 cells compared with controls or those treated with both DFMO and putrescine (Fig. 8). Compared with vector-transfected control cells the V12-Rac1 cells showed more stress fibers and a decrease in cortical actin. Both of these changes are indicative of more actively migrating cells. Constitutively active Cdc42 expressing cells migrated at a rapid rate compared with DFMO-treated vector cells, and as one would predict, the actin cytoskeleton of polyamine-depleted cells was similar to V12-Rac1 cells (data not shown).

Activation of RhoA, Rac1, and Cdc42 in Normal and Polyamine-depleted V12-Rac1, V14-RhoA, and F28L-Cdc42-expressing Cells—To understand the hierarchy of activation of the Rho GTPases in epithelial cells, we determined the activities of RhoA, Rac1, and Cdc42 in cells expressing constitutively active RhoA, Rac1, and Cdc42 grown to confluence for 4 days in control, DFMO, and DFMO plus putrescine-containing medium. Results in Fig. 9 show that activated Rac1 is significantly reduced in vector-transfected polyamine-depleted cells. V12-Rac1 (constitutively active)-expressing cells grown in the presence of DFMO showed the expected increase in activated Rac1. Because V12-Rac1 prevented the cytoskeletal changes and the inhibition of migration associated with polyamine depletion and because RhoA and Cdc42 are essential to cell migration, we examined RhoA and Cdc42 activity in Rac1-transfected cells. Vector-transfected cells had basal levels of RhoA and Cdc42 activity essential for normal migration, whereas polyamine depletion significantly decreased RhoA and Cdc42 protein levels and activities. As expected, V12-Rac1 cells had increased RhoA and Cdc42 activity in both control and polyamine-depleted cells. Although, polyamine-depleted cells had low levels of RhoA and Cdc42 protein, a high proportion of existing RhoA and Cdc42 protein was in the active state in Rac1-transfected cells compared with Vector-transfected cells (Fig. 9). Thus, Rac1 activated RhoA and Cdc42 in the presence or absence of polyamines in IEC-6 cells. In contrast, Rac1 activity was not increased in V14-RhoA-transfected cells, whereas Cdc42 activation was stimulated (Fig. 10). Constitutively active Cdc42 (F28L)-expressing cells had significant increases in both RhoA and Rac1 activity in normal as well as polyamine-depleted cells (Fig. 10).

DISCUSSION

Although polyamines are required for such basic cell processes as proliferation and migration, the actual mechanism of their action at the cellular and molecular levels is poorly understood. There is agreement, however, that the effects of these compounds depend on their strong positive charges. Putrescine, spermidine, and spermine contain 2, 3, or 4 amine groups, respectively, with pK values above 9, so they are polycations at physiological pH and strongly bind to negatively charged macromolecules, particularly nucleic acids and proteins. The fact that these are not point charges but are fixed along a flexible carbon chain allows polyamines to interact with macromolecules in structurally specific ways. Spermine molecules, for example, bind to phosphate groups of the DNA helix, occupying the small groove and stabilizing the helix by binding the two strands together (31, 32). The most frequently studied mechanisms of action of polyamines concern membranes (17) and growth-regulated genes (33). Polyamines stabilize cell membranes (34) including those of organelles such as the Golgi (35). The roles of polyamines in regulating membrane-bound enzymes,
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Spermine and spermidine block the inward rectifying K⁺ channel and, thus, perhaps act as physiological regulators of its activity (36, 37). Wang and co-workers (38, 39) have recently shown that polyamines may increase K⁺ channel-mediated Ca²⁺ influx, which is required for cell migration, and concluded that this is related to the levels of RhoA protein. There is also accumulating evidence that polyamines can influence the phosphorylation and, hence, the activation of kinases (40).

The most important single finding in our study is that the activation of Rac1 and, to a lesser extent, Cdc42 allowed the migration of polyamine-depleted intestinal epithelial cells. In fact, the depletion of polyamines had no discernible effect on either the migration or the cytoskeletal structure of cells constitutively expressing Rac1 activity. As shown in Fig. 2 polyamine depletion inhibited the activities of all three Rho-GTPases. We have previously shown that RhoA activity is essential for cell migration of this same cell line (26). However, unlike Rac activity, RhoA activity is not sufficient for migration as polyamine depletion inhibited cell migration in cells constitutively expressing activated RhoA to the same extent that it inhibited migration of wild type and vector-transfected cells (27). These results imply that polyamines are required for the direct activation of Rac1 or for a step upstream from, and leading to, the activation of Rac1. Once Rac is activated cell migration is independent of polyamines.

Rao et al. (39) show that increased Ca²⁺ influx results in the synthesis of RhoA and propose that polyamine-dependent cell migration is mediated by the expression of RhoA. They found decreased RhoA protein levels in polyamine-depleted cells similar to our current finding (Fig. 1) and to our previous report (27), but they did not measure the activity of RhoA, so it is not known whether Ca²⁺ influx was correlated with the actual activity of the protein. Signal transduction pathways are characterized by changes in the activation of preexisting proteins rather than by stimulating their synthesis or decreasing their degradation in response to rapid changes in extra- and intracellular signals. Fig. 9 shows that polyamine depletion decreased the level of RhoA protein in constitutively active Rac1 cells to the same extent that it did in the control vector-transfected cells. Because these cells migrated normally, it means that the levels of RhoA protein have little to do with the inhibition of migration after polyamine depletion.

Interestingly, in Fig. 2, Rac1 protein levels remained normal in polyamine-depleted cells, whereas the levels of RhoA and Cdc42 decreased to 60 and 45% that of normal, respectively. Thus, protein levels of Rac1 remain normal throughout the time course of polyamine depletion (Fig. 3A), and Rac1 is affected only by an approximate 60% inhibition of activity occurring between day 3 and day 4 of exposure to DFMO. It is between days 3 and 4 that spermine levels decrease significantly (6). At this same time there are similar decreases in the activities of RhoA and Cdc42. This time course parallels that for the significant decrease in cell migration, which occurs after incubation with DFMO (27). Earlier Yuan et al. (41) showed that restoration of migration was most effectively achieved by spermine and to a lesser extent by spermidine and that putrescine was ineffective. These observations clearly establish the role of polyamines in the synthesis of RhoA and Cdc42 proteins and their activities. It is also important to note that normal migrating IEC-6 cells had high levels of active Rac1 compared with RhoA and Cdc42 (Fig. 1) and that polyamine depletion decreased active protein levels significantly below basal levels (Fig. 2). Fig. 7 depicts results that are important controls regarding our findings on Rac1 and Cdc42 activity. First, polyamine depletion had little effect on the activities of either

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**Fig. 9.** Constitutively active V12-Rac1 activates RhoA and Cdc42 in polyamine-depleted IEC-6 cells. Extracts of vector and V12-Rac1 transfected-cells grown as control (C), DFMO (D), or DFMO plus putrescine (DP) groups for 4 days were subjected to the GST-PAK and GST-PKN pull-down assay described in the under "Experimental Procedures." Western blot analysis was performed using RhoA-, Rac1-, and Cdc42-specific antibodies to determine the levels of activated Rac1, RhoA, and Cdc42 proteins as well as total protein. Representative Western blots from three observations are shown.

**Fig. 10.** Activities of Rho GTPases in normal and polyamine-depleted cells expressing constitutively active RhoA and Cdc42. Vector and V14-RhoA- and F28L-Cdc42-transfected cells were grown for 4 days in control medium (C) and control medium with DFMO (D) and DFMO plus putrescine (DP). Cell extracts were used for pull-down assays for RhoA, Rac1, and Cdc42 as described in Fig. 9. Western blot analysis was performed using RhoA-, Rac1-, and Cdc42-specific antibodies to determine the levels of activated Rac1, RhoA, and Cdc42 proteins. Representative Western blots from three observations are shown.
protein in cells transfected with the corresponding constitutively active constructs. Second, DFMO caused a decrease in enzyme activity in cells transfected with vector alone that was similar to the inhibition produced in wild-type cells. And, third, polyamine depletion had no effect on vector-transfected cells that had been activated with GTPγS. This indicates that the absence of polyamines does not interfere with the ability of the Rho GTPases to be activated.

Another important finding in the current studies is the demonstration for the first time that Rac1 and Cdc42 activities are essential for optimal epithelial cell motility. Expression of dominant negative Rac1 resulted in an approximate 40% inhibition on migration, whereas Rac and Cdc42 were shown to be required for the formation of lamellipodia and filopodia, respectively (12). There is also evidence that Rac1 is involved in stress fiber formation as well (16). As indicated in the introduction, there is significant cross-talk between the Rho family GTPases. Cdc42 can activate Rac and, thus, induce filopodia in association with lamellipodia (41). Rac can activate Rho, although this does not appear to be a strong response in fibroblasts (15, 16). Nothing is known about the hierarchy of activation of the Rho GTPases in epithelial cells, and our previous data (26, 27) along with that reported here are the first to correlate the activity of polyamines in migration. First, polyamines either activate Rac1 or inhibit Rac1 activity, whereas Rac1 activities were unchanged (Fig. 9). This was predictable since RhoA activity is essential for migration (26, 27). Thus, the activation of RhoA is downstream from Rac1 and does not require polyamines directly. Fifth, although polyamine depletion decreases the levels of Rho protein, this has no effect on migration. Cells expressing constitutively active Rac1 migrated normally despite their decreased amount of Rho protein. And, finally, Rac is necessary for a step in the migration process in addition to the activation of RhoA.

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