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Research Article

Akt-mediated GSK-3β inhibition prevents migration of polyamine-depleted intestinal epithelial cells via Rac1

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Abstract. The rapid migration of intestinal epithelial cells (IEC) is important for the healing of mucosal wounds. We have previously shown that polyamine depletion inhibits migration of IEC-6 cells. Akt activation and its downstream target GSK-3β have been implicated in the regulation of migration. Here we investigated the significance of elevated phosphatidylinositol 3-kinase (PI3K)/Akt signaling on migration of polyamine-depleted cells. Polyamine-depleted cells had high Akt (Ser473) and GSK-3β (Ser9) phosphorylation. Pretreatment with 20 μM LY294002 (PI3K inhibitor) for 30 min inhibited phosphorylation of Akt, increased migration by activating Rac1 in polyamine-depleted IEC-6 cells, and restored the actin structure similar to that in cells grown in control medium. Treatment of cells with a GSK-3β inhibitor (AR-A014418) altered the actin cytoskeleton and inhibited migration, mimicking the effects of polyamine depletion. Thus, our results indicate that sustained activation of Akt in response to polyamine depletion inhibits migration through GSK-3β and Rac1.

Keywords. Akt, GSK-3β, IEC-6, migration, polyamines.

Introduction

Healing of mucosal damage has been shown to involve two different processes. The first involves sloughing of the damaged epithelial cells, and the migration of viable cells from areas adjacent to, or just beneath, the injured surface to cover the wounded areas [1, 2]. In the second, which begins 12–16 h after injury and takes 1–2 days to complete, the process of cell division replaces lost cells, and the mucosa is returned to its normal thickness [3]. We used a normal rat intestinal epithelial cell line (IEC-6) to study the process of gastrointestinal wound healing. These cells are non-transformed and retain the undifferentiated characteristics of epithelial stem cells.

Our laboratory is involved in understanding the cellular actions of polyamines. Polyamines are involved in the organization of the cytoskeleton and cell migration. *In vitro*, polyamines stimulate rapid polymerization of G-actin and formation of bundles from F-actin, indicating a possible direct effect of polyamines on cytoskeletal organization [4]. We have reported that the early phase of mucosal healing due to cell migration requires polyamines [5, 6], and that polyamine depletion inhibits migration of IEC-6 cells due to alterations in the cytoskeleton. A significant decrease in actin stress fibers, and a corresponding increase in the density of the actin cortex, were observed in IEC-6 cells when they were depleted of polyamines by means of treatment with α-difluoromethylornithine (DFMO), a specific inhibitor of ornithine decarboxylase (ODC), the first rate-limiting enzyme in polyamine synthesis [7]. Inactivation of Rho inhibited migration and altered the cytoskeleton in a manner identical to that induced by polyamine depletion [8]. We also showed that the effect of polyamine depletion on cell migration and the actin cytoskeleton could be prevented by expression of constitutively active Rac1, and in IEC-6 cells, Rac1

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activates both RhoA and Cdc42 [9]. Our laboratory recently found that polyamine depletion prevents activation of Rac1 and RhoA by sequestering them to the nucleus, and that expression of constitutively active MEK reverses this effect, allowing the cellular localization required for Rac1 activation [10].

In eukaryotic cells, migration is a highly integrated process, which orchestrates embryonic morphogenesis, contributes to tissue repair and regeneration and drives disease progression in cancer, atherosclerosis and immune diseases [11]. Cell migration is initiated in response to various extracellular responses such as growth factors, and extracellular matrix adhesion requires dynamic and spatially regulated changes of the actin cytoskeleton and microtubules. These functions are mediated and coordinated by many different intracellular signaling molecules, which include the Rho family of small GTPases, Ca^{2+}-regulated proteins, mitogen-activated protein kinases (MAPKs), protein kinase C, phosphatidylinositide kinases, and tyrosine kinases [12, 13]. Akt is a serine/threonine kinase activated downstream of class I phosphatidylinositol 3-kinase (PI3K) and various receptors. Evidence indicates that Akt promotes cell motility in mammalian fibroblasts and tumor cells [14, 15]. Akt is known to be involved in migration and invasion in cells of many different organisms. However, the molecular mechanism of how Akt regulates the cytoskeleton and thereby modulates cell motility is unknown.

Glycogen synthase kinase 3 (GSK-3) is a serine/threonine kinase that was originally identified as an enzyme that regulates glycogen synthesis in response to insulin [16]. It has been also implicated in multiple biological processes. GSK-3 phosphorylates a broad range of substrates, such as c-Myc, c-Jun, and c-Myb [17], and the translation factor eIF2B [18]. GSK-3 has also been implicated in the regulation of cell fate in *Dictyostelium* [19], and is a component of the Wnt signaling pathway required for *Drosophila* and *Xenopus* development [20, 21]. In mammalian cells, upon stimulation with growth factors GSK-3α and GSK-3β are rapidly phosphorylated at Ser21 and Ser9, respectively, resulting in their inhibition [22–24]. Akt downstream of PI3K has been demonstrated to phosphorylate both of these sites *in vitro* and *in vivo*, suggesting that growth factors down-regulate GSK-3 activity through the PI3K–PKB signaling cascade [22, 23].

In this study, we investigated whether elevated PI3K/Akt signaling in response to polyamine depletion decreases cell migration by inhibiting GSK-3β.

Materials and methods

Materials. Disposable culture ware was purchased from Corning Glass Works (Corning, NY). Media and other cell culture reagents were obtained from Invitrogen Corporation (Carlsbad, CA). Dialyzed fetal bovine serum (FBS) and other chemicals were purchased from Sigma (St. Louis, MO). Rac1 antibody was from Upstate (Lake Placid, NY). Actin antibody was from Sigma. Phospho-Akt (Ser473), total-Akt, phospho-GSK-3β (Ser9) and total GSK-3β antibodies were from Cell Signaling Technology (Beverly, MA). Rhodamine-phalloidin was from Molecular Probes (Eugene, OR). DFMO was a kind gift from Ilex Oncology Inc. (San Antonio, TX). LY294002 and GSK-3β inhibitor VIII (AR-A014418) were purchased from Calbiochem, EMD Biosciences (La Jolla, CA). Enhanced chemiluminescence Western blot detection reagent was purchased from Perkin Elmer Life Sciences (Boston, MA). The IEC-6 cell line (ATCC CRL 1592) was obtained from American Type Culture Collection (Rockville, MD) at passage 13. The cell line was derived from normal rat intestine and was developed and characterized by Quaroni et al. [25]. IEC-6 cells originate from intestinal crypt cells as judged by morphological and immunological criteria. They are non-transformed and retain the undifferentiated character of epithelial stem cells. Tests for mycoplasma were performed routinely and were negative. All chemicals used were of the highest purity commercially available.

Cell culture. Stock cell culture was maintained in T-150 flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS, 10 μg/ml insulin and 50 μg/ml gentamicin sulfate. The flasks were incubated at 37 °C in a humidified atmosphere of 90% air, 10% CO_2. Stock cells were passaged once a week at 1 : 20, and medium was changed three times weekly. The cells were restarted from original frozen stock after every seven passages.

General experimental protocols. The general protocol for the experiments and the methods used were similar to those described previously [9]. In brief, IEC-6 cells were plated at 6.25×10^4 cells/cm^2 in medium consisting of DMEM supplemented with 5% dialyzed FBS, 10 μg/ml insulin and 50 μg/ml gentamicin sulfate (control) or in control medium containing 5 mM DFMO or DFMO plus 10 μM putrescine. Cells were grown at 37 °C in a humidified atmosphere of 90% air and 10% CO_2. They were fed every other day and serum-starved for 24 h before harvesting or before the cell migration assay.

Cell migration assay. Cells were grown under control, DFMO, and DFMO plus putrescine conditions in 35-mm plates and were serum-starved for 24 h before experiments (during day 3). Plates containing a confluent monolayer of cells were marked in the center by drawing a line along the diameter of the plate with a black marker. Wounding of the monolayer was carried out perpendicular to the marked line using a gel-loading micro-tip. Plates

were washed and the area of migration was photographed with a CCD camera system using NIH Image software (Version 1.58) at the intersection of the marked line and wound edge at 0 h (WW_0) and at desired time intervals (WW_T). Cell migration was calculated as wound width covered at time t ($WW_0 - WW_T$). Each experiment was carried out three times in duplicate, and each plate was wounded twice. Therefore, n was considered to be 6 even though results are the means of 12 observations.

Rac1 activation assay. Biological activity of Rac1 protein was assayed by pull-down assay following the method of Kranenburg et al. [26]. GST-PAK fusion protein was prepared by lysing bacteria (*Escherichia coli* BL21-DE-3pLysE strain transformed with GST-PAK plasmid construct) in a buffer containing 1% Nonidet P-40, 50 mM Tris, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$ and 10% glycerol supplemented with protease inhibitors. Cell lysate was sonicated and clarified by centrifugation at 10 000 *g* for 15 min. The fusion protein was recovered by the addition of glutathione-agarose beads to the supernatant. The beads were washed three times in cell lysis buffer and resuspended before the addition of the cell lysates (100 µg). After 1 h of tumbling at 4 °C, beads were washed with lysis buffer, and the amount of Rac1 protein bound to GST-PAK protein was analyzed by SDS-PAGE and Western blot using a Rac1-specific antibody. From each sample, 20 µg proteins was resolved by SDS-PAGE to determine the level of total Rac1 protein.

Western blot analysis. Protein samples were separated on SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane by electroblotting. The membranes were then probed with appropriate primary and secondary antibodies. Immunocomplexes were visualized by enhanced chemiluminescence detection reagent.

Fluorescence microscopy. Cells were seeded on 35-mm plates and grown as described earlier. Cells were fixed with 3.7% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 10 min and washed with PBS. Cells were blocked with 3% BSA in PBS for 20 min and then incubated with rhodamine-phalloidin for 2 h. Cells were then washed with 0.1% BSA in PBS for 20 min. Images were taken using a Nikon Diaphot inverted fluorescence microscope with appropriate filters.

Statistical analysis. Data are means ± SE. All experiments were repeated three times ($n = 3$). Western blots illustrated are representative of three experiments. ANOVA with appropriate post-hoc testing was used to determine the significance of the differences between means. Values of $p < 0.05$ were regarded as significant.

Results

Polyamine depletion induces activation of Akt. As Akt is a major effector of PI3K [27, 28], we investigated its possible implication in the signaling pathway triggered by polyamines. We analyzed the levels of phosphorylated Akt (pSer473-Akt). Figure 1 shows pSer473-Akt, total Akt and actin in IEC-6 cells grown in control, polyamine-depleted (DFMO) and DFMO plus putrescine conditions. IEC-6 cells grown in control and DFMO plus putrescine conditions showed low levels of pSer473-Akt compared with the polyamine-depleted cells. There were no differences in total-Akt in the same lysate. The membrane was re-probed with anti-actin antibody as a loading control and levels of actin were found to be equal in all three conditions. Since activated Akt phosphorylates Ser9 of GSK-3β and inactivates it, we determined the level of pSer9-GSK-3β. Figure 1 shows that pSer9-GSK-3β was increased by DFMO treatment compared with both other groups with no changes in the level of GSK-3β.

Effect of inhibition of PI3K on migration of IEC-6 cells. We used LY294002 (LY), a specific inhibitor of PI3K to determine the role of PI3K/Akt on migration of IEC-6 cells. Cells were pretreated for 30 min with 20 µM LY before wounding, and the migration assay was performed as described in Methods. As expected, polyamine depletion significantly decreased cell migration. LY treatment for 30 min significantly increased migration of polyamine-depleted cells, which was more or less similar to that seen in the control and DFMO plus putrescine groups (Fig. 2a). Results in Figure 2b show that the basal level of phopho-Akt (Ser473) was significantly higher in

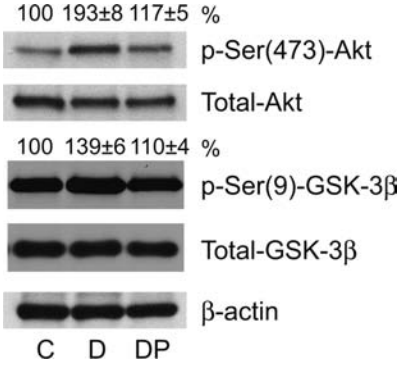

Figure 1. Akt (Ser473) and glycogen synthase kinase 3β (GSK-3β; Ser9) phosphorylation. Intestinal epithelial cell line-6 (IEC-6) cells were grown in medium (C) ± 5 mM α-difluoromethylornithine (DFMO; D) or DFMO + 10 µM putrescine (DP) for 4 days, and lysed in the presence of protease inhibitors. Proteins from each sample were separated by SDS-PAGE, followed by Western blot analysis using antibodies specific for phospho-Akt (Ser473), Akt, phospho-GSK-3β (Ser9), GSK-3β and β-actin. Representative blots and relative densitometry readings from three observations are shown.

polyamine-depleted cells compared with the cells from control and DFMO plus putrescine groups. Akt (Ser473) phosphorylation was completely inhibited by LY in cells grown in all three conditions (control, DFMO and DFMO plus putrescine). These observations suggest that short-term treatment with LY (20 μM) is sufficient to inactivate Akt and the subsequent signaling cascade. The activation of Akt was completely dependent on PI3K, as it was abrogated by preincubation of the cells with LY, confirming the upstream position of PI3K relative to Akt in the signaling pathway.

Effect of PI3K inhibition on actin cytoskeleton and Rac1 activity. Previous reports from our laboratory have shown that polyamine depletion alters the actin cytoskeletal structure. Inhibition of migration is attributed to the presence of dense cortical actin bundles that fail to reorganize into actin stress fibers essential for the migration [8–10]. Preincubation with LY increased migration of polyamine-depleted cells, so we examined the organization of the actin cytoskeleton at the migrating edge of the wounded monolayer using rhodamine-conjugated phalloidin, which specifically binds to F-actin. Figure 3a shows F-actin staining of control and polyamine-depleted cells with and without LY pretreatment. Control cells had lamellipodia and remarkable stress fibers in confluent areas as well as at the migrating edge. Polyamine-depleted cells were characterized by cortical actin and few or no stress fibers at the migrating edge and in confluent areas. When polyamine-depleted cells were pretreated with LY, stress fibers and lamellipodia formation at the migrating edge, and stress fibers were also observed in confluent

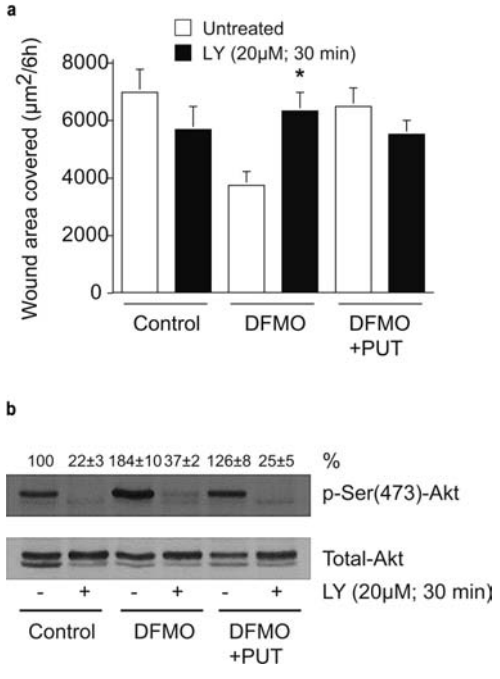

Figure 2. Effect of 20 μM LY294002 (LY) on migration. IEC-6 cells were grown in medium ± 5 mM DFMO or DFMO + 10 μM putrescine (PUT) for 4 days. Confluent cells were treated with DMSO as vehicle or pretreated with 20 μM LY for 30 min followed by wounding with a gel-loading tip in the center of plates marked to localize the wound site. Plates were washed and further incubated with fresh media. (*a*) Quantitative analysis of migration showing wound width covered compared with initial wound size (0 h) using NIH Image analysis software. Values are means ± SE of six observations. $*p < 0.05$, significantly different from the respective untreated group. (*b*) Cells grown and treated as above were lysed in the presence of protease inhibitors. Equal amounts of protein from each sample were separated by SDS-PAGE followed by Western blot analysis using antibodies specific for phospho-Akt (Ser473) and Akt. Representative blots from three observations are shown.

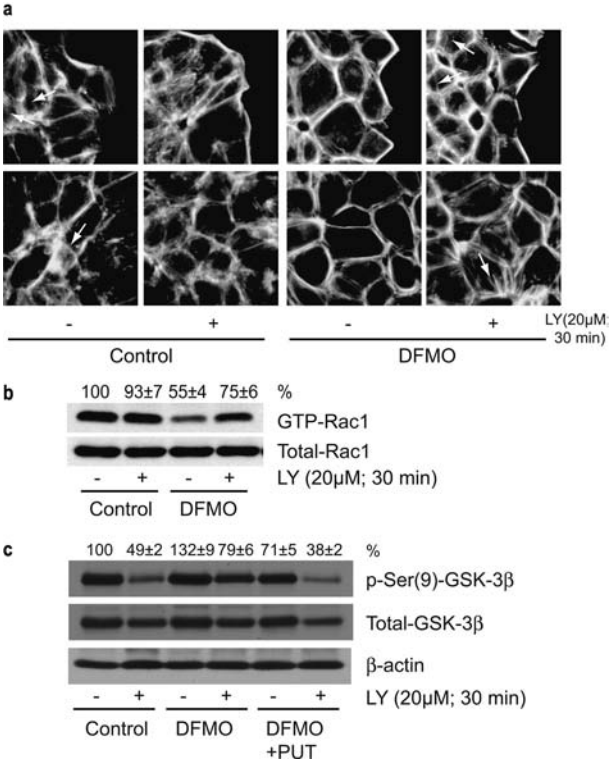

Figure 3. Effect of 20 μM LY on actin cytoskeletal structure and Rac1. (*a*). IEC-6 cells were grown and treated for the migration assay as previously described. After 6 h migration, plates were washed, fixed and stained with rhodamine-conjugated phalloidin for visualization of F-actin. Top row shows cells along the migrating edge, while bottom row depicts confluent areas of cells. Arrows indicate stress fibers. Images are representative of three observations. (*b*) Cells were lysed in the presence of protease inhibitors and subjected to the glutathione *S*-transferase-p21-activated kinase fusion protein (GST-PAK) pull-down assay as described in Methods. Active (GTP-Rac1) and total Rac1 were detected using a Rac1-specific antibody. (*c*) IEC-6 cells were grown in medium ± 5 mM DFMO or DFMO + 10 μM putrescine (PUT) for 4 days. Confluent cells were treated with DMSO as vehicle or pretreated with 20 μM LY for 30 min and lysed in the presence of protease inhibitors. Proteins from each sample were separated by SDS-PAGE, followed by Western blot analysis using antibodies specific for phospho-GSK-3β-(Ser9) and GSK-3β and β-actin. Representative blots and relative densitometry readings from three observations are shown.

areas. Pretreatment of polyamine-depleted cells with LY caused reorganization of actin to form extensive stress fibers typical of actively migrating cells.

Integrins and soluble factors relay signals to the cytoskeleton by signal transduction pathways involving a subgroup of the Rho superfamily of small GTP-binding proteins Rho, Rac and Cdc42 [29]. Activation of these proteins by GTP-binding and their subcellular localization are crucial to migration. Previously, our laboratory has shown that polyamine depletion of IEC-6 cells caused reduction of Rac1 activity [9]. Rac1 activity proved to be necessary and sufficient for migration of polyamine-depleted IEC-6 cells. In this study we determined Rac1 activity by measuring the levels of GTP-Rac1 (Fig. 3b). Polyamine depletion caused a dramatic reduction in Rac1 activity compared with the activity in cells grown in control medium, and LY pretreatment of the polyamine-depleted cells significantly increased Rac1 activity. Total Rac1 protein was found to be unchanged. We also determined the level of pSer473-Akt and total-Akt to validate the effect of LY. When control and polyamine-depleted cells were pretreated with 20 μM LY, pSer473-Akt was decreased, while total-Akt protein remained unchanged in all four groups (data not shown). We also determined the levels of pSer9-GSK-3β, total GSK-3β and actin. The levels of pSer9-GSK-3β and total GSK-3β protein significantly decreased in response to LY in all three groups (Fig. 3c). However, the effect of 20 μM LY on GSK-3β was more pronounced in control and DFMO plus putrescine groups compared with DFMO group (Fig. 3c). These results indicate that PI3K/Akt regulates levels of GSK-3β protein and its activity. Furthermore constitutively active PI3K/Akt signaling prevents Rac1 activation in polyamine-depleted cells.

Effect of GSK-3β inhibitor on migration and Rac1 activity. Since polyamine depletion-mediated Akt inhibits GSK-3β, we studied the effect of AR-A014418, an inhibitor of GSK-3β, on the migration of IEC-6 cells (Fig. 4). When IEC-6 cells grown in control, polyamine-depleted and DFMO plus putrescine conditions were treated with 10 μM AR-A014418 for 24 h, migration was inhibited in all groups.

Since the migration is dependent on actin reorganization, we examined the actin cytoskeleton of cells from control and DFMO groups with and without the GSK-3β inhibitor (AR-A014418) (Fig. 5a). Control cells had well-developed stress fibers. Polyamine-depleted cells contained cortical actin and few or no stress fibers. Control cells treated with the GSK-3β inhibitor developed an actin cortex and lost stress fibers, resembling the actin cytoskeletal structure of polyamine-depleted cells.

Since the GSK-3β inhibitor inhibited the migration of control cells and altered the actin cytoskeleton, we measured Rac1 activity under these conditions. We observed

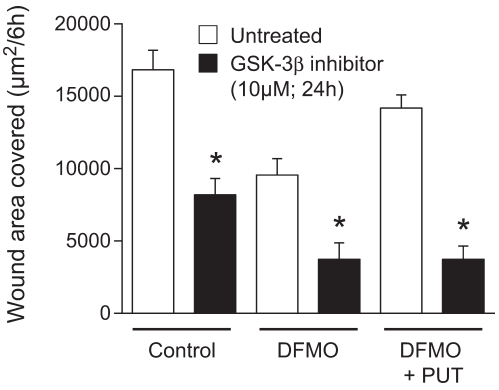

Figure 4. GSK-3β inhibition inhibits migration. IEC-6 cells were grown in medium ± 5 mM DFMO or DFMO + 10 μM putrescine (PUT) for 4 days. Confluent cells were treated with DMSO as vehicle or pretreated with 10 μM AR-A014418 (GSK-3β inhibitor) for the final 24 h, followed by wounding with a gel-loading tip in the center of plates marked to localize the wound site, washing and further incubation with fresh media. Quantitative analysis of migration shows wound width covered compared with initial wound size (0 h) using NIH Image analysis software. Values are means ± SE of six observations. *$p < 0.05$, significantly different from respective untreated group.

that cells treated with the GSK-3β inhibitor had 62% Rac1 activity compared with untreated controls (Fig. 5b).

We also studied the effect of AR-A014418 on phosphorylation of Akt to confirm its specificity. AR-A014418 had no significant effects on the levels of pSer473-Akt and total-Akt in control and polyamine-depleted cells (Fig. 5c).

Discussion

It is well known that PI3K activity is required for actin filament remodeling and cell migration in various cell-signaling pathways. Attention has been focused on identifying the downstream signaling targets of PI3K in mediating changes in cell migration and structure. The phospholipid products of phosphoinositide kinases such as phosphoinositides play critical roles in actin filament remodeling and cell migration [30]. In the present study, we examined the role of PI3K/Akt in the migration of polyamine-depleted cells. One of the major downstream targets of PI3K is the serine-threonine kinase, Akt. Akt is an essential target of PI3K that transmits survival signals from growth factors [31]. Activated Akt has a variety of biochemical functions, including the remodeling of actin filaments during VEGF-mediated endothelial cell migration [15] and the stimulation of migration of chicken embryo fibroblasts [32].

In the current study, we observed a constitutively elevated level of pSer473-Akt in polyamine-depleted cells (Fig. 1). We inhibited PI3K with LY to study the role of PI3K and Akt in migration. LY (20 μM) pretreatment for

30 min completely inhibited Akt in IEC-6 cells in control, DFMO, and DFMO plus putrescine groups. These results are in agreement with reports of Bhattacharya et al. [33] and Zhang et al. [34]. LY pretreatment led to an increase in cell migration of polyamine-depleted cells, which suggests that there is a different mechanism by which PI3K regulates cell migration in polyamine-depleted cells.

It has been reported that urokinase-induced smooth muscle cell migration requires PI3K activation [35]. PI3K is also important for the chemotactic migration of colonic smooth muscle cells [36], and combined with signaling through ERKs, PI3K/Akt is required for Met-triggered cortical neuron migration [37]. Our results suggest that LY pretreatment caused the inhibition of PI3K, leading to accumulation of phosphoinositide di-phosphate, inhibi-

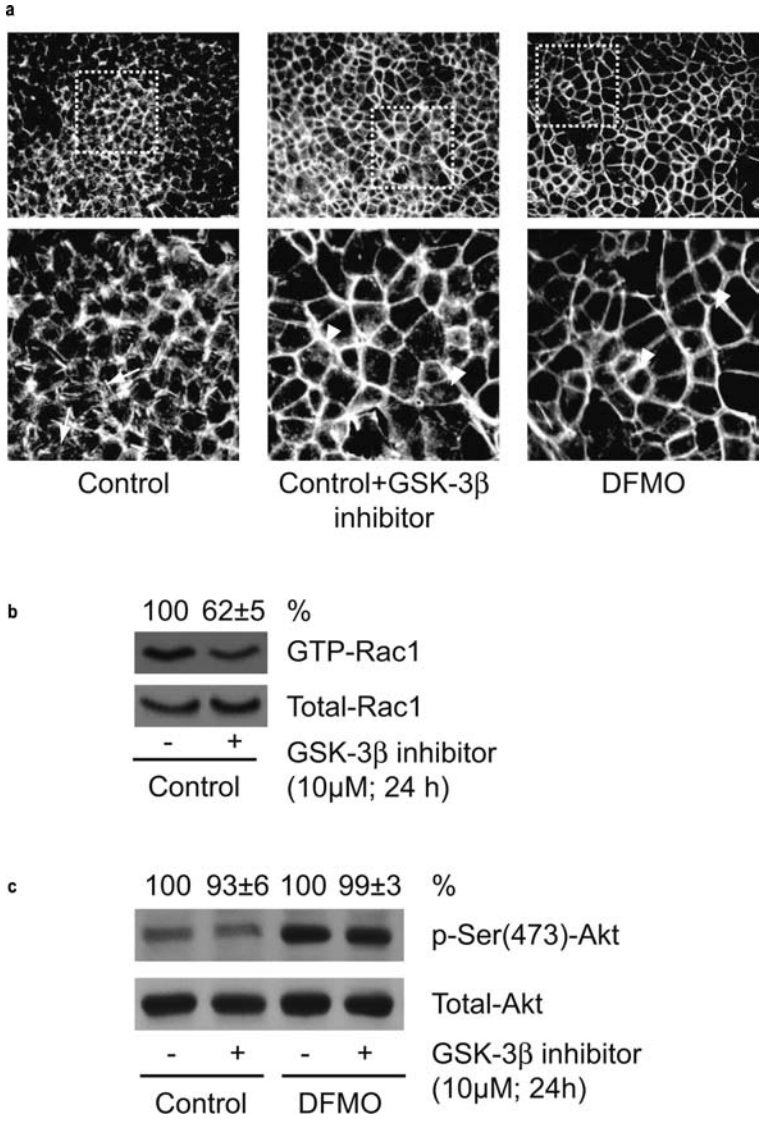

Figure 5. Effect of GSK-3 inhibitor (AR-A014418) on actin cytoskeleton and Rac1 activity. (*a*) IEC-6 cells were grown in medium ± 5 mM DFMO for 4 days. Confluent cells were treated with DMSO as a vehicle or pretreated with 10 μM AR-A014418 for the final 24 h. Cells were washed, fixed, permeabilized and stained with rhodamine-conjugated phalloidin for the visualization of F-actin. Arrows indicate stress fibers and arrowhead indicate cortical actin. Images are representative of three observations. (*b*). IEC-6 cells were grown for 4 days. Confluent cells were treated with DMSO as a vehicle or pretreated with 10 μM AR-A014418 (GSK-3*β* inhibitor) for the final 24 h. Cells were washed and lysed in the presence of protease inhibitors and Rac1 activity was determined by a glutathione *S*-transferase-p21-activated kinase fusion protein (GST-PAK) pull-down assay as described in Methods. Active (GTP-Rac1) and total Rac1 were detected by using a Rac1-specific antibody. Representative blots from three observations are shown. (*c*). IEC-6 cells were grown in medium ± 5 mM DFMO for 4 days. Confluent cells were treated with DMSO as a vehicle or pretreated with 10 μM AR-A014418 (GSK-3*β* inhibitor) for the final 24 h. Cells were lysed in the presence of protease inhibitors. Equal amounts of protein from each sample were separated by SDS-PAGE followed by western blot analysis using antibodies specific for phospho-Akt (Ser473) and Akt. Representative blots from three observations are shown.

tion of Akt activity and decreased migration of cells from control and DFMO plus putrescine groups. A recent publication by Yoeli-Lerner et al. [38] shows that when three breast cancer cell lines, transiently transfected with either wild-type Akt or a constitutively active allele (Myr.Akt), were subjected to Matrigel invasion assays, all cell lines with activated Akt potently blocked invasion compared with either vector alone or wild-type Akt.

Since pretreatment with LY increased migration of polyamine-depleted cells, we examined the actin cytoskeleton. LY had no effect on the actin cytoskeletal structure of IEC-6 cells grown in control medium. Actin is present as a cortical ring in polyamine-depleted cells, and LY led to dissolution of cortical actin and a marked increase in the number of actin stress fibers. This observation is in agreement with previous findings of Ray et al. [9] and Vaidya et al. [10], where constitutive expression of Rac1 and MEK1 restored the actin cytoskeletal structure in polyamine-depleted cells identical to that of controls. Lim et al. [39] have shown that treatment of *Pten*$^{-/-}$ mouse embryonic fibroblasts with LY led to an approximately 20–30% reduction in the number of cells exhibiting cortical F-actin compared with cells treated with vehicle. They speculated that the formation of the actin cortex was caused by a loss of lipid phosphatase activity of PTEN (phosphatase and tensin homologue deleted on chromosome 10), which led to accumulation of phosphoinositide 2,3,4 tri-phosphates. Yoeli-Lerner et al. [38] observed decreases in stress fiber formation and the inhibition of cell motility when constitutively active Akt was expressed in a breast cancer cell line, SUM-159-PT.

Rac1 activity has been reported to play an important role in the migration of IEC-6 cells, and constitutive expression of Rac1 in polyamine-depleted cells restores migration [9]. Therefore, we measured Rac1 activity in IEC-6 cells pretreated with LY and found that LY had no effect on Rac1 activity in control cells but it increased it in polyamine-depleted cells to 75% of the control level. Sanchez-Martin et al. [40] also reported an increase in Rac1 activity in T lymphoblasts when treated with LY in the presence of integrin lymphocyte function-associate antigen-1 (LFA-1 or $\alpha_L\beta_2$). LFA-1 activated Akt, leading to the inhibition of Rac1 activity, and LY treatment reversed the Akt-mediated inhibition of Rac1 activity. The mechanism by which PI3K/Akt inhibition activates Rac1 is presently unclear. Rac1, Cdc42 and RhoA possess a serine residue preceded by arginine that resembles the consensus sequence for Akt substrates (RLRPLSY), but according to the algorithm developed by Yaffe et al. [41], it is unlikely that this sequence is phosphorylated by Akt *in vivo*, compared with other well established substrates like GSK-3, Bad, eNOS or FOXO-3a. The crystal structure of Rac1 described by Hirshberg et al. [42] indicates that Ser71 is not exposed on the surface of this small GTPase, therefore its accessibility by Akt is difficult. Sanchez-Martin et al. [40] did not detect any difference in Rac1 phosphorylation in cells transfected with constitutively active Akt or treated with LY. Polyamine-depleted cells have constitutively active Akt, and LY treatment leads to inhibition of Akt and activation of Rac1.

In this study we also found that GSK-3β-phosphorylation at the Ser9 residue was high in polyamine-depleted cells. When we treated IEC-6 cells with a GSK-3β inhibitor, migration was inhibited, which suggests that GSK-3β activity is required for the migration of IEC-6 cells. Constitutive activation of Akt in polyamine-depleted cells resulted in increased phosphorylation of GSK-3β at Ser9, which made it inactive and affected migration. Koivisto et al. [43] also observed that suppression of GSK-3 activity by $LiCl_2$ or by a specific chemical inhibitor, SB-415286, blocked both staurosporine- and EGF-induced keratinocyte migration in scratch-wounded cultures. Staurosporine is a serine/threonine kinase inhibitor and it may inhibit upstream kinases of GSK-3 and increase the activity of GSK-3 and migration of keratinocytes [43]. In our studies inhibition of GSK-3β inhibited cell migration dependent on Rac1 activity. When we observed the actin cytoskeleton following inhibition of GSK-3β in control cells, cortical actin was identical to that observed in polyamine-depleted cells. These data support our finding that polyamine depletion inhibits GSK-3β activity. We also established that AR-A014418 at 10 μM for 24 h did not have a significant effect on Akt or phosphorylation of Akt (Fig. 5c). Therefore, the effect we observed with AR-A014418 was solely due to the inhibition of GSK-3β, not to the inhibition of Akt. Bhat et al. [44] characterized this inhibitor and showed that AR-A014418 does not inhibit Cdk2 or Cdk5 or 26 other related kinases ($IC_{50} \geq 100$ μM) and demonstrated the specificity for GSK-3β.

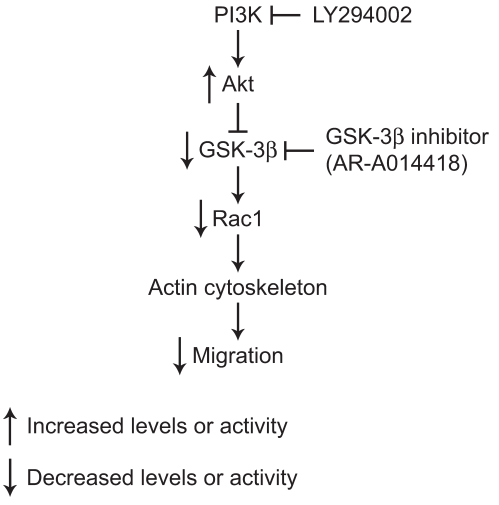

Figure 6. Schematic representation of PI3K/Akt/GSK-3β mediated migratory signaling pathway in polyamine-depleted IEC-6 cells.

In summary, the presented work shows that in polyamine-depleted cells, inhibition of PI3K with LY294002 results in inactivation of Akt. Akt phosphorylates GSK-3β and decreases its biological activity. Inactivation of Akt leads to increased GSK-3β activity, which increases Rac1 activity and migration. When IEC-6 cells grown in the control condition are treated with GSK-3β inhibitor, Rac1 activity is inhibited and actin cortex is formed, preventing migration, a phenomenon resembling that in polyamine-depleted cells (Fig. 6).

Although, PI3K/Akt is required for the migration, the extent and duration of activation largely determines whether it leads to stimulation or inhibition of migration. Our data show that polyamine-depleted cells have constitutively activated Akt, which inhibits GSK-3β activity and prevents Rac1-mediated migration by stabilizing the actin cortex.

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