Hic-5 promotes endothelial cell migration to lysophosphatidic acid

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1Department of Microbiology and Immunology, 2Sol Sherry Thrombosis Research Center, 3Department of Medicine, 4Biostatistics Consulting Center, 5Fels Institute For Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, Pennsylvania; and 6Cancer Biology Program, Harvard Medical School, Boston, Massachusetts

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Avraamides C, Bromberg ME, Gaughan JP, Thomas SM, Tsygankov AY, Panetti TS. Hic-5 promotes endothelial cell migration to lyso phosphatic acid. Am J Physiol Heart Circ Physiol 293: H193–H203, 2007. First published March 27, 2007; doi:10.1152/ajpheart.00728.2006.—Endothelial cell migration is critical for proper blood vessel development. Signals from growth factors and matrix proteins are integrated through focal adhesion proteins to alter cell migration. Hydrogen peroxide-inducible clone 5 (Hic-5), a paxillin family member, is enriched in the focal adhesions in bovine pulmonary artery endothelial (BPAE) cells, which migrate to lysophosphatidic acid (LPA) on denatured collagen. In this study, we investigate the role of Hic-5 in LPA-stimulated endothelial cell migration. LPA recruits Hic-5 to the focal adhesions and to the pseudopodia in BPAE cells plated on collagen, suggesting that recruitment of Hic-5 to focal adhesions is associated with endothelial cell migration. Knockdown of endogenous Hic-5 significantly decreases migration toward LPA, confirming involvement of Hic-5 in migration. To address the role of Hic-5 in endothelial cell migration, we exogenously expressed wild-type (WT) Hic-5 and green fluorescent protein Hic-5 C369A/C372A (LIM3 mutant) constructs in BPAE cells. WT Hic-5 expression increases chemotaxis of BPAE cells to LPA, whereas migration toward LPA of the green fluorescent protein Hic-5 C369A/C372A-expressing cells is similar to that shown in vector control cells. Additionally, ERK phosphorylation is enhanced in the presence of LPA in WT Hic-5 cells. A pharmacological inhibitor of MEK activity inhibits LPA-stimulated WT Hic-5 cell migration and ERK phosphorylation, suggesting Hic-5 enhances migration via MEK activation of ERK. Together, these studies indicate that Hic-5, a focal adhesion protein in endothelial cells, is recruited to the pseudopodia in the presence of LPA and enhances migration.

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ANGIOGENESIS, THE PROCESS of blood vessel formation, is essential for proper embryonic development and wound healing (15). Abnormal angiogenesis, however, may lead to pathological conditions such as macular degeneration (14). In addition, tumors depend on new vasculature for growth and metastasis (15). Endothelial cell migration is a critical component of angiogenesis (15). Understanding signaling events leading to endothelial cell migration will aid in the design of new targets for antiangiogenic therapy.

Endothelial cell migration is regulated by growth factors and the extracellular matrix (23). Previously, our group (35) demonstrated that lysophosphatidic acid (LPA), a bioactive lipid, promotes migration of fetal bovine heart endothelial cells on a fibronectin matrix through a balance of G; and Rho. LPA binds to and alters cellular responses through LPA1–LPA3 G-protein-coupled receptors (33). Production of LPA occurs enzymatically during platelet activation with a serum concentration between 1 and 5 μM (36). LPA is also produced by the cell surface lysophospholipase D activity of autotaxin (41). Autotaxin, an angiogenic factor (29), acts as a chemoattractant through the conversion of lysophosphatidylcholine to LPA, promoting cell motility (8). LPA also stimulates ovarian tumor growth by increasing angiogenesis (21), and LPA is detected at high levels in the ascites of ovarian carcinoma patients (45). Therefore, it is important to understand how LPA signals and controls cell migration.

In addition to growth factor signaling, the extracellular matrix affects endothelial cell migration through integrin signaling (23). Collagen, the most abundant extracellular matrix protein present in the blood vessel walls, provides the framework for endothelial cell attachment (2). Given the abundance and importance of collagen, we use collagen for the adhesive substrate to study endothelial cell migration. Furthermore, we examine the effects of LPA-stimulated endothelial cell migration because serum provides migratory signals from growth factors, including LPA (27). Bovine pulmonary artery endothelial (BPAE) cells express the collagen-binding integrins α1β1 and α2β1 (34) and the G-protein-coupled receptor LPA1 (35).

Focal adhesion proteins transduce signals from integrin receptors to the cytoskeleton to regulate cell migration (6). Hydrogen peroxide-inducible clone 5 (Hic-5) was isolated as transforming growth factor-β (TGF-β) and a hydrogen peroxide-inducible gene and was shown to localize to the focal adhesions (38, 40). Hic-5 is expressed in the developing heart (7), mature lung, mature spleen, endothelial cells, and platelets (19, 38, 47). Hic-5, a member of the paxillin family, contains four LIM domains for targeting to the focal adhesions and four LD domains to promote protein-protein interactions (40). Hic-5 and paxillin share a number of the same binding partners, including focal adhesion kinase (FAK), protein tyrosine phosphatase-PEST, and G protein-coupled receptor kinase-interacting target (GIT1) (16, 30, 31); however, they are not redundant because Hic-5 does not overcome the lethality of the paxillin-null mouse (18). Interaction of Hic-5 with FAK or GIT1 decreases cell spreading on fibronectin in NIH/3T3 cells (31, 32), and tyrosine phosphorylation of Hic-5 by EGF decreases lamellipodia formation in COS-7 cells (20). However, overexpression of Hic-5 enhances migration in epithelial cells (43), and knockdown of Hic-5 in endothelial cells decreases
migration toward VEGF (46). Nevertheless, the signal transmission pathways utilized by Hic-5 to promote endothelial cell migration are not understood.

Previously, our group (34) demonstrated that LPA stimulates BPAE cell migration on denatured collagen, not fibronectin, through strength of adhesion and localization of Hic-5 to detergent insoluble structures. Therefore, we tested the hypothesis that Hic-5 promotes endothelial cell migration to LPA on native collagen. In this study, we show that Hic-5 localization to the focal adhesions correlates with BPAE cell migration on collagen. LPA-stimulated migration is dependent on Hic-5 because knockdown of Hic-5 decreases migration. Exogenous wild-type (WT) Hic-5 enhances BPAE cell migration to LPA, which is decreased by a mutant unable to localize to the focal adhesions and by inhibition of MEK activity. MEK inhibition also decreases ERK phosphorylation. These findings show that Hic-5 is crucial in LPA-stimulated endothelial cell migration.

METHODS

Antibodies. Antibodies directed against Hic-5, paxillin, FAK (Transduction Laboratories, Lexington, KY), phosphotyrosine (4G10; Upstate USA, Charlottesville, VA), green fluorescent protein (GFP; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-active phosphoERK (Promega, Madison, WI) were used. Anti-mouse and anti-rabbit IgGs conjugated to horseradish peroxidase, rhodamine, or FITC were purchased from Santa Cruz Biotechnology. Rhodamine phalloidin, β-actin, anti-ERK (total MAPK), vinculin, and control mouse IgG were purchased from Sigma (St. Louis, MO). Anti-phospho-MEK was purchased from Cell Signaling (Danvers, MA). Phosphoserine was purchased from Abcam (ab9332) (Cambridge, MA). Anti-histone (H2B) monoclonal antibody was a gift from Dr. Marc Monestier (Temple University, Philadelphia, PA).

Cell culture and transfections. BPAE cells were purchased from VEC Technologies (Rensselaer, NY) and cultured in a mixture of MCDB-131 complete medium (VEC Technologies) and DMEM (Mediatech, Herndon, VA), supplemented with 20% FBS (Atlanta Biologicals, Norcross, GA). Cells were grown in a humidified incubator at 37°C with 5% CO2 and BPAE cells were used in experiments between passages 9 and 22.

The full-length mouse WT Hic-5 GFP-tagged construct is in the pBabe puro vector (40), with GFP at the NH2 terminus with a four-amino acid linker (Glu-Phe-Arg-Ala). pEGFP (Clontech Laboratories, Mountain View, CA) was amplified and subcloned into the pBabe puro vector (40), with GFP at the NH2 terminus with a four-amino acid linker (Glu-Phe-Arg-Ala). pEGFP (Clontech Laboratories, Mountain View, CA) was amplified and subcloned into the pBabe puro vector (40), with GFP at the NH2 terminus with a four-amino acid linker (Glu-Phe-Arg-Ala). pEGFP (Clontech Laboratories, Mountain View, CA) was amplified and subcloned into the pBabe puro vector (40), with GFP at the NH2 terminus with a four-amino acid linker (Glu-Phe-Arg-Ala). pEGFP (Clontech Laboratories, Mountain View, CA) was amplified and subcloned into the pBabe puro vector (40), with GFP at the NH2 terminus with a four-amino acid linker (Glu-Phe-Arg-Ala). pEGFP (Clontech Laboratories, Mountain View, CA) was amplified and subcloned into the pBabe puro vector (40), with GFP at the NH2 terminus with a four-amino acid linker (Glu-Phe-Arg-Ala). pEGFP (Clontech Laboratories, Mountain View, CA) was amplified and subcloned into the pBabe puro vector (40), with GFP at the NH2 terminus with a four-amino acid linker (Glu-Phe-Arg-Ala), supplemented with 20% FBS (Atlanta Biologicals, Norcross, GA). Cells were grown in a humidified incubator at 37°C with 5% CO2, and BPAE cells were used in experiments between passages 9 and 22.

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DharmaFECT1, respectively, in a 60-mm dish. Migrations were started 48 h later.

Statistical analysis. The dependent variables (cell counts) were treated as continuous variables for all analyses. All data were tested for normality using the Shapiro-Wilk’s test (1). A “normalized-rank” transformation was applied to the nonnormal data and analyzed with a general linear model ANOVA (10). Multiple pair-wise comparisons were made with the Dunn-Bonferroni adjustment (1). Differences between means (using two-tailed tests) were considered significant if the probability was ≤0.05. All fluorescent microscopy experiments were performed on living cells using epifluorescent microscopy.
were repeated at least two times. All other experiments were repeated at least three times.

RESULTS

**Hic-5 localizes to the focal adhesions in migrating cells and is redistributed by LPA to the pseudopodia.** To test whether Hic-5 regulates endothelial cell migration, the cellular localization of Hic-5 in migrating and nonmigrating cells was examined. BPAE cells were grown to confluence, scratch wounded, and 15 h later fixed and stained for Hic-5 (Fig. 1A, top) and F-actin (Fig. 1A, bottom). The confluent monolayer of cells was defined as nonmigrating due to cell-cell contact-dependent inhibition, and Hic-5 was distributed diffusely in the cytoplasm of contact-inhibited cells (Fig. 1A1i). In the migrating cells found at the edge of the wound, Hic-5 was localized to small punctate structures (Fig. 1Aii). To show that the cells were in a nonmigrating region rather than migrating at the wound edge, the cytoskeleton of the cells was stained with F-actin (Fig. 1A).

The punctate pattern of Hic-5 staining in the migrating cells suggested recruitment of Hic-5 to focal adhesions. To examine this possibility, BPAE cells were stained with Hic-5 and actin, and the localization of Hic-5 with respect to actin was observed by confocal microscopy. Actin stress fibers were stained with rhodamine phalloidin (red) and terminated at Hic-5, shown by punctate patches of green. The enlarged images of two different cells clearly demonstrate Hic-5 at the ends of actin stress fibers (Fig. 1B and insets), suggesting that Hic-5 is a focal adhesion protein.

Pseudopodia are analogous to lamellipodia and filopodia (9), which are critical in cell migration. Therefore, we tested whether LPA redistributes Hic-5 to the pseudopodia. BPAE cells were plated on inserts with 3-μm pores, too small for the cells to migrate through; however, when stimulated with LPA, BPAE cells send protrusions through the filter, and these are defined as the pseudopods. Purification of the pseudopodia from cell body shows three times as much Hic-5 found in the pseudopodia after LPA stimulation than found under control (FAF-BSA) conditions (Fig. 1C). Phospho-ERK is also found in the pseudopodia, consistent with previous observations (3, 9). Total ERK shows equal loading of the pseudopodia lanes and presence of protein in the control (FAF-BSA) lane. Histone immunoblot shows that no nuclear contamination is present in the pseudopodia. This is the first report showing that LPA recruits Hic-5 to the pseudopodia.

**WT Hic-5 localizes to the focal adhesions.** To address the role of Hic-5 in LPA-stimulated endothelial cell migration, we overexpressed GFP-tagged WT Hic-5 in BPAE cells. BPAE cells were retrovirally transduced with WT Hic-5, and a stable heterogeneous population was obtained. Western blot analysis shows that GFP-tagged WT Hic-5 was present in endothelial cells (Fig. 2A). Endogenous Hic-5 was expressed in both BPAE and GFP-tagged WT Hic-5 cells (Hic-5 immunoblot), whereas exogenous GFP-tagged WT Hic-5 was only present in WT Hic-5 cells (Fig. 2A, GFP and Hic-5 immunoblot). To ensure that the overexpressed WT Hic-5 was a focal adhesion protein, we show colocalization of WT-Hic-5 with FAK (Fig. 2B) and vinculin (Fig. 2C) by indirect immunofluorescence.

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**Fig. 2.** Wild-type (WT) Hic-5 is present in the focal adhesions. A: Western blot confirming WT Hic-5 expression in the BPAE cells. IB, immunoblot. BPAE cells were retrovirally transduced to express WT Hic-5. WT Hic-5 and BPAE cell lysates were analyzed by Western blot using anti-GFP (top left) or anti-Hic-5 (top right). β-Actin shows approximate protein loading in the lanes (bottom). Colocalization of focal adhesion kinase (FAK; B) or vinculin (C) with WT Hic-5 cells was visualized by epifluorescent microscopy. Bar = 10 μm.
Fig. 3. WT Hic-5 is present in the focal adhesions and pseudopodia. 

A: BPAE, WT Hic-5, and green fluorescent protein (GFP)-BPAE cells were plated on collagen for 2 h and subsequently left unstimulated (control) or stimulated with LPA (30 min). Hic-5 is detected in BPAE cells by indirect immunofluorescence followed by epifluorescent microscopy. WT Hic-5 is detected by confocal microscopy. GFP is visualized by epifluorescent microscopy. Bar = 10 μm. 

B: Pseudopodia experiment was performed as described in Fig. 1. Amount of WT Hic-5 in the pseudopodia fraction (in arbitrary units) shown in B was determined by densitometry.
Endogenous GFP fluorescence shows that WT Hic-5 is present in punctate structures (green) and colocalizes with FAK (red), as shown by the merged images (yellow) (Fig. 2B). Similarly, WT Hic-5 colocalizes with vinculin (Fig. 2C). These data suggest that the GFP-tagged WT Hic-5 localizes to the focal adhesions similar to endogenous Hic-5.

**WT Hic-5 is present in focal adhesions and pseudopodia.** We observed that LPA stimulated endogenous Hic-5 recruitment to the focal adhesions of BPAE cells plated on collagen at 30 min (Fig. 3A, top). Thus we wanted to examine the effect of LPA on WT Hic-5 recruitment to the focal adhesions at 30 min. Surprisingly, under nonstimulated (control) conditions, WT Hic-5 was found in the focal adhesions (Fig. 3A, bottom). This suggests that exogenous expression of Hic-5 promotes Hic-5 localization into the focal adhesions even under nonstimulated conditions (Fig. 3A). GFP alone was not recruited to the focal adhesions (Fig. 3A) even in the presence of LPA for 30 min. Because LPA recruited endogenous Hic-5 to the pseudopodia (Fig. 3B), we next examined the localization of WT Hic-5 to the pseudopodia. We found Hic-5 (50 kDa) and WT Hic-5 (75 kDa) in the pseudopodia of both FAF-BSA (control) and LPA-stimulated cells (Fig. 3B) and found that twice as much WT Hic-5 was recruited after LPA stimulation. Phospho-ERK and total ERK were also found in nonstimulated and LPA-stimulated conditions. There is no nuclear contamination in the pseudopodia, as shown by the histone immunoblot. These data suggest that exogenous WT Hic-5, similar to endogenous Hic-5, localizes to pseudopodia with LPA stimulation.

**WT Hic-5 enhances endothelial cell migration to LPA.** The effect of WT Hic-5 expression on cell migration was examined. On collagen, WT Hic-5 expression in BPAE cells increased migration to LPA compared with that shown in parental BPAE cells and control GFP BPAE cells (Fig. 4). GFP BPAE cells migrate in a manner similar to BPAE cells, suggesting GFP does not alter endothelial cell migration. S1P acts through distinct G-protein-coupled receptors S1P1 and S1P3 in endothelial cells and thus was used as a positive control for endothelial cell migration. Therefore, overexpression of WT Hic-5 increases BPAE cell migration to LPA on collagen.

**GFP Hic-5 C369A/C372A does not localize to the focal adhesions or promote migration toward LPA.** We generated the LIM3 mutant GFP Hic-5 C369A/C372A, which has previously been shown not to localize to the focal adhesions (20).
To determine the importance of subcellular localization of Hic-5 in endothelial cell migration, we overexpressed the GFP Hic-5 C369A/C372A mutant in the BPAE cells (Fig. 5A). As previously shown, this mutant does not localize to the focal adhesions (Fig. 5B) (20). This effect was specific to the LIM3 (GFP-Hic-5 C369A/C372A) mutant, as these cells were able to form vinculin focal adhesions (Fig. 5C). Next, we looked at GFP Hic-5 C369A/C372A localization to the pseudopodia. Surprisingly, the LIM3 mutant was able to localize to the pseudopodia, most likely because it is already present in the cytoplasm. However, with LPA stimulation, recruitment of GFP Hic-5 C369A/C372A to the pseudopodia is minimal, suggesting that LPA-stimulated recruitment was impaired. BPAE and WT Hic-5 cells show an increase of Hic-5 in the LPA-stimulated pseudopodia (Fig. 1B and 3B).

To demonstrate that there is a direct effect between localization of Hic-5 to the focal adhesions and migration, we used the LIM3 mutant in migration assays. The LIM3 mutant migrates to LPA in a manner similar to the control GFP cells, whereas WT Hic-5 shows enhanced migration toward LPA (Fig. 6A). This suggests that the localization of the WT Hic-5 to the focal adhesions is critical for migration. To show a direct effect between Hic-5 expression and migration, we used siRNA to the overexpressed mouse WT Hic-5. siRNA to the WT Hic-5 decreased Hic-5 expression (Fig. 6B, inset) and did not affect endogenous bovine Hic-5 (lower band in the Hic-5 blot). Migration toward LPA was decreased compared with the control siRNA. These studies demonstrate that Hic-5 must localize to the focal adhesions to enhance migration and that Hic-5 expression enhances LPA-stimulated BPAE cell migration.

WT Hic-5 enhances ERK phosphorylation. To understand changes in signaling in WT Hic-5 cells, we examined serine/threonine phosphorylation. Western blots of lysates are from BPAE, WT Hic-5, and GFP-BPAE cells plated on collagen for 2 h and stimulated with LPA (L) or S1P (S) (1 μM) for 5 min or left in FAF-BSA [control (C)]. A: detection of phosphoserine (p-serine) by Western blot. B: Western blots for P-ERK and for total ERK (top) and densitometry analysis for this experiment (bottom), where the control BPAE is normalized to 1. Densitometry analysis compares P-ERK with total ERK. C: Western blots for P-MEK and for total ERK as the loading control (top) and densitometry showing ratio between P-MEK and the loading control total ERK (bottom), where the control is normalized to 1.
Fig. 8. MEK inhibitor decreases ERK but not MEK phosphorylation. BPAE, WT Hic-5, and GFP BPAE cells were plated on collagen, and cells were untreated or treated with PD-98059 or U-0126 (25 μM) before stimulation with LPA or S1P as indicated. Left: untreated lysates. Right: PD-98059-treated lysates. A: Western blots for P-ERK and total ERK (top) and densitometry analysis for this experiment (bottom), where control BPAE is normalized to 1 (ratio of P-ERK to total ERK). Open bars, untreated conditions; solid bars, PD-98059-treated condition. B: Western blots for P-MEK and total ERK (top) and densitometry analysis (bottom). Open bars, no treatment; solid bars, PD-98059 treatment (ratio of P-MEK to total ERK). C: Western blots for P-ERK and total ERK (top) with U-0126 treatment (right) and densitometry analysis (bottom). Open bars, no treatment; solid bars, U-0126 treatment (ratio of P-ERK to total ERK).
pretreated for 15 min with the MEK inhibitor (U-0126; 25 μM) before addition to the migration chamber. Migration of WT Hic-5 to LPA was significantly decreased in the presence of U-0126 (Fig. 9). This suggests that one mechanism by which Hic-5 enhances migration is via ERK activity.

Knockdown of endogenous Hic-5 decreased LPA-stimulated BPAE cell migration. To confirm that endogenous Hic-5 is important in LPA-stimulated cell migration, we used siRNA to knockdown endogenous Hic-5. For the control siRNA, we used human GAPDH-specific siRNA, which does not decrease bovine GAPDH (Fig. 10, inset) and therefore can appropriately be used as a control. Because the ability of the BPAE cells to migrate toward LPA appears to depend on different cultures of cells and cell passage number (migration decreases with longer passage number), we chose cells that were in culture for <10 passages and had a significant migration toward LPA. Knockdown of endogenous Hic-5 in these cells significantly decreased LPA-stimulated migration, confirming our data obtained in the overexpressed system (Fig. 10). Therefore, we show that not only overexpressed Hic-5 enhances LPA-stimulated migration but that endogenous Hic-5 is important in promoting LPA-stimulated migration.

**DISCUSSION**

The novel finding of this study is that LPA recruitment of Hic-5 to the focal adhesions promotes endothelial cell migration due to an increase in ERK activity. We specifically show that localization of Hic-5 to the focal adhesions is critical for migration. In addition, the increase in ERK activity is clearly caused by overexpression of Hic-5 because neither the BPAE nor the GFP cells show enhanced ERK activity in the presence of LPA. Therefore, one mechanism by which Hic-5 enhances migration is via MEK and ERK activation.

In agreement with others, we show that WT Hic-5 and endogenous Hic-5 are focal adhesion proteins colocalizing with FAK and vinculin (46). Neither endogenous Hic-5 nor WT Hic-5 is detected in the nucleus of BPAE cells (data not shown), consistent with previous reports (40). GFP does not localize to the focal adhesions, suggesting that GFP does not bind to Hic-5 or other proteins in the focal adhesions. We

**MEK inhibitor decreased migration.** Because LPA enhances ERK phosphorylation, we tested the effects of a MEK inhibitor on migration. The WT Hic-5 cells were pretreated with U-0126 before addition to the migration chamber. Migration of WT Hic-5 to LPA was significantly decreased in the presence of U-0126 (Fig. 9). This suggests that one mechanism by which Hic-5 enhances migration is via ERK activity.

Next, we blocked MEK activity using pharmacological inhibitors to MEK, either the U-0126 or the PD-98059 compound. We observed that both PD-98059 and U-0126 decreased ERK phosphorylation (Fig. 8, A and C). Densitometry shows that ERK phosphorylation is decreased fourfold by PD-98059 in LPA-stimulated WT Hic-5 cells (Fig. 8A) and that U-0126 blocked ERK phosphorylation threefold in WT Hic-5 cells (Fig. 8C). However, PD-98059 did not block MEK phosphorylation (Fig. 8B). These data suggest that WT Hic-5 enhanced MEK activity, which then enhanced ERK activity. The MEK inhibitor did not completely block MEK phosphorylation but presumably blocked subsequent kinase activity. Therefore, some ERK activity may remain due to low-level MEK activity.

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The novel finding of this study is that LPA recruitment of Hic-5 to the focal adhesions promotes endothelial cell migration due to an increase in ERK activity. We specifically show that localization of Hic-5 to the focal adhesions is critical for migration. In addition, the increase in ERK activity is clearly caused by overexpression of Hic-5 because neither the BPAE nor the GFP cells show enhanced ERK activity in the presence of LPA. Therefore, one mechanism by which Hic-5 enhances migration is via MEK and ERK activation.

In agreement with others, we show that WT Hic-5 and endogenous Hic-5 are focal adhesion proteins colocalizing with FAK and vinculin (46). Neither endogenous Hic-5 nor WT Hic-5 is detected in the nucleus of BPAE cells (data not shown), consistent with previous reports (40). GFP does not localize to the focal adhesions, suggesting that GFP does not bind to Hic-5 or other proteins in the focal adhesions. We
specifically show that Hic-5 is recruited to focal adhesions in migrating cells and that this recruitment is critical for migration. Paxillin localization to the focal adhesions is necessary for Crk-dependent lamellipodia formation and cell spreading (26). In addition, paxillin mutants, not localizing to the focal adhesions (4), inhibit Chinese hamster ovary cell migration (5). Hic-5 must be targeted to the focal adhesions to decrease lamellipodia formation in fibroblasts (20). We demonstrate that lack of Hic-5 recruitment to the focal adhesions decreases migration toward LPA, suggesting that Hic-5 localization to the focal adhesions is critical for migration.

In BPAE cells plated on collagen, we demonstrate that, after LPA stimulation, Hic-5 is recruited to pseudopodia where it may regulate signaling. WT Hic-5 localizes to focal adhesions in BPAE cells attached to fibronectin similar to the endogenous Hic-5 (Ref. 34 and data not shown), suggesting that exogenous expression is not disrupting the normal localization. Previously, we observed that endogenous Hic-5 was present in the focal adhesions of BPAE cells plated on gelatin or fibronectin independent of LPA stimulation (34). In contrast, we observed that, on collagen, endogenous Hic-5 localizes to focal adhesions after LPA stimulation, suggesting that integrin activation affects localization of focal adhesion proteins into the focal adhesions independent of chemoattractant. We also observed that WT Hic-5 localizes to focal adhesions in the absence of LPA, suggesting that increased expression of Hic-5 enhances localization to the focal adhesions. Nevertheless, BPAE cells are a good system to study the effects of Hic-5 in migration because they express endogenous Hic-5 and the relevant cell signaling molecules to mediate the downstream effects of WT Hic-5 expression. The exogenous WT Hic-5 in the focal adhesions is likely utilizing the same signaling pathway as endogenous Hic-5 to promote migration. Hic-5 was initially identified in a screen for TGF-β inducible genes (38) and was shown to be induced by TGF-β in NMuMG cells during epithelial mesenchymal transformation (43). Therefore, increases in Hic-5 expression may be physiologically relevant.

To demonstrate a role for Hic-5 in chemotaxis, we exogenously expressed WT Hic-5 in BPAE cells. In support of our hypothesis, we found that exogenous WT Hic-5 enhances migration to LPA on collagen. GFP does not alter cell migration, consistent with previous observations (37). We also showed that knockdown of endogenous Hic-5 decreases chemotaxis. Therefore, we demonstrated that Hic-5 is a positive regulator of endothelial cell migration to LPA. Prior studies have shown that Hic-5 decreases cell spreading and lamellipodia formation in fibroblasts (20, 31). Hic-5 decreases cell spreading by competing with paxillin for binding to FAK, thus decreasing both paxillin and FAK phosphorylation (32). Tyrosine phosphorylation of Hic-5 by EGF decreases lamellipodia formation (20). In addition, Nishiya et al. (31) suggested that Hic-5 may decrease cell migration because Hic-5 decreases cell spreading and complexes with GIT1 in a different binding mode than paxillin. Despite prior studies suggesting that Hic-5 has a negative role in cell migration, enhanced expression of Hic-5 in epithelial cells increases migration through an unknown mechanism (43). Furthermore, in endothelial cells, Hic-5 interacts with Traf-4 to promote VEGF-mediated chemotaxis (46). Decreasing Hic-5 with siRNA blocked the interaction with Traf-4 and partially decreased migration (46), suggesting that Hic-5 may be involved in multiple signaling pathways to regulate migration. We demonstrated that one possible pathway that Hic-5 uses to enhance endothelial cell migration is through an ERK signaling pathway.

ERK promotes migration of many different cell types, including endothelial cells (39). The MEK inhibitors decrease the migration of cells in response to matrix proteins, such as collagen (24), and growth factors, such as VEGF (13), similar to dominant-negative ERK mutant (24, 25). In BPAE cells, we observed that MEK inhibitors decrease cell migration and ERK phosphorylation, suggesting that the ERK/MAPK pathway is involved in Hic-5-mediated cell migration to LPA. Potential downstream targets of ERK that affect cell migration are myosin light-chain kinase (MLCK) (24) and calpain (22). Inhibition of the ERK pathway decreases MLCK phosphorylation and cell migration (24). Furthermore, ERK phosphorylates MLCK and calpain II and increases MLCK and calpain II activity (17, 24). MLCK and calpain activation may be involved in focal adhesion turnover, which is important for cell migration (17, 42, 44).

Endothelial cell migration is important in both tumor angiogenesis and wound healing (15, 27). LPA present in the ascites of ovarian cancer patients (45) may enhance endothelial cell migration by activating ERK through Hic-5 recruitment to the focal adhesions. Furthermore, mechanical injury to the vascular wall, for example, in the treatment of atherosclerosis by angioplasty, leads to endothelial cell proliferation and migration (12). Hic-5 expression may be increased during tissue injury by TGF-β or under oxidizing conditions that may regulate endothelial cell function to promote cell migration and vessel repair. Therefore, possible changes in Hic-5 expression in the vascular cells during atherosclerosis may regulate endothelial cell migration. Understanding signaling events that alter cell migration will aid in the present understanding of angiogenesis and antiangiogenic therapies, important both in treatment of restenosis after angioplasty and in tumor biology, respectively.

In summary, there are four novel points to this study. Migration regulates cellular localization of endogenous Hic-5 to the focal adhesions; endogenous and exogenous Hic-5 is recruited to the pseudopodia after LPA-stimulation; and Hic-5 expression enhances migration to LPA and ERK activity. Taken together, these studies suggest that Hic-5 enhances LPA-stimulated migration by activating ERK.

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