Obligatory role for phospholipase C-γ1 in villin-induced epithelial cell migration

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Wang Y, Tomar A, George SP, Khurana S. Obligatory role for phospholipase C-γ1 in villin-induced epithelial cell migration. Am J Physiol Cell Physiol 292: C1775–C1786, 2007. First published January 17, 2007; doi:10.1152/ajpcell.00420.2006.—While there is circumstantial evidence to suggest a requirement for phospholipase C-γ1 (PLC-γ1) in actin reorganization and cell migration, few studies have examined the direct mechanisms that link regulators of the actin cytoskeleton with this crucial signaling molecule. This study was aimed to examine the role that villin, an epithelial cell-specific actin-binding protein, and its ligand PLC-γ1 play in migration in intestinal and renal epithelial cell lines that endogenously or ectopically express human villin. Basal as well as epidermal growth factor (EGF)-stimulated cell migration was accompanied by tyrosine phosphorylation of villin and its association with PLC-γ1. Inhibition of villin phosphorylation prevented villin-PLC-γ1 complex formation as well as villin-induced cell migration. The absolute requirement for PLC-γ1 in villin-induced cell migration was demonstrated by measuring cell motility in PLC-γ1−/− cells and by downregulation of endogenous PLC-γ1. EGF-stimulated direct interaction of villin with the Src homology domain 2 domain of PLC-γ1 at the plasma membrane was demonstrated in living cells by using fluorescence resonance energy transfer. These results demonstrate that villin provides an important link between the activation of phosphoinositide signal transduction pathway and epithelial cell migration.

CELL MIGRATION PLAYS A CRUCIAL role in epithelial biology. Most cancers are carcinomas, and increased cell migration is a major determinant of metastasis. In addition, epithelial cell injury as a result of osmotic fluctuations, exposure to bacteria, viruses, and necrotic agents is repaired rapidly and continually by cell migration. Intestinal epithelial injury occurs frequently even in undisturbed gut without any ensuing clinical disease (33). Cellular migration along the crypt-villus axis is also critical to the normal turnover of the epithelial lining. Perturbation of cell migration alters cell death programs and may promote chronic mucosal inflammation and even adenoma formation (18). Epithelial cells migrate by a complex process with a poorly understood molecular basis. In addition, no therapeutic modalities facilitating epithelial mucosal repair have been established so far. Growth factors and cytokines have been demonstrated as major regulators of epithelial cell migration (14, 39). The intestinal phenotype of the epidermal growth factor (EGF) receptor-null mouse and the mouse that have been shown to be an actin-binding protein (11). This is relevant, since cell migration is driven by actin polymerization. What is not known is how stimulation of cell surface receptors is linked to actin polymerization. To this end, it has been suggested that EGFR and F-actin may facilitate the formation of signaling complexes that include phospholipase C-γ1 (PLC-γ1), thus sensing extracellular stimuli (12, 45). The activation of PLC-γ1 is linked to virtually all growth factor receptors. Abrogation of the cytoskeletal reorganization has been shown to disrupt PLC-actin association, PLC-γ translocation, and/or PLC-γ activation (10, 11, 46). Thus the cytoskeleton plays an important role in both growth factor- and PLC-γ-mediated intracellular signaling.

Villin is a tissue-specific actin-binding protein that is expressed in most significant amounts in the renal and gastrointestinal epithelial cells, where it is localized to the microvillar core and the terminal web (5). In addition, villin has also been identified in exocrine glands of endodermic lineage such as the thymus, as well as those associated with the gastrointestinal tract such as pancreatic and biliary duct cells. Villin expression has been noted in other epithelial cells including brush cells (19), taste receptor cells (20), and osteocytes (26). Villin is unique among the actin regulatory proteins in that it can nucleate, cap, sever, and bundle actin filaments. Villin regulates epithelial cell migration; however, the cellular and molecular mechanisms of villin-induced cell migration have not been determined (43). Our group (43) has previously demonstrated that tyrosine phosphorylation of villin is essential to its function in cell migration. In addition, from in vitro studies in our laboratory using recombinant villin protein (30, 36), we know that tyrosine-phosphorylated villin interacts with PLC-γ1 and regulates PLC-γ1 catalytic activity, thus modifying the phosphoinositide signal transduction pathway. On the basis of these earlier studies, we hypothesized that interaction of tyrosine-phosphorylated villin with PLC-γ1 may be mechanistically important to its role in cell migration. This suggests that tyrosine phosphorylation of villin in response to receptor activation may be coordinated with actin remodeling by villin, where it serves to integrate and transduce PLC-γ1-mediated signals, thus regulating epithelial cell migration. In this study, we have tested this hypothesis and demonstrated that PLC-γ1 is a biologically important ligand of villin that is indispensable for villin’s function in cell migration.

MATERIALS AND METHODS

Materials. Caco-2 cells (clone C2BBel) were purchased from American Type Culture Collection (ATCC; Rockville, MD). Madin-
Darby canine kidney (MDCK Tet-Off) cells were a kind gift from Dr. Keith E. Mostov (University of California, San Francisco, CA); mouse embryo fibroblasts with (PLC-γ1/+) and without (PLC-γ1−/−) PLC-γ1 were a kind gift from Dr. Graham Carpenter (Vanderbilt University, Nashville, TN) (22). The Src homology domain 2 (SH2) domains of PLC-γ1 cloned in pEGFP-C1 vector, GFP-γ1SA, was a generous gift from Dr. Matilda Katan (The Institute of Cancer Research, London, UK) (31). PLC-γ1 small interference RNA (siRNA) and a negative control siRNA SMARTpool were purchased from Dharma-Upstate. c-Src dominant negative (K296R/Y528F mutations) cDNA was purchased from Upstate Biotechnology (Lake Placid, NY). All other chemicals were obtained from BD Biosciences, Sigma, Calbiochem, or Invitrogen.

Cell transfection and culture. SEYFP-, DsRed2-, or influenza A virus hemagglutinin (HA)-tagged wild-type and mutant villin proteins were cloned and stably transfected in MDCK Tet-Off cells as described previously (George S, Tomar A, Wang T, Mathew S, and Khurana S; unpublished observations). Caco-2 cells were cultured as described previously (18). Cells were used between 2 and 3 wk postconfluence. Cells provided by ATCC were at postconfluence. Cells provided by ATCC were at

Migration 7 h postwounding in cells transfected/infected with vector/control siRNA. Caco-2 cell migration was measured in the presence of mitomycin C (50 μg/ml). All cell migration experiments were done in media containing 1% fetal bovine serum (FBS), which had no effect on cell proliferation but maintained cell viability; 1% FBS also had no effect on villin phosphorylation or cell migration rates. Comparisons between mean values were made using one-way repeated-measures analysis of variance and Tukey’s modified t-test (Bonferroni criteria) with P < 0.05 considered significant.

Invasion assays were performed using 1 × 104 cells/well plated in six-well invasion chambers coated with Matrigel. EGF (100 ng/ml) was added to the lower chamber. The invasion chambers were incubated for 24 h, after which the Matrigel membrane was removed. To examine the transmigrated cells on the lower surface of the membrane, we stained filters using the DIFF-Quick staining kit. In the absence of EGF, no transmigration of cells was seen in 24 h.

Immunoprecipitation and Western blot analysis. Cells were lysed in buffer containing 20 mM HEPES, pH 7.2, 1% Triton X-100, 150 mM NaCl, 1 mM sodium orthovanadate, 50 mM NaF, and protease inhibitors. Tyrosine-phosphorylated proteins and PLC-γ1 were immunoprecipitated from Triton-soluble cell extracts by using monoclonal antibodies to PLC-γ1, phosphotyrosine, or a phospho-villin polyclonal antibody (VP-70782) as described previously (27, 43). Western blot analysis was performed using monoclonal antibodies to villin, phosphotyrosine (PY-20), PLC-γ1, or polyclonal phospho-villin antibody VP-70782. The Western blots were quantified using a CCD camera and imaging system (Bio-Rad). Quantitative densitometric analysis was performed using the image software Scion Image.

Fluorescence resonance energy transfer analysis in living cells. Fluorescence resonance energy transfer (FRET) analysis was performed in MDCK Tet-Off cells coexpressing EGFP-tagged SH2 domains of PLC-γ1 (GFP-γ1SA) and DsRed2-tagged villin proteins. Cells were serum-starved overnight and treated without or with EGF (100 ng/ml). FRET calculations were made using the method of sensitized FRET. FRET was calculated from at least seven cells in three independent experiments. DsRed2 and EGFP were excited with the 543- or 488-nm lines of a helium-neon or argon ion laser, respectively, and fluorescence was recorded with a 560- or 505-nm long-pass filter, respectively. Calculation of corrected FRET (FRETc) was carried out on a pixel-by-pixel basis for the entire image. The bleed of EGFP and DsRed2 through the FRET filter channel was corrected by applying the following equation: FRETc = raw FRET – (A+ acceptor) – (B+ donor), where A+ and B+ are coefficients A and B, respectively. The fluorescence was analyzed using confocal laser scanning microscopy (LSM 5 PASCAL, Zeiss, Thornwood, NY). The overall intensity of FRET was calculated using the Metamorph software. Images are displayed in pseudocolor mode, where white and black areas display high and low values of FRET in the range of 0–200 relative light units, respectively.

Downregulation of PLC-γ1, PLC-ζ, and the control siRNA were purchased from Dharmacon-Upstate. Approximately 70% confluent MDCK Tet-Off cells were transfected with the RNA oligonucleotides (200 nM) using Lipofectamine 2000 reagent. Western blot analysis using PLC-γ1 monoclonal antibody was performed to assess gene silencing. All experiments were performed 48 h posttransfection.

Cell proliferation and cell viability measurements. Caco-2 cell proliferation was measured by bromodeoxyuridine (BrdU) labeling by quantitating BrdU incorporation into newly synthesized DNA of replicating cells, using the BrdU in situ detection kit according to the instructions of the manufacturer (BD Pharmingen). Cell viability was measured using propidium iodide (2 μg/ml in PBS). Flow cytometric measurements were made on a BD LSRII flow cytometer.
RESULTS

Direct interaction of villin with PLC-γ1 and localization of villin-PLC-γ1 complex in living cells. Our group (27, 36) has previously reported that in vitro and in vivo tyrosine-phosphorylated villin forms a complex with PLC-γ1. To determine whether the interaction between villin and PLC-γ1 was a direct association and to identify the intracellular localization of the villin-PLC-γ1 complex, we elected to use FRET in cells co-expressing EGFP-tagged mutant PLC-γ1 (GFP-γ1SA) and DsRed2-tagged villin. Using this PLC-γ1 mutant protein expressing the SH2 domains of PLC-γ1 (GFP-γ1SA), Matsuda et al. (31) previously demonstrated that the SH2 domains of PLC-γ1 are sufficient for the translocation of this enzyme to the plasma membrane. We (George S, Tomar A, Wang T, Mathew S, and Khurana S, unpublished observations) report that the DsRed2-villin is fully functional by examining the intracellular distribution of DsRed2-tagged villin and EGF-stimulated redistribution of DsRed2-tagged villin to the developing lamellipodia in MDCK Tet-Off cells as well as in an in vitro wound assay in which villin-induced increase in cell migration by DsRed2-tagged villin was compared with HA-tagged villin protein. In MDCK cells coexpressing GFP-γ1SA and DsRed2-villin, a strong FRET signal (FRET efficiency of 15%, P < 0.01, n = 7) was seen at the cell surface in response to EGF treatment (Fig. 1B), which was absent in untreated cells (Fig. 1A). It may be noted that the FRET signal obtained in these studies may be an underestimate of the villin-PLC-γ1 interaction, since the interaction between DsRed2-tagged villin and PLC-γ1 includes the interaction with both the endogenous PLC-γ1 as well as GFP-γ1SA. Following EGF treatment, there was a redistribution of PLC-γ1 as well as a significant pool of villin to the cell surface in developing lamellipodia and membrane ruffles (Fig. 1C, c and d, arrowheads). However, only a small portion of this total villin pool interacted with PLC-γ1. This is consistent with our group’s previous biochemical studies (27), which demonstrated that the villin present in the detergent-soluble cell extracts in complex with PLC-γ1 was fourfold less than the villin present in the detergent-insoluble fraction. Since our group (27, 36) has previously established that only tyrosine-phosphorylated villin interacts with PLC-γ1 and, furthermore, since the only known ligands of SH2 domains are tyrosine-phosphorylated proteins, we made the assumption that the villin that interacts with the SH2 domain of PLC-γ1 in living cells is tyrosine phosphorylated. Coimmunoprecipitation of tyrosine-phosphorylated villin with PLC-γ1 from these cells in response to EGF treatment supports this assumption (Fig. 1D). Together with our previous study, data presented in this study allows us to conclude that in living cells in response to receptor activation, a small pool of the total villin associates with the SH2 domain of PLC-γ1 at the plasma membrane. The fact that both proteins interact at the plasma membrane suggests that villin can participate with PLC-γ1 in communicating cell surface signals to the microfilament structure.

Villin regulates intestinal cell migration. Hepatocyte growth factor (HGF) and EGF are potent motogens and chemoattractants for epithelial cells (3, 13). These receptor tyrosine kinases lead to the activation of several signal transduction pathways including the activation of PLC-γ1 (6, 17). To test our hypothesis that villin may integrate the signals originating at the cell surface with the microfilament structure to initiate changes in cell locomotion, we characterized basal and growth factor-induced cell migration in the human colonic adenocarcinoma cell line Caco-2. Cell migration was recorded between times 0 and 24 h postwounding for Caco-2 cells cultured in the presence of 1% FBS (which inhibits cell proliferation but maintains cell viability) and mitomycin C (50 μg/ml; to prevent cell proliferation) (6). Rates of cell proliferation and cell viability during this period were recorded using BrdU and propidium iodide staining and showed no significant difference between controls and treated groups (data not shown). Both EGF (10 ng/ml) and HGF (50 ng/ml) significantly increased Caco-2 cell migration (P < 0.001, n = 24) (Fig. 1E). Increased cell migration was accompanied by tyrosine phosphorylation of villin and its association with PLC-γ1 (Fig. 1F). Villin was tyrosine phosphorylated and associated with PLC-γ1 in wounded monolayers even in the absence of growth factors (Fig. 1F, wounded). FBS (1%) had no effect on villin phosphorylation (Fig. 1F, control). These data demonstrate that tyrosine phosphorylation of villin and its association with PLC-γ1 was temporally related to increased intestinal cell migration. These data also suggest that phosphorylation of villin and its association with PLC-γ1 may not be growth factor specific. Since both EGF and HGF resulted in tyrosine phosphorylation of villin and association of phospho-villin with PLC-γ1, we elected to limit our study henceforth to the use of EGF.

To establish a direct role for villin in cell migration and to examine the molecular mechanism of villin-induced cell migration, an obvious approach would be to knock down villin in an intestinal cell line. Caco-2 cells express a high level of villin endogenously. However, suppression of villin expression in Caco-2 cells impairs brush border assembly and alters cell morphology (9). Therefore, we reasoned that overexpression of a phosphorylation site mutant of villin (lacking all 10 identified phosphorylatable tyrosine residues) in confluent monolayers of Caco-2 cells should have a dominant negative effect, thus allowing us to determine the role of villin in intestinal cell migration (43). Furthermore, such an approach would have the added advantage of allowing us to examine the cellular and molecular mechanisms of phosphorylated villin in cell migration. Hence, to document a direct connection among tyrosine phosphorylation of villin, its association with PLC-γ1, and increased cell migration, we overexpressed the phosphorylation site mutant of villin (VIL/AIFM) in differentiated Caco-2 cells. Two-week postconfluence Caco-2 cells were infected with the adenovirus-expressing vector alone or VIL/AIFM. The mutant villin protein was expressed fused to the influenza A virus HA tag, which allowed us to follow the expression of the mutant villin protein relative to the endogenous villin protein in Caco-2 cells (Fig. 2A). Expression of VIL/AIFM or vector alone had no effect on the brush border or Caco-2 cell morphology (data not shown). Overexpression of VIL/AIFM had a dominant negative effect over the endogenous villin protein and significantly inhibited both basal and growth factor-induced Caco-2 cell migration (P < 0.001, n = 12, Fig. 2B). Expression of VIL/AIFM also decreased the total pool of tyrosine-phosphorylated villin (50% compared with Caco-2 cells infected with vector alone, P < 0.01, n = 3) as well as the pool of phospho-villin associated with PLC-γ1 (58% compared
Fig. 1. Association of villin with phospholipase C-γ₁ (PLC-γ₁) in response to wounding or growth factor treatment. A: Madin-Darby canine kidney (MDCK) Tet-Off cells were cotransfected with enhanced green fluorescent protein (EGFP)-tagged PLC-γ₁ mutant protein (GFP-γ₁SA; a) and DsRed2-tagged villin (b). Images show the signal in untreated cells in the donor, EGFP channel (a) and in the acceptor, DsRed2 channel (b), and the corrected fluorescence resonance energy transfer (FRET) signal (c). B: positive FRET signal 5 min after the addition of epidermal growth factor (EGF; 100 ng/ml). Images show the signal in the EGFP channel (a) and in the DsRed2 channel (b) and the corrected FRET signal (c). The corrected FRET image is shown in pseudocolor, and the color scale shows the relationship between color and pixel value. Image is representative of 7 other cells that were analyzed with similar results. Bar, 5 μm. C: spatiotemporal dynamics of villin and PLC-γ₁ redistribution following EGF treatment. Images show the signal in the EGFP channel (PLC-γ₁) before (a) and after EGF treatment (c) and in the DsRed2 channel (villin) before (b) and after EGF treatment (d). Arrowheads indicate distribution of both villin and PLC-γ₁ to the developing lamellipodia following EGF treatment. Bar, 5 μm. D: MDCK Tet-Off cells described in A–C were used to coimmunoprecipitate PLC-γ₁ and tyrosine-phosphorylated villin [using phospho-villin antibody VP-70782, described previously (43)]. Tyrosine-phosphorylated villin and PLC-γ₁ coimmunoprecipitate from cells treated with EGF (+) but not from untreated cells (−). This blot is representative of 3 other experiments with similar results. IP, immunoprecipitate; IB, immunoblot. E: 2-wk-old postconfluent Caco-2 cells were denuded with a sharp blade, and migration of the remaining cells into the wound was measured as distance migrated from the original wound at 24 h postwounding. Cell migration was measured in the absence or presence of EGF (10 ng/ml) or hepatocyte growth factor (HGF; 50 ng/ml). Data were normalized to control values (set as 100) and expressed as percent change in migration compared with control. Control refers to cell migration at 24 h postwounding in the absence of growth factors. Values are means ± SE (n = 24). *P < 0.001, statistically significant compared with control cells. F: Triton-soluble cell extracts from unwounded monolayers in the absence of growth factors (control), wounded monolayers in the absence of growth factors (wounded), or wounded monolayers treated with EGF or HGF were used to immunoprecipitate tyrosine-phosphorylated villin (using phospho-villin antibody VP-70782) and PLC-γ₁. Immunoprecipitated proteins were probed with monoclonal phospho-tyrosine antibody PY-20 or phospho-villin polyclonal antibody VP-70782. Western blots are representative of 5 other experiments with similar results.
with Caco-2 cells infected with vector alone, \( P < 0.01, n = 3 \) in migrating Caco-2 cells (Fig. 2C). Complete inhibition of villin phosphorylation and formation of villin-PLC-\( \gamma_1 \) could not be achieved in Caco-2 cells, since the endogenous expression of both PLC-\( \gamma_1 \) and villin is very high in this cell line.

Since the villin mutant VIL/AYFM could not completely abolish the effects of endogenous villin in Caco-2 cells, we elected to confirm these observations by ectopically expressing wild-type (VIL/FL) and phosphorylation site mutant of villin (VIL/AYFM) in MDCK Tet-Off cells. Unlike Caco-2, MDCK cells are nontransformed epithelial cells. MDCK cells have a well-developed brush border and exhibit many properties of a polarized monolayer but do not express villin. Stable transfection of VIL/FL increased MDCK cell migration (Fig. 3, A and B).

In contrast, MDCK Tet-Off cells stably transfected with VIL/AYFM migrated like villin-null cells. VIL/FL was tyrosine phosphorylated and associated with PLC-\( \gamma_1 \) in wounded monolayers of MDCK cells treated with EGF (Fig. 3C). Likewise, villin was tyrosine phosphorylated and associated with PLC-\( \gamma_1 \) in wounded monolayers in the absence of EGF (data not shown). Growth factor treatment enhanced MDCK cell migration, and expression of VIL/FL in these cells further augmented the effect of EGF on cell migration. This is consistent with our previous report that expression of villin is sufficient to enhance cell migration and that villin increases EGF-induced cell motility, thus acting as a regulator of EGF-stimulated cell migration (43). The villin mutant VIL/AYFM had no effect on cell migration, suggesting that the increase in cell migration observed with VIL/FL requires phosphorylatable tyrosine residues in villin. A similar observation was made in IEC-6 cells transfected with full-length villin (data not shown). Together these studies demonstrate, first, that villin regulates epithelial cell migration, and second, that tyrosine phosphorylation of villin and its association with PLC-\( \gamma_1 \) correlates with villin’s effects on cell migration.

Tyrosine phosphorylation by Src kinase regulates the villin-PLC-\( \gamma_1 \) complex formation. To identify the specific tyrosine kinase(s) that regulates villin-PLC-\( \gamma_1 \) complex formation, we first characterized the tyrosine kinase regulating Caco-2 cell migration by measuring cell migration in the absence or presence of the Src family kinase inhibitor PP2 and its inactive analog, PP3. This choice was based on previous in vitro studies in our laboratory (36, 47) where we determined that in vitro villin can be tyrosine phosphorylated by c-Src kinase. As shown in Fig. 4A, PP2 inhibited basal cell migration (43% at 24 h postwounding compared with untreated control cells, \( P < 0.01, n = 24 \)). PP2 also significantly inhibited EGF-stimulated Caco-2 cell migration (\( \sim 30\% \) at 24 h postwounding compared with EGF-treated cells, \( P < 0.001, n = 24 \)). PP3 had little effect on basal or EGF-stimulated Caco-2 cell migration. Biochemical analysis revealed that inhibition of Caco-2 cell migration by PP2 was accompanied by inhibition of villin phosphorylation as well as inhibition of phospho-villin-PLC-\( \gamma_1 \).
complex formation (Fig. 4B). These data demonstrate that activation of Src family tyrosine kinase(s) phosphorylates villin in Caco-2 cells and is required for the association of villin with PLC-γ₁. It may be noted that although tyrosine kinase inhibition has been previously implicated in the regulation of both basal and EGF-stimulated intestinal cell migration, neither the kinase(s) nor the phosphorylated substrates involved were identified (2).

To further validate these data, we used a dominant negative construct of c-Src (Ad-DN-c-Src). Caco-2 cells were infected with control vector or with Ad-DN-c-Src, and both the formation of villin-PLC-γ₁ complex formation and cell migration were measured. The expression of dominant negative c-Src in Caco-2 cells was determined by Western blot analysis using a c-Src monoclonal antibody (Fig. 4C). A lower band of ~57 kDa was seen in the Western blots and determined to be a degradation fragment of c-Src. Overexpression of dominant negative c-Src inhibited the formation of phospho-villin-PLC-γ₁ complex. (Fig. 4C). These studies confirm the role of Src kinase in the association of villin with PLC-γ₁. As shown in Fig. 4D, dominant negative c-Src also inhibited both basal and growth factor-stimulated cell migration (an average of 46% compared with untreated control cells and 70% compared with EGF-treated cells, P < 0.001, n = 12). Together, these data demonstrate that villin’s ability to associate with PLC-γ₁ requires the activation of Src kinase. The effect of dominant negative c-Src on c-Yes and/or c-Fyn, two other tyrosine kinases of the Src family that are also expressed in Caco-2 cells, cannot be ruled out. Furthermore, although these data do not allow us to determine whether the effects of c-Src on villin phosphorylation are direct, in previous studies (36, 47) our group has demonstrated that in vitro villin is a substrate of c-Src kinase. Based on these previous reports, together with the data presented in this study, we suggest that villin may be phosphorylated by c-Src kinase, thus regulating both villin’s association with PLC-γ₁ and the villin-induced increase in cell migration.

PLC-γ₁ is required for villin-induced increase in cell migration. In previous experiments, by disrupting villin phosphorylation by PP2, overexpression of dominant negative c-Src, and expression of VIL/AYFM, we have demonstrated that tyrosine phosphorylation of villin is required for its association with PLC-γ₁ and, furthermore, that this association correlates with enhanced cell motility. To characterize the role of PLC-γ₁ in villin-induced cell migration, we performed the following experiments. Caco-2 cell migration was measured in the absence or presence of the PLC-γ₁-specific inhibitor U-73122 and its inactive analog, U-73343. In the presence of 5 μM U-73122, basal cell migration was significantly reduced (~51% compared with untreated control cells, P < 0.001, n = 24), whereas cells treated with U-73343 behaved like control cells (Fig. 5A). EGF-induced Caco-2 cell migration was likewise inhibited by U-73122 but not by U-73343 (65% compared with EGF treated cells, P < 0.001, n = 24). These data suggest that PLC-γ₁ activation regulates both basal and EGF-stimulated Caco-2 cell migration, indicating that the interaction of tyrosine-phosphorylated villin with PLC-γ₁ may be functionally significant in intestinal cell migration.

To obtain direct proof for the requirement for PLC-γ₁ in villin-induced cell migration, we elected to use two strategies: 1) downregulate endogenous PLC-γ₁, and 2) use PLC-γ₁−/− cells. Transfection of PLC-γ₁ siRNA successfully reduced the expression of endogenous PLC-γ₁ in MDCK Tet-Off cells expressing VIL/FL (98%, n = 6, P > 0.001, Fig. 5B). In the absence of significant endogenous PLC-γ₁, villin-induced cell migration was inhibited with cells expressing control siRNA in the absence or presence of EGF (n = 12, P < 0.001, Fig. 5C). A similar observation was made in HeLa Tet-Off cells expressing full-length villin (data not shown). Together, these data validate our observation that villin’s function in cell migration requires PLC-γ₁ and that this effect is also not cell type specific. To further substantiate these data, we used PLC-γ₁−/− cells to examine villin-induced cell migration. PLC-γ₁−/− null embryos were used to generate immortalized fibroblasts genetically deficient in PLC-γ₁, and retroviral infection of these cells was used to derive PLC-γ₁−/− expressing cells (Fig. 6A) as described previously (22, 24). Both cell lines were infected with adenoviral constructs to express either wild-type (VIL/FL) or phosphorylation site mutant (VIL/AYFM) villin proteins (Fig. 6B). Expression of
villin in PLC-γ1−/− cells did not result in enhanced cell migration in the absence or presence of EGF (Fig. 6C). In contrast, expression of VIL/FL in PLC-γ1+/+ cells significantly (compared with PLC-γ1+/+ cells infected with vector alone, P < 0.01, n = 12) enhanced cell migration in the absence and presence of EGF (Fig. 6C). Cells expressing VIL/AyFM migrated like PLC-γ1−/− cells and PLC-γ1+/+ cells in the absence of villin. Expression of villin in PLC-γ1−/− cells had no effect on the tyrosine phosphorylation of villin (Fig. 6D). These data demonstrate that villin does not require PLC-γ1 for its tyrosine phosphorylation. Absence of PLC-γ1 also had no effect on the intracellular distribution of villin at or near the cell surface (Fig. 6E). These data confirm our previous observations that tyrosine phosphorylation of villin determines its intracellular distribution at the cell surface (43). PLC-γ1+/+ cells migrate faster in the presence of VIL/F1L, and tyrosine-phosphorylated villin forms a complex with PLC-γ1 in these cells in the absence or presence of EGF (Fig. 6F). It may be noted that inhibition of PLC by U-73122 has been shown to inhibit the activity of the actin regulatory protein coflin and to delay the initiation of protrusions (35). Thus, like coflin, villin’s function in cell migration requires PLC-γ1. Together, these data demonstrate that tyrosine phosphorylation of villin alone is not sufficient for villin-induced cell migration and that phosphovillin-stimulated cell migration requires PLC-γ1.

Several studies have demonstrated that increased cell migration does not always imply increased invasiveness (21, 41). Hence, we elected to examine the role of tyrosine-phosphorylated villin and PLC-γ1 in transmembrane migration of cells through Matrigel. PLC-γ1+/+ cells expressing VIL/F1L were more invasive than PLC-γ1−/− cells expressing VIL/FL (Fig. 7). Thus expression of wild-type villin in PLC-γ1−/− cells enhanced cell invasion. Expression of VIL/AyFM in PLC-γ1−/− cells enhanced cell invasion compared with its expression in PLC-γ1−/− cells (P < 0.05). However, the number of VIL/AyFM expressing PLC-γ1+/+ cells invading through the Matrigel was still threefold less than that of PLC-γ1+/+ cells expressing VIL/FL. These data demonstrate that tyrosine phosphorylation of villin and association of phospho-villin with PLC-γ1 correlate with enhanced cell invasion. Together, these studies provide convincing evidence for the absolute requirement for PLC-γ1 and tyrosine-phosphorylated villin for villin’s function in cell migration and cell invasion. Inhibition of the downstream effectors of PLC-γ1 activation, namely, protein kinase C (PKC) and intracellular Ca2+, inhibited Caco-2 cell migration but had no effect on either the tyrosine phosphorylation of villin or its association with PLC-γ1, suggesting that these events occur upstream of PLC-γ1 activation and are independent of PKC and Ca2+-dependent pathways (see Supplemental Fig. 1).
(The online version of this article contains supplemental data.)

DISCUSSION

In this study we have described how cell surface receptor activation is communicated to actin-binding proteins, thus regulating cell function. Specifically, in this report we have demonstrated that villin is tyrosine phosphorylated by both EGF and HGF and that tyrosine phosphorylation of villin recruits PLC-γ1 as a binding partner. The phosphorylation of villin and its association with PLC-γ1 was neither growth factor (seen with both EGF and HGF) nor cell type specific, suggesting that this function may be well conserved in all epithelial cells that express villin. Although our group has previously demonstrated in vitro (27, 36), using recombinant PLC-γ1 protein and pull-down assays as well as coimmunoprecipitation from rabbit intestinal cell extracts, that tyrosine phosphorylated villin can associate with PLC-γ1, what was not known was whether this association was direct and whether this association had any biological relevance. In this study using FRET analysis, we have demonstrated for the first time the direct interaction of villin with PLC-γ1 in living cells. The Förster distance, $R_o$, which is the donor acceptor distance at which FRET is 50% efficient for DsRed/EGFP, is 57.6 Å (37). Thus a positive FRET signal with EGFP-PLC-γ1 and DsRed2-villin suggests that the two proteins must come close enough at a distance of <100 Å. These studies also identified the domain in PLC-γ1 that determines its association with villin, namely, the SH2 domain of PLC-γ1. More importantly, examining the interaction of villin with PLC-γ1 in living cells allowed us to determine that the two proteins interact at the plasma membrane in response to receptor activation.

With the use of fibroblasts genetically deficient in PLC-γ1, it has been determined that there is no absolute requirement for PLC-γ1 for cell adhesion, cell spreading, or cell migration but, rather, that PLC-γ1 facilitates these cell functions (23, 44), thus suggesting that other proteins/factors participate with PLC-γ1 in the regulation of these cell functions. In this study, we report that PLC-γ1 interacts with villin and that both PLC-γ1 and villin regulate intestinal and renal epithelial cell migration. Inhibition of villin phosphorylation by either the Src family kinase inhibitor PP2 or dominant negative c-Src inhibited tyrosine phosphorylation of villin, its association with PLC-γ1, and cell migration. The role of tyrosine phosphorylation in these events was confirmed by overexpression of a phosphorylation site mutant of villin, VIL/AYFM. Ectopic expression of this mutant in MDCK cells had no effect on cell migration, whereas overexpression of this mutant in Caco-2 cells (which also endogenously express villin) had a dominant negative effect, inhibiting both basal and EGF-stimulated cell migration. To demonstrate that PLC-γ1 was required for villin’s effects on cell migration, we used siRNA to downregulate endogenous PLC-γ1 as well as a PLC-γ1-deficient cell line derived from PLC-γ1 null embryos. PLC-γ1 was not required for either the

Fig. 5. Activation of PLC-γ1 is essential for basal and growth factor-stimulated epithelial cell migration. A: both control and EGF-stimulated migrating cells were treated with the PLC-γ1-specific inhibitor U-73122 (5 μM) or its inactive analog, U-73343 (5 μM). Inhibition of PLC-γ1 with U-73122 inhibited both basal and EGF-induced cell migration. The inactive analog U-73343 had no effect. Data were normalized to control values (set as 100) and expressed as percent change in migration compared with control. Control refers to cell migration at 24 h postwounding in the absence of growth factors or inhibitors. Values are means ± SE. *P < 0.05, statistically significant compared with control cells; †P < 0.05, statistically significant compared with EGF-treated cells. B: downregulation of endogenous PLC-γ1 inhibits villin-induced cell migration. Approximately 70% confluent MDCK Tet-Off cells expressing wild-type villin were transiently transfected with PLC-γ1 small interference RNA (siRNA) or a control oligonucleotide (Cont; 200 nM). At 48 h posttransfection, cells were examined for PLC-γ1 expression. Blots show >90% gene silencing with PLC-γ1 siRNA, whereas the control oligonucleotide had no effect on PLC-γ1 expression, in MDCK cells treated with or without EGF. Also shown are quantitative Western blots of cell extracts probed with monoclonal antibodies to villin and actin. C: cell migration was measured in MDCK cells expressing VIL/FL 48 h posttransfection with PLC-γ1 or control siRNA in the absence or presence of EGF. Downregulation of PLC-γ1 significantly inhibited villin-induced cell migration in the absence ($P < 0.001$) or presence ($P < 0.001$) of EGF. Data were normalized to control values (set as 100) and expressed as percent change compared with control. Control refers to cell migration 7 h postwounding in MDCK Tet-Off cells expressing VIL/FL transfected with control siRNA. Values are means ± SE (n = 12).
tyrosine phosphorylation of villin or its intracellular distribution in cells, but it was required for villin’s function in cell migration. Together, these studies demonstrate that villin participates in responding to extracellular stimuli together with PLC-γ1, thus serving to integrate and transduce signals involved in cell migration. In epithelial cells, villin may provide the connection between PLC activation and EGF-stimulated chemotaxis/cell motility, which may be fulfilled by related proteins in other cells (7).

Expression and activation of PLC-γ1 in rat intestine is the greatest during weaning, which is characterized by the most significant changes in intestinal morphology and a period of maximum migration of intestinal cells along the crypt-villus axis (28, 40). Villin also appears as an early marker of the endodermal cell lineage and a marker of cells arising from mesenchymal/epithelial conversion in the developing intestine and kidney (4, 32). Thus villin and PLC-γ1 expression are maintained during periods of most substantial cell migration. It
may be noted that villin expression is also maintained during tumorigenesis independent of the morphological differentiation of the tumor cells (42). In this study we have demonstrated that overexpression of the villin phosphorylation site mutant VIL/AYFM significantly retards both basal and EGF-stimulated cell migration in a colon adenocarcinoma cell line. Likewise, we have demonstrated that cells expressing villin migrate faster following EGF treatment compared with cells that do not express villin, thus suggesting that in epithelial cells, villin is recruited to the lamellipodia to enhance basal and growth factor-stimulated cell migration. On the basis of these observations, we suggest that villin-induced cell migration/cell invasion could play a significant role during embryogenesis in restitution as well as in the dissemination of metastatic tumor cells.

The defining event in cell motility is rapid actin polymerization and extension of the lamellipodia in the direction of cell movement. Actin polymerization can be initiated by severing or uncapping of preexisting actin filaments (8, 49). Villin is required for both actin severing and cell migration (15, 43). However, the molecular mechanisms that regulate either the actin-severing function of villin or villin’s function in cell migration are not very well understood. Work in our laboratory (30, 43, 47, 48) has previously demonstrated that tyrosine phosphorylation of villin is essential to both its functions, namely, actin severing as well as cell migration. In this report, we extend our previous studies to demonstrate that the ligand-binding properties of tyrosine-phosphorylated villin, specifically its interaction with PLC-γ1, is also essential to villin’s role in cell migration. Together with data from our previous studies, data presented in this report demonstrate that tyrosine phosphorylation of villin allows the redistribution of villin to the cell surface, where it can form a complex by interacting with the SH2 domain of PLC-γ1 (43). This interaction of tyrosine phosphorylated villin with PLC-γ1 is required for villin’s function in cell migration.

Villin binds phosphatidylinositol 4,5-bisphosphate (PIP2) with a high binding affinity ($K_d = 39.4 \mu M$) compared with the reported binding affinity of PLC-γ1 for PIP2 ($K_d = 1 \text{mM}$) (25, 30). Thus, by virtue of its ability to bind PIP2, villin could regulate the PLC-γ1 catalytic activity. Consistent with this idea, our group (36) has previously demonstrated that in vitro villin can regulate the catalytic activity of PLC-γ1 by sequestering its substrate PIP2. Furthermore, we determined that inhibition of PLC-γ1 catalytic activity by villin could be reversed by tyrosine phosphorylation of villin, which prevents villin’s association with PIP2 (36). Thus tyrosine phosphorylation of villin and its association with PLC-γ1 regulate the catalytic activity of PLC by modifying villin’s ability to sequester PIP2. Tyrosine phosphorylation of villin prevents villin from associating with PIP2, which vacates
the F-actin binding sites in villin (30, 36). In addition, tyrosine phosphorylation of villin per se enhances its actin-severing activity (29, 48). Thus phosphorylation of villin correlates with increased actin severing by villin. On the basis of these observations and previous studies done with cofilin, we speculate increased actin severing by villin. On the basis of these observations and previous studies done with cofilin, we speculate increased actin severing by villin. On the basis of these observations and previous studies done with cofilin, we speculate increased actin severing by villin. On the basis of these observations and previous studies done with cofilin, we speculate increased actin severing by villin.

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