# PKCδ and cofilin activation affects peripheral actin reorganization and cell-cell contact in cells expressing integrin α5 but not its tailless mutant

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## Summary

**Integrin-mediated cell adhesion transduces signaling activities for actin reorganization, which is crucially involved in cellular function and architectural integrity. In this study, we explored the possibility of whether cell-cell contacts might be regulated via integrin-α5β1-mediated actin reorganization. Ectopic expression of integrin α5 in integrin-α5-null intestinal epithelial cells resulted in facilitated retraction, cell-cell contact loss, and wound healing depending on Src and PI3K (phosphoinositide 3-kinase) activities by a reagent that affects actin organization. However, cytoplasmic tailless integrin α5 (hereafter referred to as α5/1) expression caused no such effects but rather sustained peripheral actin fibers, regardless of Src and PI3K signaling activities. Furthermore, integrin α5 engagement with fibronectin phosphorylated Ser643 of PKCδ, upstream of FAK and Src and at a transmodulatory loop with PI3K/Akt. Pharmacological PKCδ inactivation, dominant-negative PKCδ adenovirus or inactive cofilin phosphatase (SSH1L mutant) retrovirus infection of α5-expressing cells sustained peripheral actin organization and blocked the actin reorganizing-mediated loss of cell-cell contacts. Meanwhile, wild-type PKCδ expression sensitized α5/1-expressing cells to the actin disruptor to induce cell scattering. Altogether, these observations indicate that integrin α5, but not α5/1, mediates PKCδ phosphorylation and cofilin dephosphorylation, which in turn modulate peripheral actin organization presumably leading to an efficient regulation of cell-cell contact and migration.**

Key words: PKCδ, Cofilin, Actin organization, Cell contacts, Integrin

## Introduction

Epithelial monolayer integrity is crucial for the function and homeostasis of epithelia. The epithelial monolayer is maintained by cell-cell contacts between adjacent cells through homophilic interactions between adhesion molecules including E-cadherin and others, and cell adhesions via integrin engagements to ECM proteins within basement membranes (Thiery, 2003). Cell-cell contact (i.e. tight and adherens junctions) and cell adhesion sites (i.e. focal adhesions) are linked to intracellular actin filaments through diverse protein-protein interactions. Therefore, epithelial monolayer integrity and intracellular actin organization influence each other bi-directionally. That is, disruption of cell contacts and/or adhesions can cause alterations in actin organization, and conversely aberrant actin organization can cause changes in cell contacts and/or adhesions. Disruption of this monolayer integrity can cause not only the functional impairment of normal epithelium but also the dissemination of cancerous cells from a primary tumor cell body during the early stages of metastasis (Hirohashi and Kanai, 2003; Thiery, 2002). However, the mutual regulatory linkages between cell-cell contacts and cell adhesions are largely unknown, but mechanistic investigations on the regulatory linkages between cell-cell contacts and cell adhesions are being expanded (Boyer et al., 2000).

Integrins, a group of cell adhesion receptors, are composed of an α and a β subunit. The integrins participate in the activation of diverse intracellular signaling molecules and in the reorganization of actin filaments (Brakebusch and Fassler, 2003; Carragher et al., 2003; Juliano et al., 2004). This participation is accomplished through direct signaling via integrin-mediated engagements of cells to ECM proteins, and by indirect signaling in collaboration with other membrane receptors (e.g. growth factor receptors, G-protein-coupled receptors or cytokine receptors) (Bhowmick et al., 2001; Eliceiri, 2001; Lee et al., 2004; Short et al., 2000; Yamada and Even-Ram, 2002). Interaction between integrins and ECMs at focal adhesions causes the clustering of integrins and the recruitment of signaling molecules and actin filaments to integrin cytoplasmic tails, and leads to the activation of integrin-mediated intracellular signal transduction (Hynes, 2002). Diverse intracellular signaling molecules, including focal adhesion kinase (FAK), Src family kinase (SFK), Erk, Akt/protein kinase B (PKB) and Rho GTPase family members including RhoA, Rac1 and CDC42 can be activated by integrin-mediated cell adhesion (Gilcrease, 2007; Juliano,

2002). Rho GTPase-mediated reorganization of actin filaments can involve their downstream effector molecules, which include LIMK, cofilin, MLCK and ROCK that affect actin polymerization and myosin light chain (MLC) phosphorylation-mediated intracellular contractility (Schmitz et al., 2000). Cofilin is known to sever actin filaments when it is dephosphorylated (Galkin et al., 2003). Cofilin Ser3 is dephosphorylated by Slingshot phosphatase (SSH) or chronophin (Huang et al., 2006; Ohta et al., 2003). These important molecules have been shown to regulate morphological changes via actin reorganization in diverse cell types.

Meanwhile, cell-cell contacts in epithelium involve homophilic interactions between E-cadherin at adherence junctions, claudin or occludin at tight junctions, or desmocollin or desmoglein at desmosomes (Thiery, 2003). These adhesion molecules recruit adaptors or signaling molecules at their cytoplasmic tails, so that the protein complexes are formed for their connections to actin or intermediate filaments (Weis and Nelson, 2006). Cell-cell contact loss can be caused by dramatic actin reorganization even without E-cadherin suppression or downregulation, however, the mechanistic aspects of the process remain largely unknown. Integrins are known to reorganize actin filaments, as explained above, but the contribution made by integrin-ECM engagement to the regulation of cell-cell contacts is not well understood. It was previously shown that Fer tyrosine kinase and Rab1 are involved in cross-talk between cell-cell contacts and focal adhesions (Balzac et al., 2005; Retta et al., 2006).

In this study, we investigated the significance of integrin-signaling-mediated actin organization in regulation of cell-cell contacts. Normal rat intestinal epithelial cells ectopically expressing wild-type (WT) or tailless (i.e. lacking for the cytoplasmic tail of the C-terminal 27 amino acids, including GFFKR residues) integrin α5 were used to define the integrin signaling. We observed that α5-expressing cells dynamically showed loss of cell-cell contacts, when they were treated with an actin-affecting reagent, whereas α5/1-expressing cells sustained peripheral actin bundles, such that the reagent-mediated cell-cell contact loss was not observed. Furthermore, the inactivation of PKCδ or cofilin caused persistent peripheral actin organization, and thus blocked the cell-cell contact loss of α5-expressing cells. These observations indicate that a reagent-mediated actin-reorganization leads to loss of cell-cell contact, through integrin α5, PKCδ phosphorylation-dependent and cofilin dephosphorylation-dependent peripheral actin reorganization.

## Results

### Actin-reorganization-mediated loss of cell-cell contacts in RIE1 cells expressing integrin α5 but not its tailless mutant

To study how integrin signaling may regulate cell-cell contacts, we utilized stable cell lines ectopically expressing wild-type (WT) or mutant integrin α5 (RIE1-α5 or RIE1-α5/1, respectively). The mutant integrin α5 (α5/1) lacks the C-terminal 27 amino acids of the cytoplasmic tail, leaving one amino acid proximal to the transmembrane domain. RIE1-α5 and RIE1-α5/1 cells generally showed polygonal epithelial morphologies with cell-cell contacts (Fig. 1A). The comparable expressions of the intact or tailless α5 integrin

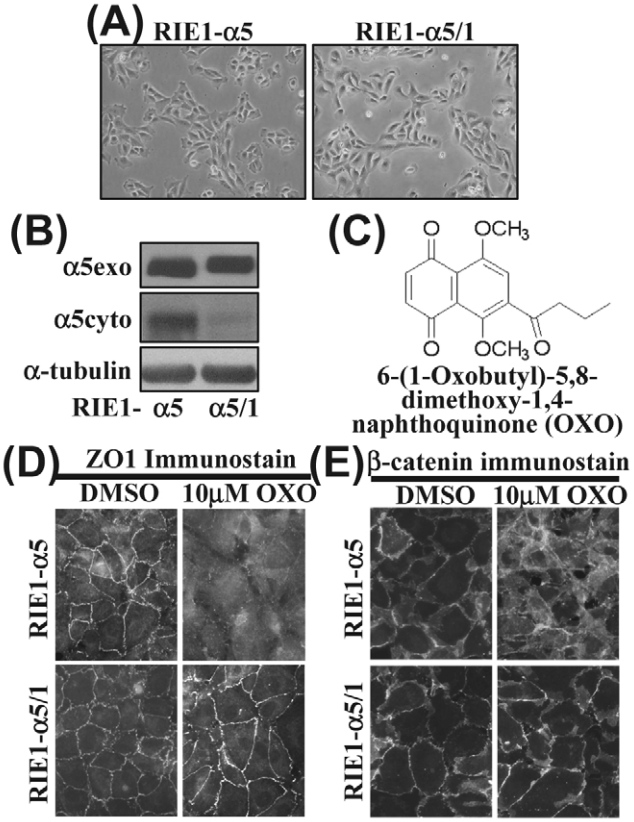


**Fig. 1.** Cell-cell contact loss of RIE1-α5, but not RIE1-α5/1, cells after OXO treatment. (A) Stable cell lines ectopically expressing integrin α5 (RIE1-α5) or tailless α5 (RIE1-α5/1) were maintained, as described in Materials and Methods. Images for subconfluent cells in normal culture media were taken using a microscope equipped with a digital camera. Both cell lines generally form colonies. (B) Whole cell lysates from subconfluent cells were prepared for immunoblotting using an antibody against an extracellular region (α5exo) or the cytoplasmic tail (α5cyto) of the human integrin α5 subunit, or α-tubulin. Data shown represent at least three independent experiments. (C) Chemical structure of 6-(1-oxobutyl)-5,8-dimethoxy-1,4-naphthoquinone (OXO). (D,E) Cells were seeded onto 10% FBS-DMEM-H-coated glass coverslips. Once confluent monolayers had been formed by incubation in 5% $CO_2$ at 37°C, cells were treated with either vehicle (DMSO) or OXO (10 μM) for 24 hours, prior to being immunostained for ZO1 (D) or β-catenin (E). Data shown are representative of three different experiments.

subunit in these cells were confirmed by immunoblotting (using anti-integrin α5 antibody recognizing an extracellular region or the cytoplasmic tail; Fig. 1B). The surface expression of both these subunits was confirmed by FACS analysis (data not shown), as shown previously (Lee and Juliano, 2000). The stable cells also showed similar growth rates (i.e. doubling times of 18±2 hours). Since, (1) we failed to find any growth factors (including 100 ng/ml hepatocyte growth factor) or cytokines capable of causing RIE1-α5 cells to scatter and (2) cell scattering involves cell-cell contact disruption, presumably via actin-reorganization-based morphological changes, we investigated if a reagent-mediated actin reorganization might cause cell-cell contact loss in cells with integrin α5 WT or its tailless mutant expression. A putative anti-tumorigenic reagent (Lee et al., 2007), 6-(1-oxobutyl)-5,8-dimethoxy-1,4-

naphthoquinone (OXO; Fig. 1C), was used because we have found it effective as an actin-disrupting reagent (see below). When RIE1 cells were treated with OXO at 10 μM, cells grew well with no significant change in doubling time (data not shown). However, treatment of RIE1-α5 cells with OXO (at 10 μM for 24 hours) caused loss of β-catenin and ZO1 from cell-cell contact sites, although this did not occur in RIE1-α5/1 cells (Fig. 1D,E). These observations indicate that OXO treatment caused a loss of cell-cell contacts in RIE1 cells with intact integrin α5, but not those with tailless integrin α5. Unfortunately, we were not able to immunostain for E-cadherin, despite using three different commercial anti-E-cadherin antibodies and diverse protocols including different fixation methods, probably because endogenous E-cadherin levels were not high enough to be sensitized by the antibodies during the immunofluorescent staining approaches. However, its expression was detected by immunoblotting and found to be unchanged by OXO (data not shown).

We next examined whether OXO treatment induced α-smooth muscle actin (SMA, a marker for mesenchymal cell types) in RIE1-α5, but not in RIE1-α5/1, cells. As was expected, SMA expression was enhanced only in RIE1-α5 cells by OXO treatment (Fig. 2A), indicating that OXO-mediated EMT was integrin α5 dependent. The activities of signaling molecules in cells left untreated or treated with OXO were then examined to explore the molecular basis underlying OXO-mediated EMT in RIE1-α5, but not in RIE1-α5/1, cells.

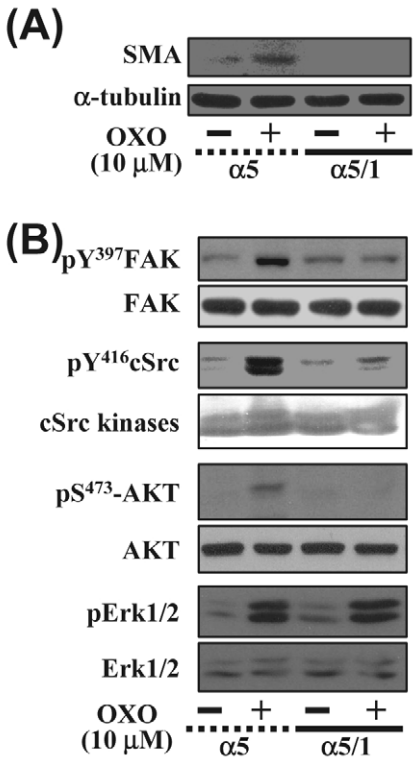


**Fig. 2.** Signal activity specifically impaired in RIE1-α5/1 cells by OXO treatment. (A,B) Subconfluent (70~80% confluent) cells in 60 mm culture dishes were treated with either DMSO or 10 μM OXO for 24 hours. Protein amounts in whole cell lysates were normalized before standard western blotting for the indicated molecules. Data shown are representative of three independent experiments.

Whereas RIE1-α5 cells showed enhanced activities of FAK, SFK and Akt/PKB after OXO treatment, RIE1-α5/1 cells did not (Fig. 2B). However, Erk1/2 activity was increased by OXO in both cell lines, which is consistent with the comparable growth rates of these cell lines, indicating a certain specificity of integrin signaling impairment in RIE1-α5/1 cells (Fig. 2B).

Since SFK and phosphoinositide 3-kinase (PI3K)/Akt are known to be important for EMT (Avizienyte and Frame, 2005), and their phosphorylations were increased by OXO treatment that also caused loss of cell-cell contacts (Fig. 2B), we next performed pharmacological inhibitor studies to determine if SFK or PI3K/Akt activity was involved in OXO-mediated EMT in RIE1-α5 cells. Cells on coverslips precoated with normal culture medium were pretreated with DMSO, LY294002 (a specific PI3K inhibitor which consequently causes Akt/PKB inhibition), PP2 (a specific SFK inhibitor, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine), or PP3 (a negative control for PP2, 4-amino-7-phenylpyrazol[3,4-*d*]pyrimidine), 30 minutes before 10 μM OXO treatment for 24 hours. Cell-cell contacts were then analyzed by immunostaining of ZO1 at contact sites. OXO-mediated RIE1-α5 cell-cell contact loss was abolished by the pharmacological inhibition of SFK or PI3K/Akt, but not by PP3 treatment, whereas RIE1-α5/1 cell-cell contact was maintained regardless of the reagent treatments (Fig. 3).

## Persistent actin organization at RIE1-α5/1 cell peripheries

It was considered that OXO-mediated cell-cell contact loss in RIE1-α5, but not in RIE1-α5/1, cells might involve OXO-mediated differential actin reorganization dependent on intact integrin α5. To test this possibility, we treated cells with OXO at a higher concentration (30 μM) prior to actin staining. RIE1-α5 cells showed morphological retraction after treatment with 30 μM OXO for 30 minutes, whereas RIE1-α5/1 cells showed no significant changes (Fig. 4A). Since OXO is a naphthoquinone compound, which are known to cause cytoskeletal alterations via the generation of reactive oxygen species (ROS) (Bellomo et al., 1990; Mirabelli et al., 1989), we examined the possibility that OXO might generate ROS. However, ROS were not found to be generated in RIE1 cells by OXO treatment (at 10 μM for 30 minutes or 24 hours), whereas a positive control of $H_2O_2$ treatment showed effective ROS generation (Fig. 4B and data not shown). Since cofilin is known to sever actin filaments when it is dephosphorylated (Galkin et al., 2003), the effects of OXO treatment on cofilin Ser3 phosphorylation (i.e. $pS^3$cofilin) were then examined in both cell lines. Consistent with the observed effects of pharmacological inhibitors on OXO-mediated cell-cell contact loss, inhibition of SFK or PI3K/Akt blocked OXO-mediated $pS^3$cofilin decrease in RIE1-α5 cells. This suggests that OXO-mediated actin filament disruption cause the observed contact loss, which could be blocked by additional SFK or PI3K/Akt preinhibition. However, basal and OXO-mediated $pS^3$cofilin levels in RIE1-α5/1 cells were much higher than those in RIE1-α5 cells, and unchanged regardless of pharmacological inhibitions (Fig. 4C). Another well-known actin-disrupting reagent cytochalasin D, which is much more potent than OXO, was examined to determine if RIE1-α5/1 cells could be less susceptible to actin disruption. As was expected, RIE1-α5 cells showed barely detectable basal and cytochalasin-D-mediated

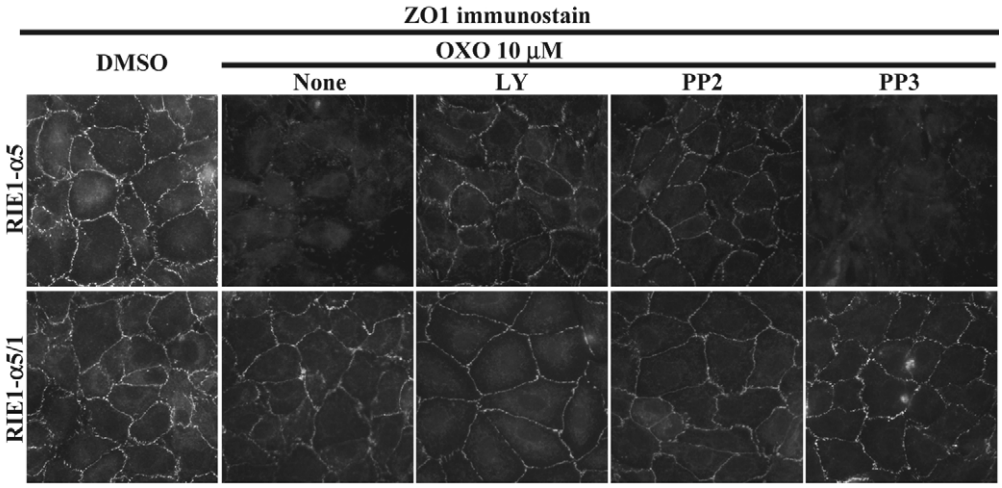


**Fig. 3.** Inhibition of PI3K and SFK blocked OXO-mediated scattering of RIE1-α5 cells, but did not affect cell-cell contacts between RIE1-α5/1 cells. Cells were seeded onto 10% FBS-DMEM-H-coated glass coverslips. Once cells formed monolayers, they were not pretreated or pretreated with LY294002 (LY, 20 μM), PP2 (10 μM), or PP3 (10 μM), 30 minutes before DMSO or 10 μM OXO treatment. Cells were processed for immunostaining against ZO1, 24 hours later, as described in Materials and Methods. Data shown are representative of three independent experiments.

$pS^{3}$cofilin levels, whereas $pS^{3}$cofilin levels in RIE1-α5/1 cells were easily detected (Fig. 4D). Moreover, when RIE1-α5 or RIE1-α5/1 cells were treated with cytochalasin D at low doses (i.e. 0.05 or 0.2 μM), actin filament organization was found to be disrupted in RIE-α5, but not in RIE1-α5/1 cells, indicating that somehow RIE1-α5/1 cells are able to maintain an intact actin filament organization (Fig. 4E). Actin filaments in RIE1-α5 or RIE1-α5/1 cells left untreated or treated with OXO alone or plus pharmacological reagent were then stained. RIE1-α5 cells treated with OXO alone were found to lose cell-cell contacts with cellular retractions. However, pre-inhibition with SFK or PI3K abolished this OXO-mediated retraction of RIE1-α5 cells, whereas PP3 pretreatment did not (Fig. 4F, upper panel). Again, RIE1-α5/1 cells did not show any significant changes in actin organization, regardless of these inhibitions, and prominent peripheral actin organizations were observed under all conditions (Fig. 4F, lower panel). These experiments were not possible with cytochalasin D, because it appeared too potent and treatment led to sudden cell collapse (data not shown). Therefore, the maintenance of cell-cell contacts between RIE1-α5/1 cells even in the presence of OXO appeared to be related to a persistent actin polymerization along cell peripheries.

### PKCδ inactivation sustained peripheral actin organization and blocked OXO-mediated cell-cell contact loss in RIE1-α5 cells

It is possible that persistent peripheral actin organization in RIE1-α5/1 cells might protect cell-cell contacts from OXO treatment. We next tried to identify signaling molecules that supported peripheral actin organization by using pharmacological reagents. Among the reagents tested, we found that treatment with rottlerin, a specific PKCδ inhibitor, enhanced peripheral actin organization in RIE1-α5 cells (Fig. 5A). Therefore, we pretreated RIE1-α5 cells with rottlerin prior to OXO treatment, and then examined β-catenin localization at cell-cell contact sites. Rottlerin pretreatment of RIE1-α5 cells did maintain cell-cell contacts even after OXO treatment (Fig. 5B). To validate the role of PKCδ in the blockade of OXO-mediated cell-cell contact loss, RIE1-α5 cells were infected with adenovirus for dominant negative (DN) K376A PKCδ (Hirai et al., 1994) for 8 hours. Infected cells were then treated with DMSO or OXO for 24 hours prior to immunofluorescent staining. ZO1 or β-catenin immunostaining showed that the expression of DN PKCδ in RIE1-α5 cells abolished their OXO-mediated losses at cell-cell contact sites (Fig. 5C), consistent with the finding of the rottlerin inhibitor study. Next, we conversely examined if activation of PKCδ in RIE1-α5/1 cells facilitated cell-cell contact loss by OXO treatment. Infection of RIE1-α5/1 cells with adenovirus for WT PKCδ [overexpression of which enhances $pS^{643}$PKCδ (Lee et al., 2005)] caused cell-cell contact loss after OXO treatment (Fig. 5D). However, WT PKCδ overexpression alone (without OXO treatment) did not cause loss of contact between RIE1-α5/1 cells, presumably indicating that OXO treatment involves the activations of other signaling pathways or molecules, such as cortactin, MLC and/or Fer (see Discussion) in addition to PKCδ. It has previously been reported that A375-SM melanoma cell adhesion via integrin α5β1 engagement to fibronectin requires activation of PKCα, but not of PKCδ (Mostafavi-Pour et al., 2003). Therefore, we examined if PKCα was also involved in the OXO-mediated loss of cell-cell contact between RIE1-α5 cells. Unlike PKCδ, infection of RIE1-α5 or RIE1-α5/1 cells with DN or WT PKCα adenovirus, respectively, did not affect OXO-mediated effects on ZO1 localization (Fig. 5C,D, bottom panels), indicating that PKCα is not involved in the OXO-induced loss of contacts between RIE1-α5 cells. Furthermore, when actin organization was examined, the infection of RIE1-α5 cells with DN PKCδ adenovirus was found to sustain peripheral actin organization even after OXO treatment (Fig. 5E, left). However, in the case of RIE1-α5/1 cells, infection with WT PKCδ adenovirus failed to sustain peripheral actin organization after OXO treatment (Fig. 5E, right). Meanwhile, the phosphorylation of PKCδ Ser643 but not of Thr505 (i.e. $pS^{643}$PKCδ and $pT^{505}$PKCδ, respectively) appeared to be correlated with OXO-mediated cell-cell contact loss in RIE1-α5 cells. In other words, $pS^{643}$PKCδ was enhanced or remained unchanged when RIE1-α5 or RIE1-α5/1 cells were treated with OXO, respectively, although its basal level was marginally higher in RIE1-α5/1 cells (Fig. 5F, lanes 1, 2, 4, and 5). Furthermore, DN PKCδ expression in RIE1-α5 cells reduced OXO-mediated $pS^{643}$PKCδ level, whereas WT PKCδ expression in RIE1-α5/1 cells increased, as was expected (Fig. 5F, lanes 2, 3, 5, and 6). In addition, OXO-enhanced SMA expression level in RIE1-α5 cells was decreased by DN PKCδ

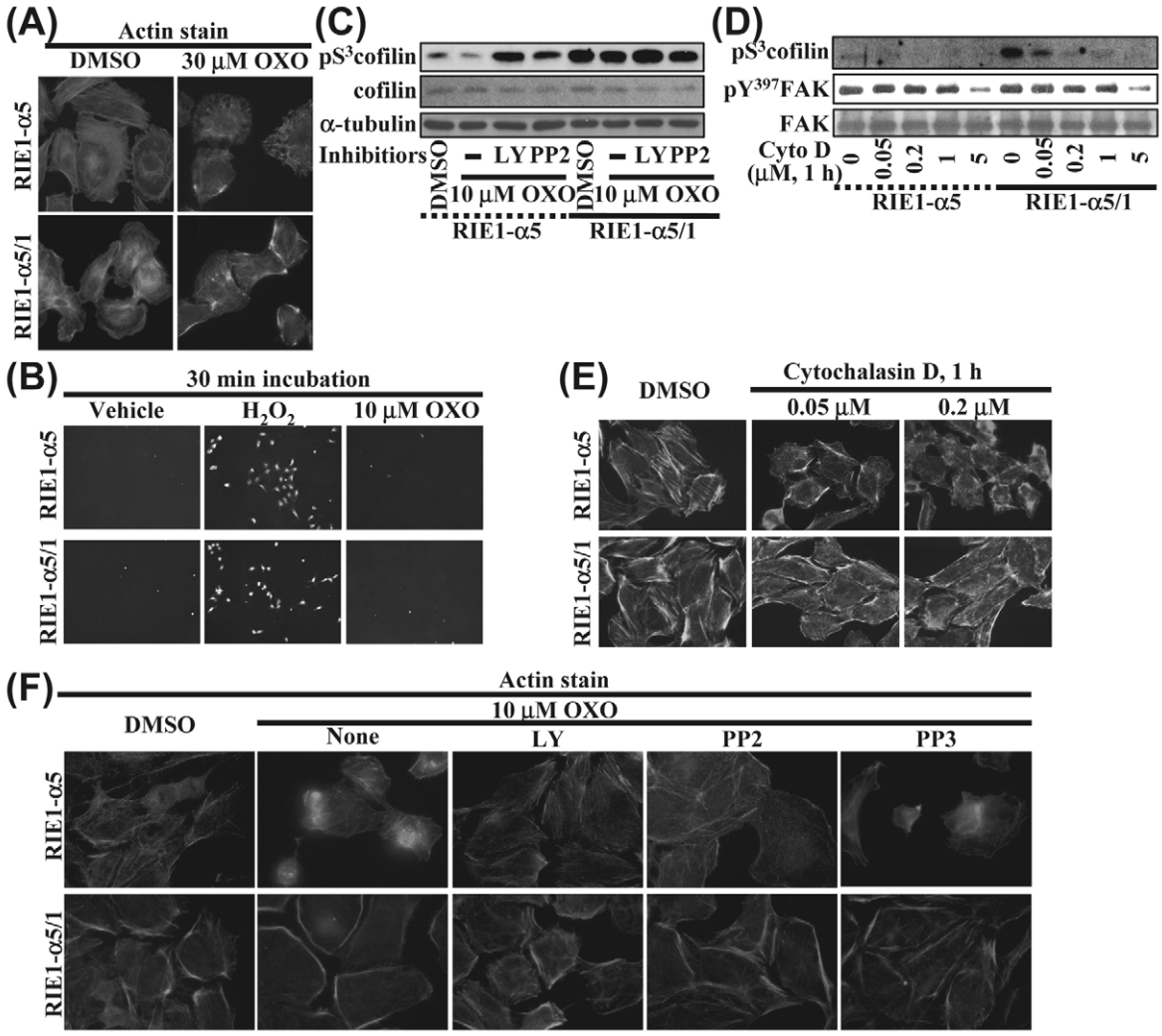


**Fig. 4.** Differential cofilin phosphorylation and peripheral actin reorganization in RIE1-α5 and RIE1-α5/1 cells. (A) Cells were seeded onto 10% FBS-DMEM-H-coated glass coverslips. After cells had adhered and spread typically, they were treated with either DMSO or 30 μM OXO for 30 minutes. Cells were then processed for actin staining using phalloidin-conjugated with Rhodamine, as described in Materials and Methods. Note that 30 μM OXO treatment disrupted actin filament organization only in RIE1-α5 cells. (B) Cells on normal culture medium-coated coverslips were incubated with 20 μM DCHF-PBS for 30 minutes, prior to washing and visualization of ROS-positive cells by fluorescent microscopy. (C,D) Cells (at 70~80% confluence) in 60 mm culture dishes were not pretreated or pretreated with the indicated pharmacological inhibitors, as in Fig. 3, prior to treatment with DMSO or 10 μM OXO for 24 hours (C) or with DMSO (i.e. 0 μM of cytochalasin D) or cytochalasin D at the indicated concentrations for 1 hour (D). Whole cell lysates were then prepared and normalized protein amounts were used in standard western blotting for the indicated molecules. Note that RIE1-α5/1 cells showed more sustained $pS^3$cofilin levels than RIE1-α5 cells did, even after treatment with cytochalasin D. (E) Cells on normal culture medium-coated coverslips were treated with DMSO or cytochalasin D (0.05 or 0.2 μM) for 1 hour, prior to actin staining as in A. (F) Cells were manipulated as described in the legend of Fig. 3, with the exception of staining for actin using phalloidin-conjugated with Rhodamine. Data shown are representative of three independent experiments.

pre-infection (Fig. 5F, bottom, lanes 1, 2 and 3), whereas SMA level, unchanged by OXO treatment of RIE1-α5/1, was increased by WT PKCδ expression (Fig. 5F, bottom, lanes 4, 5 and 6). $pS^{643}$PKCδ-dependent cell-cell contact loss by OXO treatment appeared to be specific for RIE1-α5 cells, since the pre-infection of RIE1 cells overexpressing integrin α4 (RIE1-α4) with DN PKCδ adenovirus did not block OXO-induced cell contact loss (Fig. 5G).

## Peripheral actin organization via cofilin phosphorylation

The phosphorylation of cofilin Ser3 (i.e. $pS^3$cofilin) was also affected by PKCδ activity in RIE1 cells, and changes in $pS^3$cofilin were found to be correlated with patterns of peripheral actin organization, as shown in Fig. 5E. The overexpression of DN PKCδ in RIE1-α5 cells abolished OXO-mediated decrease in $pS^3$cofilin, but the overexpression of WT PKCδ, and thereby the enhancement of $pS^{643}$PKCδ, in RIE1-α5/1 cells (Lee et al., 2005) decreased $pS^3$cofilin upon OXO treatment, although $pS^3$cofilin level in OXO-treated RIE1-α5/1 was higher than that in OXO-treated RIE1-α5 cells (Fig. 6A). Basal $pS^3$cofilin level of RIE1-α5/1 was also higher than that of RIE1-α5 cells. Cofilin Ser3 is dephosphorylated by

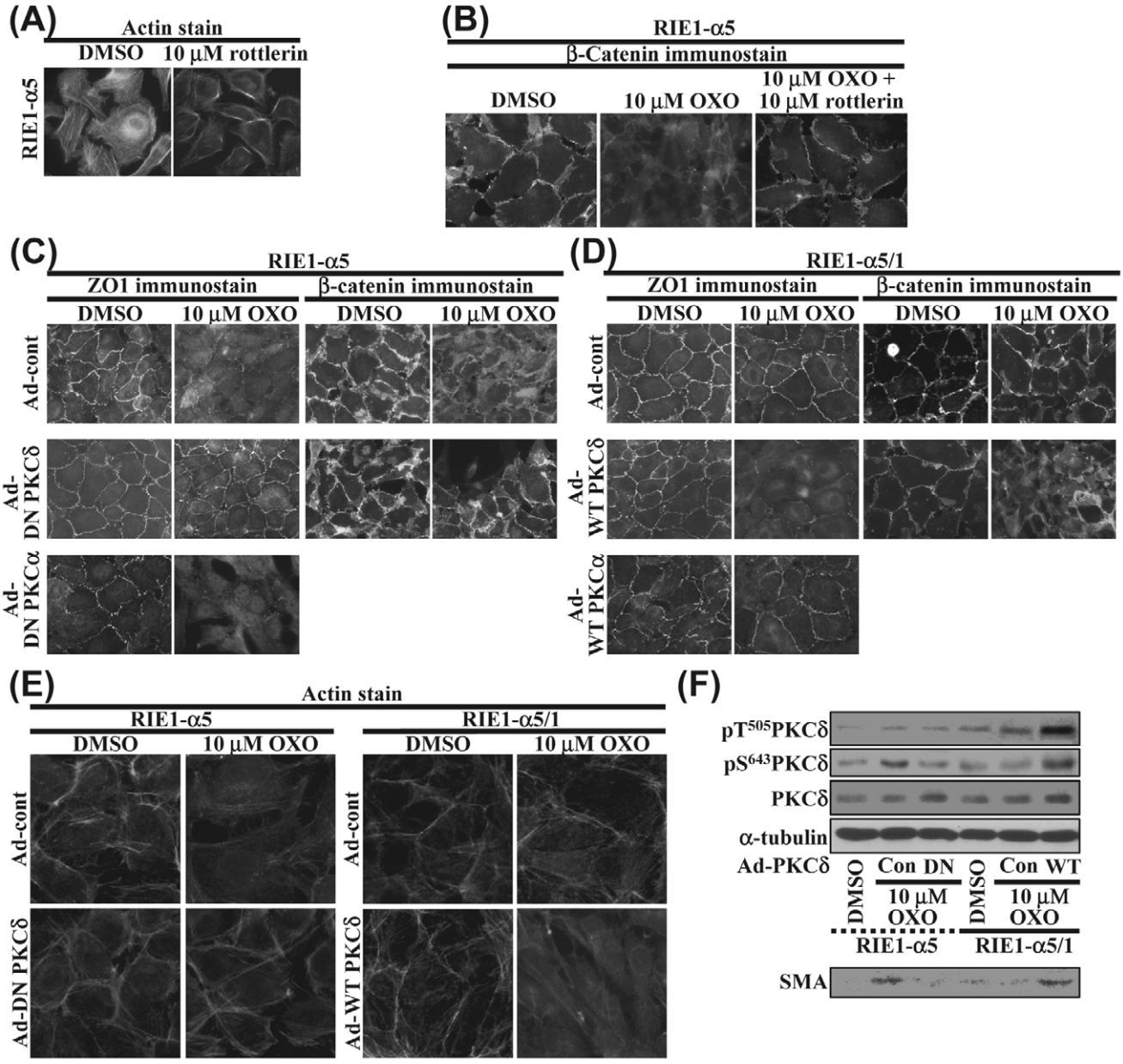


**Fig. 5.** OXO-mediated cell-cell contact loss of RIE1-α5 cells involves pS$^{643}$PKCδ-dependent dynamic peripheral actin reorganization. (A,B) RIE1-α5 cells (at 70~80% confluence) on 10% FBS-DMEM-H-coated coverslips were treated with DMSO or 10 μM rottlerin for 24 hours (A), or with DMSO, 10 μM OXO alone or OXO plus rottlerin (10 μM OXO + 10 μM rottlerin) for 24 hours (B). Cells were then stained for actin (A) or immunostained for β-catenin (B), as explained in Materials and Methods. (C-E) Cells were infected with adenovirus for GFP (Ad-cont), dominant negative K376A PKCδ (Ad-DN PKCδ) or K368R PKCα (Ad-DN PKCα), or wild-type PKCδ (Ad-WT PKCδ) or PKCα (Ad-WT PKCα) for 8 hours. After infection, media were replaced with fresh culture media. DMSO or 10 μM OXO was then treated for 24 hours, prior to immunofluorescent staining against ZO1 (left panels) or β-catenin (right panels; C,D), or stained for actin using phalloidin-conjugated Rhodamine (E). (F) Cells in 60 mm culture dishes were infected with adenovirus for GFP (Con), K376A DN PKCδ (DN), or WT PKCδ (WT), for 8 hours. After the viruses had been washed out, cells were treated with DMSO or 10 μM OXO for 24 hours, before preparation of whole cell lysates. An equal amount of proteins was subjected to standard western blotting for the indicated molecules. (G) RIE1-α4 cells were manipulated as in (C) prior to ZO1 immunofluorescent staining. Data shown are representative of three independent experiments.

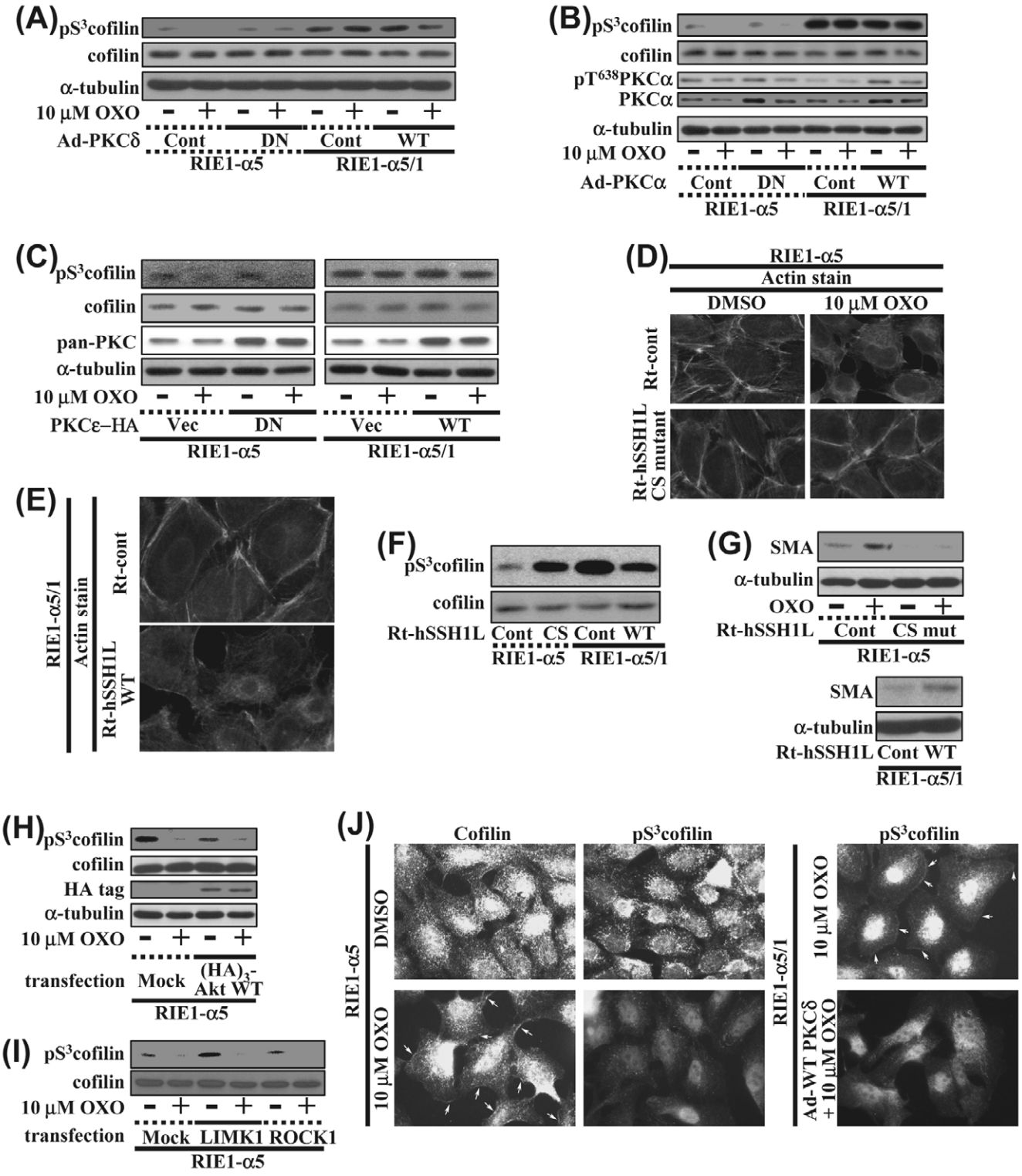


**Fig. 6.** Dynamic reorganization of peripheral actin filament and cell-cell contact loss of RIE1-α5 cells depend on PKCδ-dependent cofilin dephosphorylation after OXO treatment. (A-C) Cells in 60 mm culture dishes were infected with adenovirus for GFP (Cont), K376A DN PKCδ (DN), or WT PKCδ (WT), for 8 hours (A). Cells were infected or transiently transfected with adenovirus for K368R DN or WT PKCα (B) or K437R or WT PKCε-HA (C), respectively. (D-I) Cells on 10% FBS-DMEM-H-coated glass coverslips (D,E) or in 60 mm culture dishes (F,G) were infected with retrovirus for control empty vector (Rt-cont), inactive hSSH1L-CS mutant (Rt-hSSH1L-CS mutant), or wild-type hSSH1L (Rt-hSSH1L WT) for 24 hours. RIE1-α5 cells were transiently transfected with control (Mock), $(HA)_3$-Akt WT (H), LIMK1, or ROCK1 (I) plasmids, 24 hours before OXO treatment. After 24 hours without or with OXO treatment, cell lysates were prepared. An equal amount of proteins was used in standard western blotting for the indicated molecules (A,B,C,F,G,H,I). Cells on coverslips were stained for actin (D,E) or immunostained for cofilin or $pS^3$cofilin (J). Data shown are representative of three independent experiments.

Slingshot phosphatase (SSH) thus activating it for actin filament severing (Huang et al., 2006; Ohta et al., 2003), and pS[3]cofilin can be increased by LIMK, ROCK (Schmitz et al., 2000) or PTEN (phosphatase and tensin homologue deleted in chromosome 10) protein level or activity (Nishita et al., 2004). Interestingly, however, SSH1L mRNA and LIMK1, ROCK1 and PTEN protein levels were similar in RIE1-α5 and RIE1-α5/1 cells (data not shown). Furthermore, other stable RIE1-α5/1 cell lines showed consistently and generally higher pS[3]cofilin levels than other stable RIE1-α5 cell lines (data not shown). Being consistent with the observations in Fig. 4C-E, these findings indicate that impaired integrin α5 signaling may somehow lead to a well polymerized actin status, probably via activated LIMK1 (see below) or other molecules. Nonetheless, it is possible that a reduction in pS[3]cofilin by OXO treatment in RIE1-α5 cells, as shown in Fig. 4C, may lead to dynamic retractile actin organization.

In a manner consistent with no OXO-induced cell scattering via PKCα, as shown in Fig. 5C, PKCα activity regulation did not further affect OXO-mediated pS[3]cofilin (Fig. 6B). In addition, PKCε K437R dominant mutant or WT overexpression in RIE1-α5 or RIE1-α5/1 cells, respectively, did not further alter OXO-mediated pS[3]cofilin levels, indicating that PKCε is not involved in the OXO-mediated effects on RIE1 cells (Fig. 6C).

To determine if indeed peripheral actin organization was regulated by cofilin phosphorylation status, RIE1-α5 cells were infected with retrovirus for control empty vector or an inactive human cofilin phosphatase CS mutant, hSSH1L-CS, treated with DMSO or 10 μM OXO, and then stained for actin. Whereas control retrovirus-infected RIE1-α5 cells showed morphological retraction and peripheral actin disruption after OXO treatment, hSSH1L-CS retrovirus-infected cells showed persistent peripheral actin bundles and no retraction even after OXO treatment (Fig. 6D). However, RIE1-α5/1 cells infected with hSSH1L WT retrovirus showed morphological retraction via loss of peripheral actin organization even without OXO treatment (Fig. 6E). As was expected, the expression of hSSH1L CS mutant or WT forms increased or decreased pS[3]cofilin levels of RIE1-α5 or RIE1-α5/1 cells, respectively, compared to the basal level of each cell line (Fig. 6F). The effects of hSSH1L CS mutant or WT on cell-cell contacts, as shown in Fig. 6D,E, were found to be correlated with changes in SMA levels (Fig. 6G, left or right, respectively). Therefore, the disruption of peripheral actin organization in RIE1-α5 cells by OXO treatment appeared to involve dephosphorylated, and thus active, cofilin, during OXO-mediated cell-cell contact loss.

It was previously reported that PI3K activity mediates SSH1L activation and pS[473]Akt colocalizes with cofilin but not with pS[3]cofilin, during the insulin-induced membrane protrusion of mammary MCF7 cells (Nishita et al., 2004). Therefore, we examined whether Akt was correlated with OXO-mediated decrease in pS[3]cofilin level of RIE1-α5 cells. Overexpression of Akt WT reduced the basal level of pS[3]cofilin, which was further downregulated by OXO treatment (Fig. 6H). This suggests that OXO-enhanced PI3K/Akt activity is linked to SSH1L activation for less pS[3]cofilin in RIE1-α5 cells. In addition, transient transfection experiments to reveal the upstream effector(s) of cofilin showed that LIMK1, but not ROCK1, overexpression increased basal pS[3]cofilin in RIE1-α5 cells, which was inhibited by OXO treatment (Fig. 6I). This indicates that LIMK1 is upstream of cofilin when RIE1-α5 cells are treated with OXO. It was also previously reported that a complex formation between SSH1L and LIMK1 inactivates LIMK1 and thus potentiates the cofilin dephosphorylation (Soosairajah et al., 2005), suggesting that both LIMK1 and SSH1L are upstream of cofilin during the OXO-mediated (peripheral) actin reorganization in RIE1-α5 cells. Meanwhile, OXO-mediated pS[3]cofilin of DN PKCδ-infected RIE1-α5 cells was similar or slightly lower than that of WT PKCδ-infected RIE1-α5/1 cells, as shown in Fig. 6A. The former cells blocked OXO-mediated contact loss, but the latter cells lost contacts after OXO treatment. Therefore, it is also likely that the spatial regulation of pS[3]cofilin is required for peripheral actin regulation and cell-cell contact maintenance in the presence of OXO. To test it, we examined if OXO treatment regulated the spatial localizations of cofilin and pS[3]cofilin. When RIE1-α5 cells were treated with OXO, certain nonphosphorylated cofilin was located at cell peripheries, but pS[3]cofilin was not (Fig. 6J, left and middle). Meanwhile, OXO-treated RIE1-α5/1 cells still showed pS[3]cofilin at cell peripheries, but pS[3]cofilin was no longer observed at peripheries when cells were pre-infected with WT PKCδ adenovirus (Fig. 6J, right). This indicates that the spatial localization of pS[3]cofilin is important in the PKCδ-dependent OXO effects on contact loss of RIE1-α5 cells.

### PKCδ mediates signaling from integrin α5 to focal adhesion molecules

The roles of PKCδ in adhesion-mediated signal transduction to FAK have been previously described (Vuori and Ruoslahti, 1993), although no involvement of PKCδ in adhesion-mediated FAK activation has been shown (Miranti et al., 1999). Therefore, we investigated how pS[643]PKCδ was linked with focal adhesion molecules (e.g. FAK and SFK) and PI3K/Akt during integrin α5-dependent cell-cell contact loss after OXO treatment. We examined whether extracellular liganding of integrin α5, via replating cells on fibronectin (Fn), could enhance pS[643]PKCδ. RIE1-α5 replated on Fn showed increased pS[643]PKCδ, but not pT[505]PKCδ, compared to that of suspended cells. However, RIE1-α5/1 cells did not show any significant increase in pS[643]PKCδ and pT[505]PKCδ (Fig. 7A). Intact integrin α5-dependent pS[643]PKCδ increase appeared to correlate with complex formation between Ser643-phoshorylated PKCδ and intact integrin α5, but not tailless α5, although this complex was observed at a low level (<5% of input) and reverse coimmunoprecipitation (i.e. α5 coimmunoprecipitated by anti-Ser643-phoshorylated PKCδ) failed in our protocols (Fig. 7B and data not shown). Thus, the integrin α5 cytoplasmic tail might be important for complex formation and thus for PKCδ Ser643 phosphorylation/activation after OXO treatment. Since no conclusive evidence indicates that PKCδ Ser643 phosphorylation is crucial for its kinase activity (Li et al., 1997; Stempka et al., 1999), we examined both pS[643]PKCδ and PKCδ activity without or with OXO. When immunoprecipitated PKCδ was incubated with PKCδ-depleted extracts of RIE1-α5 cells in the absence or presence of OXO to determine whether OXO itself affected PKCδ phosphorylation at Ser643, the pS[643]PKCδ level of the immunoprecipitates was found to be increased by the extracts, but not further changed by OXO (Fig. 7C, upper). In addition, in vitro PKCδ assay using recombinant PKCδ and peptide

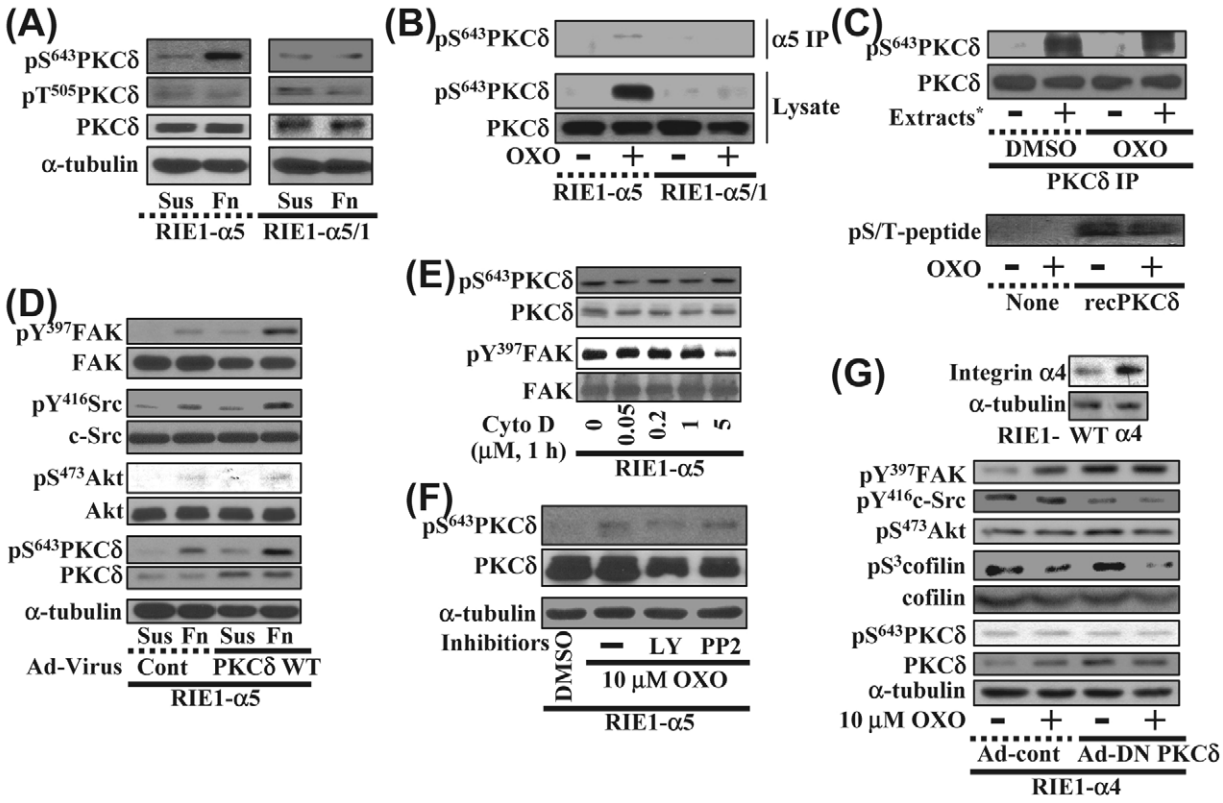


**Fig. 7.** PKCδ mediates signaling from integrin α5 to focal adhesion molecules. (A) Cells were trypsinized, collected, suspended into DMEM-H-1% BSA, and rotated (60 rpm) at 37°C for 45 minutes. Then half of cells was kept in suspension and the other half was replated onto fibronectin-coated (10 μg/ml) 60 mm dishes for 2 hours. After incubation, whole cell lysates were prepared for immunoblots for the indicated molecules. (B) Whole cell extracts from the cells without or with OXO treatment were prepared and immunoprecipitated with anti-integrin α5 (clone P1D6). The immunoprecipitates and lysates were immunoblotted for pS$^{643}$PKCδ or PKCδ. (C) Whole cell extracts from RIE1-α5 cells were immunoprecipitated with anti-PKCδ. The PKCδ immunoprecipitates were incubated in a reaction buffer (25 mM Tris, pH 7.5, 10 mM $MgCl_2$, 50 μM ATP and 1 mM DTT) without or with the PKCδ-depleted extracts (Extracts*; 10 μg protein/reaction) in the absence or presence of 10 μM OXO for 30 minutes at 25°C with shaking. After incubation, SDS-PAGE sample buffer was added to stop the reaction before immunoblotting for pS$^{643}$PKCδ and PKCδ (upper panels). For in vitro PKCδ kinase assay (lower panel), determination of phosphorylation of Ser/Thr in the substrate by recombinant PKCδ (recPKCδ) using HTScan$^{TM}$ PKCδ kinase assay kit was performed following the manufacturer's protocols. The primary antibody of the kit and proper secondary antibody were used for immunoblots. (D) RIE1-α5 cells were infected with adenovirus (Ad-Virus) encoding for a control protein (i.e. GFP, Cont) or WT PKCδ for 12 hours. Twenty four hours after the infection, cells were maintained in suspension (Sus) or replated onto fibronectin-coated (10 μg/ml; Fn) 60 mm dishes for 2 hours prior to cell lysates preparation and standard western blotting for the indicated molecules. (E,F) Whole cell lysates prepared as in Fig. 4C,D were normalized and used for standard western blotting for the indicated molecules. (G) RIE-α4 or parental RIE1 WT cells in normal culture were harvested for integrin α4 and α-tubulin immunoblots (upper panel). RIE1-α4 cells were infected with control (Ad-cont) or DN PKCδ (Ad-DN PKCδ) adenovirus and treated with OXO (10 μM for 24 hours) prior to harvesting and immunoblotting for the indicated molecules. Data shown are representative of at least three independent experiments.

substrate showed that OXO did not affect PKCδ enzyme activity (Fig. 7C, bottom), indicating that the OXO effects on PKCδ are indirect.

Furthermore, when RIE1-α5 cells were replated on Fn after PKCδ WT virus infection to increase pS$^{643}$PKCδ, pY$^{397}$FAK, pY$^{416}$Src and pS$^{473}$Akt were further enhanced (Fig. 7D), indicating that PKCδ acts upstream of them. In addition, pS$^{643}$PKCδ was not altered even when pY$^{397}$FAK was inhibited by cytochalasin D (5 μM), indicating that PKCδ is not downstream of FAK in this system (Fig. 7E). Moreover, OXO-mediated pS$^{643}$PKCδ in RIE1-α5 cells was partially or insignificantly reduced by PI3K or SFK inhibition, respectively, indicating that PKCδ is not downstream of SFK (Fig. 7F). These data indicate that PKCδ mediates signaling from integrin α5 to SFK and FAK.

RIE1-α4 cells showed a reduced pS$^{3}$cofilin level after OXO treatment (Fig. 7G), being consistent with OXO-mediated loss of contacts between RIE1-α4 cells (Fig. 5G, upper). Although basal pY$^{397}$FAK of RIE1-α4 cells was increased by DN PKCδ infection, pre-infection with DN PKCδ adenovirus did not block the OXO-mediated effects on pS$^{3}$cofilin, pS$^{643}$PKCδ, pY$^{397}$FAK, pY$^{416}$Src and pS$^{473}$Akt (Fig. 7G). These observations are consistent with no blocking of OXO-mediated cell-cell contact loss (Fig. 5G, lower). Therefore, the pS$^{643}$PKCδ dependency of the OXO effects appeared to be specific for integrin α5-expressing cells, whereas RIE1-WT or RIE1-α4 cells showed loss of cell-cell contacts after OXO treatment presumably in a PKCδ-independent manner (Fig. 5G and data not shown).

### OXO-mediated wound healing dependent on intact integrin α5 subunit

The observed OXO-mediated loss or maintenance of cell-cell contacts depending on WT or mutant integrin α5 signaling, respectively, may differentially support cell migration properties, since cell-cell contacts may resist cell migration. To test this possibility, we examined the wound healing abilities of RIE1-α5 and RIE1-α5/1 cells under diverse experimental conditions. First, when we examined the effects of OXO treatment on wound healing, we observed that wound healing by RIE1-α5 cells was slightly improved, whereas wound healing by RIE1-α5/1 cells was retarded after OXO treatment, without any improvement even after a longer period of treatment (Fig. 8A). This OXO-mediated retardation might be due to the maintenance of cell-cell contacts as well as the inhibition of basal intracellular migration machinery by OXO treatment. To determine whether wound healing abilities depend on SFK and PI3K/Akt activities and actin filaments, cells were wounded and pretreated with inhibitors against SFK (PP2 and its negative control PP3), PI3K/Akt (LY294002) or MLCK (ML9), 10 minutes before 10 μM OXO treatment. Degrees of wound healing were then compared. Pharmacological inhibition of PI3K/Akt, SFK or MLCK blocked OXO-enhanced wound healing by RIE1-α5 cells, but PP3 did not (Fig. 8B, upper). By contrast, the wound healing abilities of RIE1-α5/1 cells was unchanged under the same experimental conditions (Fig. 8B, bottom). Next, we compared wound healing abilities when cells were infected with control- or PKCδ-adenovirus. OXO-enhanced wound healing by RIE1-α5 cells was blocked by DN PKCδ expression. However, wound healing by RIE1-α5/1 cells that was unchanged by OXO treatment, was facilitated by WT PKCδ preinfection (Fig. 8C). These effects were correlated with blockades of cell-cell contact loss by pharmacological inhibition or DN PKCδ expression in RIE1-α5 cells, and with induction of cell-cell contact loss by WT PKCδ expression in RIE1-α5/1 cells (Figs 3 and 5). Thus, it is likely that the different degrees of wound healing observed after OXO treatment might be attributed to the integrin α5- and PKCδ-dependent regulations of cofilin-mediated peripheral actin organization and cell-cell contact.

## Discussion

Not only integrin-mediated cell adhesions to ECM but also cell-cell contacts at tight and adherence junctions through claudin or occludin and E-cadherin, respectively, link extracellular cues to intracellular actin filaments (Thiery, 2003). Thus, a bidirectional communication between focal adhesions and cell-cell contacts can be possible through actin cytoskeletal reorganization and signal transduction. However, mechanistic investigations of these aspects at the molecular level have been limited. This study provides evidences that actin reorganization effected by a chemical reagent could regulate cell-cell contact and SMA expression via PKCδ and cofilin phosphorylation and/or activity-mediated regulation of peripheral actin organization, differentially depending on integrin α5 WT or tailless mutant. Evidence obtained during this study suggests that PKCδ mediates signaling from integrin α5 to focal adhesion molecules (e.g. FAK and SFK) and PI3K/Akt to dynamically regulate cofilin dephosphorylation-dependent peripheral actin reorganization, and consequently EMT and wound healing ability, but that signaling via mutant integrin α5, which lacks the whole cytoplasmic tail (i.e. α5/1) cannot cause pS$^{643}$PKCδ and dynamic regulation of peripheral actin organization (Fig. 9). Intact integrin α5-dependent pS$^{643}$PKCδ increase after OXO treatment appeared to occur via OXO-mediated complex formation between integrin α5 and pS$^{643}$PKCδ. The complex formation might occur only in limited regions, such

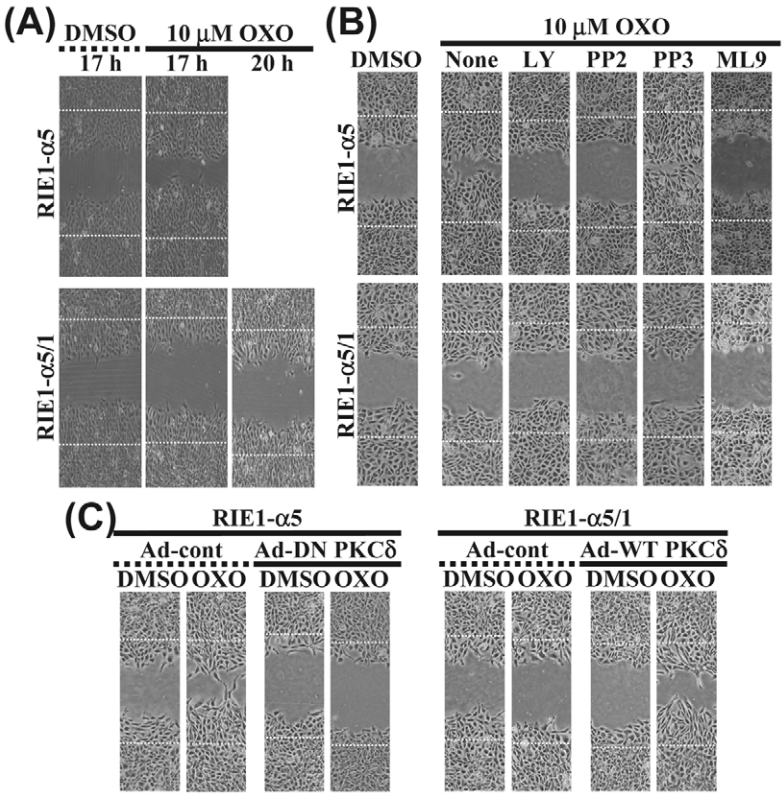


**Fig. 8.** Cell-cell contact loss from the effects of OXO on peripheral actin filaments is correlated with wound healing abilities that are dependent on integrin α5, PKCδ, SFK and Akt/PKB activities. (A) Confluent RIE1 cells seeded onto 60 mm culture dishes were wounded, washed twice with 10% FBS-DMEM-H, and treated with DMSO or 10 μM OXO, as described in Materials and Methods. Phase contrast images were taken 17 or 24 hours after wounding and treatment. (B) Confluent cells in 60 mm culture dishes were wounded, washed, and then pretreated with LY294002 (LY, 20 μM), PP2 (10 μM), PP3 (10 μM) or ML9 (20 μM) 10 minutes before 10 μM OXO treatment. Another set of cells was treated with DMSO alone in parallel. After incubation for 20 hours, phase contrast images were taken. Note that ML9 treatment caused a certain level of cytotoxicity. (C) Cells in 60 mm culture dishes were infected with adenovirus for GFP (Ad-cont), K376A DN PKCδ (Ad-DN PKCδ), or WT PKCδ (Ad-WT PKCδ) for 8 hours. After the viruses had been washed out, the cells were treated with DMSO or 10 μM OXO for 16 hours, and then images were taken of the wound area. Dotted lines indicate the starting lines for wound healing. Data shown are representative of three independent experiments.

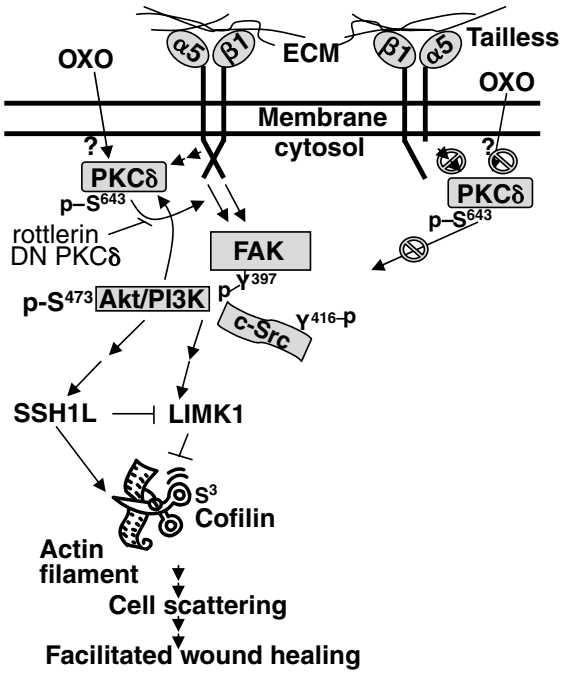


**Fig. 9.** Working model of cell-cell contact loss and wound healing through integrin α5-PKCδ signal transduction to regulate cofilin dephosphorylation and peripheral actin reorganization. When actin filaments were affected presumably by extracellular cues or reagent-mediated morphological changes, integrin α5-mediated PKCδ Ser643 phosphorylation may cause the activation of focal adhesion molecules including FAK and SFK. The PI3K/Akt pathway may be linked to PKCδ through a transmodulatory loop (refer to text). Activation of focal adhesion molecules and PI3K/Akt correlates with the dephosphorylation of cofilin Ser3 upon OXO treatment, presumably via the activation of cofilin phosphatase, SSH1L (Nishita et al., 2004) and via SSH1L activation-mediated inhibition of LIMK1 activity (Soosairajah et al., 2005). In particular, OXO treatment can also cause localization of nonphosphorylated cofilin at cell peripheries. These can result in severing of peripheral actin filaments via the dephosphorylated and thus activated cofilin. This dynamic peripheral actin reorganization may lead to cell-cell contact loss, and thereby enhance wound healing. However, specific signaling linkage(s) to dynamically regulate PKCδ, FAK, SFK, and PI3K/Akt activity, and actin reorganization, is impaired in cells expressing integrin α5 lacking its whole cytoplasmic tail (right side) which appears to be crucial for association with and signaling to PKCδ, although signaling for Erk1/2-mediated cell proliferation, probably via intact transmembrane and extracellular domains is still possible. Therefore, integrin-PKCδ-mediated dynamic regulation of cofilin activity and peripheral actin reorganization is responsible for cell-cell contact loss in response to stimuli that cause morphological changes.

as focal adhesions around cell peripheries, and only a small amount of the complex was thus formed. It cannot be ruled out that the formation of the complex may occur indirectly via another mediator such as integrin-linked kinase (ILK), syndecan or growth factor receptor.

Since we failed to find any growth factors (including hepatocyte growth factor up to 100 ng/ml) or cytokines capable of causing RIE1 cell scattering, we instead used OXO to stimulate cells, which has been shown to affect actin organization. OXO itself did not generate ROS in RIE1 cells, although naphthoquinone compounds, such as OXO, have been shown to cause cytoskeletal alteration through ROS generation (Bellomo et al., 1990; Mirabelli et al., 1989). In addition, OXO did not affect the kinase activity of recombinant PKCδ in vitro. Thus, the OXO effects may be indirect possibly through actin reorganization or disruption. Although OXO treatment might bypass certain physiological cue-mediated processes to effect actin reorganization, the observations made in this study suggest that cell-cell contact status can be regulated by integrin-ECM adhesion via PKCδ and cofilin phosphorylation/activity-mediated peripheral actin reorganization.

We observed that intact integrin α5 enhanced pS$^{643}$PKCδ, focal adhesion molecules (FAK and SFK) and Akt phosphorylation, and reduced cofilin phosphorylation presumably via activation of a cofilin phosphatase SSH1L after OXO treatment. However, integrin α4 and tailless α5/1 could not enhance pS$^{643}$PKCδ, presumably because no complex was formed between α5/1 or α4 and pS$^{643}$PKCδ. A similar integrin-subtype-specific linkage to PKC has previously been reported; signaling of integrin α5 (but not of α4) required PKC activity, especially PKCα (Mostafavi-Pour et al., 2003). However, in this current study, integrin α5-mediated signaling for actin reorganization via cofilin dephosphorylation required PKCδ, but not PKCα or PKCε, indicating specific linkages between integrin and PKC subtypes. Furthermore, it was previously reported that 4β-phorbol 12-myristate 13-acetate (PMA)-mediated cofilin dephosphorylation in neutrophils is blocked by bisindolylmaleimide I (BIM1, an antagonist of PKCα, βI, βII, γ, δ and ε), but not by Gö6976 (an antagonist of PKCα, βI, βII and μ) (Zhan et al., 2003). These previous and current studies suggest that PKCδ can regulate cofilin dephosphorylation. Consistent with the findings of this study, it was shown that PKCδ causes EMT of urinary bladder carcinoma cells, whereas PKCα/β promotes cell-cell contacts (Koivunen et al., 2004). Therefore, cell-cell contact maintenance of RIE1-α5/1 cells, even after OXO treatment, might be attributed to their specific inability to enhance pS$^{643}$PKCδ enough for dynamic regulation of peripheral actin reorganization. However, even tailless integrin α5/1 supported Erk1/2-mediated cell proliferation. This might be possible, because the transmembrane and/or extracellular domains of α5 or tailless α5/1 integrins could still transduce signaling for Erk1/2 activation through association with caveolin-1 and recruitment of Fyn (Wary et al., 1998), which might be strengthened by unidentified mechanisms in the presence of OXO, although OXO did not directly activate Erk1/2 during an in vitro Erk assay (data not shown). Alternatively, it cannot be ruled out that another unidentified target of OXO might mediate Erk1/2 activity, independently of the cytoplasmic tail of integrin α5.

How did integrin-mediated pS$^{643}$PKCδ and PKCδ activity regulate cofilin (de)phosphorylation? It is unclear at this time whether PKCδ activates SSH1L directly. PKC was previously shown to be correlated with cofilin dephosphorylation; activation of PKC in resting neutrophils by treatment of PMA, an activator of PKC, markedly dephosphorylated cofilin (Djafarzadeh and Niggli, 1997; Zhan et al., 2003). In stimulated human peripheral blood T lymphocytes, the activation of the PKC-Ras-PI3K cascade was correlated with cofilin dephosphorylation (Samstag and Nebl, 2005). Furthermore, it was previously shown that PI3K activity in MCF7 cells causes cofilin dephosphorylation via the activation

of SSH1L, and pS$^{473}$Akt colocalizes with nonphosphorylated cofilin at the cell periphery (Nishita et al., 2004). We observed enhanced pS$^{643}$PKCδ and pS$^{473}$Akt, peripheral localization of nonphosphorylated cofilin, and retractile (peripheral) actin organization after OXO treatment of RIE1-α5 (but not of RIE1-α5/1) cells, which were blocked by expression of inactive hSSH1L mutant. We also observed that the overexpression of Akt WT could reduce pS$^{3}$cofilin. Therefore, OXO-mediated pS$^{643}$PKCδ and pS$^{473}$Akt may be important for hSSH1L activation and thereby cofilin dephosphorylation at RIE1-α5 cell peripheries.

The OXO-mediated activations of FAK and SFK in RIE1-α5 cells might also result in cofilin dephosphorylation via their signaling linkages to PI3K/Akt, depending on complex formation between intact integrin α5 and pS$^{643}$PKCδ. SFK activity has been shown to activate the PI3K/Akt pathway via a direct protein interaction (Pleiman et al., 1994), phosphorylation of the regulatory p85 subunit of PI3K (Cuevas et al., 2001), and inactivation of PTEN (Lu et al., 2003). Furthermore, pY$^{397}$FAK is known to recruit Src or PI3K (Chen et al., 1996; Eide et al., 1995). This current study also showed that LIMK1 was upstream of cofilin when RIE1-α5 cells were treated with OXO. Complex formation between SSH1L and LIMK1 was shown to cause inactivation of LIMK1 and thus potentiate cofilin dephosphorylation (Soosairajah et al., 2005), suggesting that both LIMK1 and SSH1L are upstream of cofilin during OXO-mediated (peripheral) actin reorganization in RIE1-α5 cells. Also, in this study, OXO treatment caused removal of pS$^{3}$cofilin from the peripheries of RIE1-α5 cells, but not of RIE1-α5/1 cells. In addition, peripheral pS$^{3}$cofilin and sustained peripheral actin bundles of RIE1-α5/1 cells were removed by PKCδ WT overexpression. Thus, intact integrin α5-dependent pS$^{643}$PKCδ and activation of PKCδ downstream molecules including FAK, SFK and Akt might regulate SSH1L activation and LIMK1 inhibition, to cause cofilin dephosphorylation around cell peripheries to facilitate peripheral actin severing, morphological retraction, and consequent cell scattering (Fig. 9).

The roles of the different PKC isotypes and their spatiotemporal relationships at cell-cell contact sites were previously reported (Collazos et al., 2006). PKC activity has also been shown to be involved in integrin-mediated signaling. In the present study on the PKCδ-dependent EMT of RIE1 cells, the linkage between intact integrin α5 and PKCδ was found to be specific; integrin α4 and tailless α5 did not transduce signals to PKCδ and integrin α5 did not transduce signals to PKCα or PKCε. We also observed that engagement of intact integrin α5, but not of tailless α5/1, to fibronectin efficiently increased pS$^{643}$PKCδ, upstream of FAK, SFK and Akt. General PKC activation has been shown to precede integrin α5β1-mediated FAK phosphorylation and cell spreading on fibronectin, through indirect effects of PKC on FAK phosphorylation (Vuori and Ruoslahti, 1993). We also previously reported that PKCδ expression and Ser643 phosphorylation induced by treating gastric carcinoma cells with TGFβ1 led to the inductions and activations of integrins α2 and α3, which were required for FAK activity-dependent cellular spreading and metastasis potential (Lee et al., 2005). In addition, RhoA-mediated actin organization and focal adhesion of gastric carcinoma cells were enhanced by PMA in a PKCδ activity-dependent manner (Lee et al., 2006). HEK293 cell adhesion also caused integrin activation and PKCδ phosphorylation in a PTEN activity-dependent manner, whereas suspended cells did not (Parekh et al., 2000). These previous and present studies support the notion that integrin transduces signals to FAK through PKCδ mediation. Although phosphorylation of FAK and Src were found to be regulated by PKCδ in the present study, inhibition of PI3K/Akt slightly reduced pS$^{643}$PKCδ levels in the presence of OXO, indicating that the PI3K/Akt pathway partly regulates PKCδ. Thus, the relationship between PI3K/Akt and PKCδ might form a transmodulatory loop, because PKCδ could also regulate FAK, SFK and PI3K/Akt (see above). However, the significance of PKCδ regulation by Akt is not well understood at this time. The overexpression of WT PKCδ and thus enhanced Ser643 phosphorylation (Lee et al., 2005) blocked peripheral actin reorganization and caused cell-cell contact loss only after OXO treatment. This observation indicates that OXO causes more than PKCδ activation. The phosphorylation of cortactin and/or myosin light chain (MLC) might be regulated by integrin α5-dependent and PKCδ-independent signaling in the presence of OXO treatment (data not shown). Cortactin was previously shown to be involved in peripheral actin organization for cell-cell contacts (Helwani et al., 2004). Previously, non-receptor Fer tyrosine kinase released from disrupted adherence junction plaques was found to affect actin filaments and modulate integrin affinity for ECMs at focal adhesions (Arregui et al., 2000; Greer, 2002). We also observed in our system that kinase-dead Fer expression in RIE1-α5/1 cells blocked peripheral actin organization (data not shown), indicating that RIE1-α5/1 cells may have somehow highly activated Fer for persistent peripheral actin organization. It may be likely that OXO also regulates cortactin and/or Fer activity. We are currently carrying out investigations to determine how cortactin or Fer is involved in integrin α5-mediated signal transduction to regulate peripheral actin reorganization.

## Materials and Methods

### Cells

The integrin-α5-null normal rat intestinal epithelial (RIE1) cells ectopically and stably expressing human integrin α5 (RIE1-α5) or cytoplasmic tailless α5 [RIE1-α5/1; in which the cytoplasmic tail of the C-terminal 27 amino acids, including GFFKR residues, was deleted, leaving one amino acid (Lys) proximal to the transmembrane domain] were previously described (Lee and Juliano, 2000). Stable RIE1 cells overexpressing human integrin α4 (RIE1-α4) was prepared by a transfection of pcDNA3.1-α4 (a kind gift from M. H. Ginsberg, University of California, San Diego, CA, USA) to RIE1 parental cells with a little endogenous α4 expression. The stable cells were maintained in DMEM-H (Gibco-BRL) culture medium containing 10% (v/v) fetal bovine serum (FBS), 0.25 μg/ml gentamycin (Calbiochem) and 200 μg/ml G418 (A.G. Scientific Inc., San Diego, CA, USA) at 37°C and 5% $CO_2$. The RIE1-α5 cells were similar to the parental RIE1 (WT) cells in terms of actin organization and cell-cell contact formation in normal culture condition. Upon treatment with 6-(1-oxobutyl)-5,8-dimethoxy-1,4-naphthoquinone (OXO), the parental RIE1 WT cells showed cell scattering, as did RIE1-α5 cells, and OXO treatment enhanced cell scattering and wound healing ability (data not shown). Expression of integrin β1 in RIE1 WT, RIE1-α4, RIE1-α5 and RIE1-α5/1 cells was similar (data not shown).

### Immunofluorescence microscopy

Cells were replated on 10% FBS-DMEM-H-coated glass coverslips and incubated overnight at 37°C to achieve typical adhesion and spreading. RIE1-α5, RIE1-α5/1 or RIE1-α4 cells were infected with adenovirus for GFP or WT or dominant negative (DN) PKCδ (Hirai et al., 1994), or PKCα for 8 hours prior to replating. In some instances, cells were infected for 24 hours with a pLNCX retrovirus (a modified control retroviral vector) or pLNCX-hSSH1L WT- or CS mutant-myc-$(His)_6$, in which the catalytic Cys residue is replaced with Ser. A modified pLNCX was used to subclone an inactive human Slingshot (hSSH1L) CS mutant cDNA insert (a kind gift from Tadashi Uemura, Kyoto University, Kyoto, Japan) at *Hin*dIII

(5′) and *Xho*I (3′) sites. Cells were treated with vehicle (DMSO), OXO (at 10 or 30 μM in DMSO), or cytochalasin D (0.05 or 0.2 μM) for the indicated periods. In certain cases, cells were pretreated with LY294002 (20 μM; LC Laboratories®, Woburn, MA, USA), PP2 (10 μM; A.G. Scientific Inc.), PP3 (10 μM; A.G. Scientific Inc.) or rottlerin (10 μM; Calbiochem), 30 minutes before OXO treatment. Cells were then fixed with 3.7% formaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS at room temperature (RT) for 10 minutes, and washed three times with PBS. The cells were then incubated with primary antibody for 1 hour at RT and washed with PBS (three times 10 minutes). The primary antibodies for cofilin, pS[3]cofilin (Cell Signaling Technology, Beverly, MA, USA), β-catenin (Santa Cruz Biotechnology) and ZO1 (Zymed, S. San Francisco, CA, USA) were used. Cells were then incubated with anti-rabbit IgG-conjugated TRITC or FITC (Chemicon) in a dark, humidified chamber for 1 hour at RT. For actin staining, cells were stained with phalloidin-conjugated Rhodamine (Molecular Probes) for 1 hour at RT, washed three times with PBS, and mounted with a mounting solution (DakoCytomation, Germany). Mounted samples were examined using fluorescence microscopy (BX51TR, Olympus, Japan).

### Cell lysates preparation and western blots

Whole cell lysates from cells treated with DMSO or OXO at the indicated concentrations and times or from cells kept in suspension or replated on fibronectin (10 μg/ml; Chemicon) were prepared, as described previously (Lee et al., 2005). Before cells were treated with DMSO, OXO, or other pharmacological inhibitors, cells were manipulated for transfection or infection with the indicated plasmids or viruses, respectively, as above. PKCε K437R dominant mutant was generated from WT (a kind gift from J.-W. Soh, Inha University, Korea) using a QuickChange site-directed mutagenesis kit (Stratagene) and its sequence was confirmed by direct sequence analysis. Protein amounts in lysates were normalized, and then used in standard western blots using phospho-Y[397]FAK, phospho-Y[416]Src, PKCδ, Src (Santa Cruz Biotechnology), Akt/PKB, phospho-S[473]Akt/PKB, phospho-T[505]PKCδ, phospho-S[643]PKCδ, pan-PKC, Erk1/2, phospho-Erk1/2, cofilin, phospho-S[3]cofilin (Cell Signaling Technology), α-smooth muscle actin (SMA; Sigma-Aldrich), FAK, α-tubulin, integrin α5 (BD Transduction Laboratories, San Jose, CA, USA), and the cytoplasmic tail of integrin α5 (Chemicon, Temecula, CA, USA).

### ROS generation analysis

For the 2′,7′-dichlorodihydrofluorescein (DCHF) assay to examine reactive oxygen species (ROS) generation by OXO treatment, 20 μM DCHF-PBS was added to RIE1 cells on normal culture medium-coated coverslips or in 96-well plates in the absence or presence of OXO treatment for 30 minutes or 24 hours. After incubation, cells on coverslips were washed and examined for fluorescence using a BX51TR microscope (Olympus, Japan) or cells in 96-well plates were washed and fluorescence was quantified using a plate reader at 530 nm.

### Coimmunoprecipitation between integrin α5 and PKCδ

Subconfluent RIE1-α5 or RIE1-α5/1 cells without or with OXO treatment (10 μM for 24 hours) were harvested for whole cell extracts (Lee et al., 2005). An equal amount of protein was immunoprecipitated with anti-α5 mAb (clone P1D6; Chemicon) and the immunoprecipitates were immunoblotted for pS[643]PKCδ and PKCδ.

### Analysis of OXO effects on PKCδ Ser643 phosphorylation or kinase activity

Whole cell extracts were obtained from RIE1-α5 cells using a buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 50 mM NaF, 1 mM sodium pyrophosphate, 0.1% SDS and 0.1% Triton X-100) and immunoprecipitated with anti-PKCδ. The PKCδ immunoprecipitates were mixed with an equal amount of a reaction buffer (25 mM Tris, pH 7.5, 10 mM $MgCl_2$, 50 μM ATP and 1 mM DTT) without or with the PKCδ-depleted extracts (10 μg protein/reaction) in the absence or presence of 10 μM OXO for 30 minutes at 25°C with shaking. After incubation, SDS-PAGE sample buffer was added to stop the reaction before immunoblotting for pS[643]PKCδ and PKCδ. For the in vitro PKCδ kinase assay, HTScan™ PKCδ kinase assay kit (Cell Signaling Technology) was used according to the manufacturer's protocols. For determination of phosphorylation of Ser and Thr in the substrate peptide, the primary antibody of the kit and proper secondary antibody were used for immunoblots with a 0.1 μm pore size nitrocellulose transfer membrane.

### Wound healing assay

When cells in 60 mm culture dishes had formed a confluent monolayer, wounds were made by scraping through the cell monolayer with a pipette tip. Cells were then washed twice with DMEM-H containing 10% FBS. In certain cases, cells were treated with pharmacological inhibitors, as explained above, 10 minutes before OXO treatment. For PKCδ expression studies, cells were infected with adenovirus for either GFP, WT or DN PKCδ for 8 hours, prior to wounding and treatment with DMSO or 10 μM OXO. After incubation at 37°C for the indicated periods, images were taken of the area around wounds for each condition, using a microscope equipped with a digital camera (CKX41, Olympus, Japan).

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