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CD151 regulates epithelial cell–cell adhesion through PKC- and Cdc42-dependent actin cytoskeletal reorganization

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CD151, a member of the tetraspanin family proteins, tightly associates with integrin $\alpha\beta 1$ and localizes at basolateral surfaces of epithelial cells. We found that overexpression of CD151 in A431 cells accelerated intercellular adhesion, whereas treatment of cells with anti-CD151 mAb perturbed the integrity of cortical actin filaments and cell polarity. E-Cadherin puncta formation, indicative of filopodia-based adhesion zipper formation, as well as E-cadherin anchorage to detergent-insoluble cytoskeletal matrix, was enhanced in CD151-overexpressing cells. Levels of GTP-bound Cdc42 and Rac were also elevated in

CD151-overexpressing cells, suggesting the role of CD151 in E-cadherin-mediated cell–cell adhesion as a modulator of actin cytoskeletal reorganization. Consistent with this possibility, engagement of CD151 by the substrate-adsorbed anti-CD151 mAb induced prominent Cdc42-dependent filopodial extension, which along with E-cadherin puncta formation, was strongly inhibited by calphostin C, a protein kinase C (PKC) inhibitor. Together, these results indicate that CD151 is involved in epithelial cell–cell adhesion as a modulator of PKC- and Cdc42-dependent actin cytoskeletal reorganization.

Introduction

CD151 (PETA-3/SFA-1) is a member of the tetraspanins, a growing family of proteins that span the plasma membrane four times (Fitter et al., 1995; Hasegawa et al., 1996). Tetraspanins have been shown to associate with each other and with other integral membrane proteins, including integrins, the major receptors for ECM adhesive proteins (Maecker et al., 1997; Hemler, 1998). Although the physiological function of CD151 remains to be elucidated, several lines of evidence indicate that CD151 is involved in the regulation of cell motility and polarity (Yauch et al., 1998; Penas et al., 2000; Yanez-Mo et al., 2001). In epithelia, CD151 is associated with $\alpha\beta 1$ and some other integrins, and is localized not only at cell–ECM but also at cell–cell adhesion sites (Sterk et

al., 2000). Given the high affinity association of CD151 with integrin $\alpha\beta 1$ (Yauch et al., 1998; Serru et al., 1999), most, if not all, CD151 may function as a complex with integrin $\alpha\beta 1$. Although the importance of integrin $\alpha\beta 1$ in cell adhesion to the basement membrane has been well documented (Carter et al., 1991; Delwel et al., 1994; Fukushima et al., 1998), it is unknown whether this integrin, either alone or as a complex with CD151, is involved in cell–cell adhesion.

Recently, CD151 and some other tetraspanins were shown to associate with conventional PKC (cPKC; Zhang et al., 2001) and modulate integrin-dependent cell morphology (Kazarov et al., 2002). cPKC has been shown to regulate a variety of biological events, including cell–cell and cell–ECM adhesion and the inside-out activation of integrins (Shattil

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Abbreviations used in this paper: cPKC, conventional PKC; dn, dominant-negative; GST-CRIB, a fusion protein of GST to the Cdc42/Rac-interactive-binding domain of PAK1; GST-RB, a fusion protein of GST to the Rho-binding domain of Rho kinase.

and Brass, 1987; Shimizu et al., 1990). PKC α , one of the cPKCs, was shown to be involved in actin reorganization during cell–cell adhesion (Cowell and Garrod, 1999). It is generally accepted that cytoplasmic actin dynamics are regulated by the Rho family of small GTPases, often leading to the extension of the cell front as filopodia or lamellipodia, depending on the stimuli (Mackay and Hall, 1998). These dynamic processes are dependent on integrin-mediated cell–ECM adhesion, and cell adhesion to different ECM ligands leads to differential activation of Rho GTPases. Thus, cell adhesion to fibronectin via integrin $\alpha 5 \beta 1$ selectively activates Rho, whereas cell adhesion to laminin-10/11 via integrin $\alpha 3 \beta 1$ activates Rac, but not Rho (Gu et al., 2001). However, it is not known whether CD151 is involved in the ligand-dependent, differential activation of Rho GTPases through specific association with integrin $\alpha 3 \beta 1$.

The Rho family GTPases have also emerged as crucial regulators of cadherin-dependent cell–cell adhesion (Braga, 2000; Fukata and Kaibuchi, 2001). A filopodia-based mechanism of intercellular junction formation has been shown to operate during embryonic development of *Caenorhabditis elegans* (Raich et al., 1999) and *Drosophila melanogaster* (Jacinto et al., 2000). In keratinocytes, filopodium formation was reported to be the driving force of cell–cell adhesion, leading to the “adhesion zipper” model of epithelial cell–cell adhesion (Vasioukhin et al., 2000). In support of this model, E-cadherin-mediated cell–cell adhesion is associated with activation of Cdc42 (Kim et al., 2000), although the mechanisms regulating small GTPases during cell–cell adhesion are only poorly understood. We address the possible involvement of CD151 in cell–cell adhesion and its associated actin reorganization. Given the localization of CD151 at sites of epithelial cell–cell contacts as a stable complex with integrin $\alpha 3 \beta 1$, and the functional association of CD151 with cPKC, it is tempting to speculate that CD151 may play a role in epithelial cell–cell adhesion through cPKC-dependent actin reorganization. Here, we provide evidence that CD151 is a novel regulator of cPKC- and Cdc42-dependent actin reorganization, thereby stimulating E-cadherin-dependent cell–cell adhesion.

Results

Overexpression of CD151 enhances cell–cell adhesion

To investigate the role of CD151 at cell–cell contact sites, we transfected A431 human epidermoid carcinoma cells with a cDNA encoding CD151 as a GFP fusion protein, and established a panel of stable transfectants. Surface biotinylation of the transfectants and subsequent immunoprecipitation with anti-CD151 mAb showed that CD151 with a GFP tag (designated CD151-GFP) was expressed on the cell surface as a 55-kD protein (Fig. 1 A). The identification of this protein as CD151-GFP was verified by immunoblotting with an anti-GFP antibody (unpublished data). Densitometric determination of the relative intensities of the bands corresponding to CD151-GFP and endogenous CD151 indicated that the level of surface-expressed recombinant CD151 was >20 times greater than that of endogenous CD151. Immunoprecipitation of integrin $\alpha 3 \beta 1$ resulted in the coprecipitation of CD151-GFP, confirming that the re-

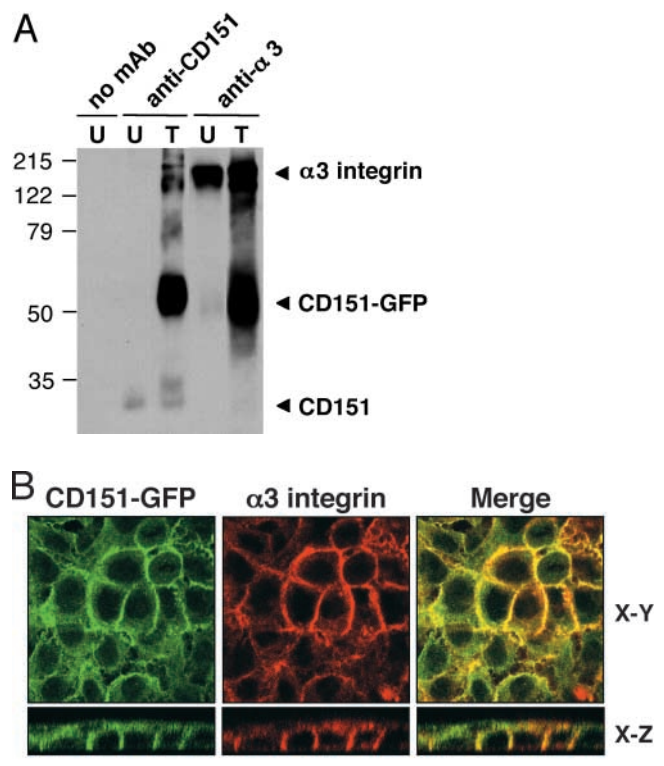


Figure 1. Surface expression and integrin $\alpha 3 \beta 1$ association of transfected CD151 with a GFP tag. (A) Stable A431 transfectants overexpressing CD151-GFP (T) or untransfected A431 cells (U) were surface-labeled with sulfo-NHS-LC-biotin, lysed in RIPA buffer, and immunoprecipitated with anti-CD151 mAb or anti- $\alpha 3$ integrin mAb. The positions of the molecular weight markers are indicated on the left. (B) Transfectants were fixed in paraformaldehyde and stained with TRITC-conjugated anti- $\alpha 3$ integrin mAb to demonstrate the colocalization of the transfected CD151-GFP (green) and $\alpha 3$ integrin (red). X-Z, vertical confocal images. Bar, 10 μ m.

combinant CD151 retained the ability to associate with integrin $\alpha 3 \beta 1$. The association of CD151-GFP with integrin $\alpha 3 \beta 1$ was further supported by their colocalization at cell–cell contact sites as demonstrated by double fluorescence detection of integrin $\alpha 3 \beta 1$ and GFP (Fig. 1 B). Vertical confocal sections indicated that the majority of CD151-GFP and integrin $\alpha 3 \beta 1$ localized at both lateral and apical surfaces with only moderate accumulation at the basal surface.

Interestingly, A431 cells overexpressing CD151-GFP gave more compact colonies than control GFP-transfected cells, when plated at a low density (Fig. 2 A). Furthermore, when cells were tracked by time-lapse video microscopy, CD151-GFP-overexpressing cells were significantly faster than the control cells in forming colonies (Fig. 2 B), endorsing the notion that intercellular adhesiveness was significantly enhanced by overexpression of recombinant CD151. Enhanced cell–cell adhesion in CD151-overexpressing cells was further supported by wound closure assays. When a monolayer of A431 cells transfected with either CD151-GFP or GFP alone was wounded by scraping the surface, wound closure was significantly retarded in cells overexpressing CD151-GFP (Fig. 2 C). The retardation of wound closure could result from either suppression of cell migration or increased intercellular adhesiveness that resisted the

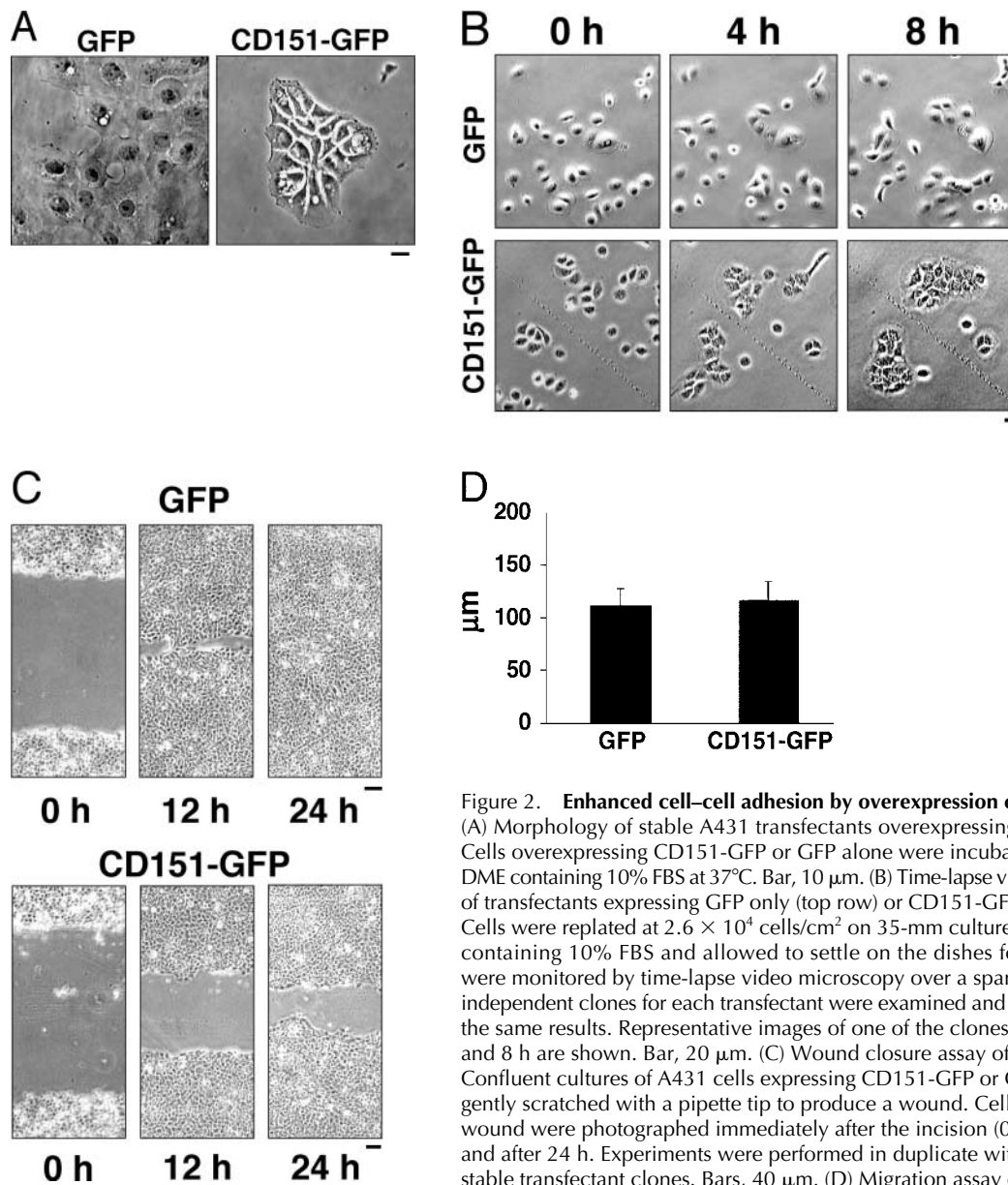


Figure 2. Enhanced cell–cell adhesion by overexpression of CD151-GFP. (A) Morphology of stable A431 transfectants overexpressing CD151-GFP. Cells overexpressing CD151-GFP or GFP alone were incubated for 12 h in DME containing 10% FBS at 37°C. Bar, 10 μm. (B) Time-lapse video microscopy of transfectants expressing GFP only (top row) or CD151-GFP (bottom row). Cells were replated at 2.6×10^4 cells/cm² on 35-mm culture dishes in DME containing 10% FBS and allowed to settle on the dishes for 12 h. Cells were monitored by time-lapse video microscopy over a span of 8 h. Three independent clones for each transfectant were examined and gave essentially the same results. Representative images of one of the clones at times 0, 4, and 8 h are shown. Bar, 20 μm. (C) Wound closure assay of transfectants. Confluent cultures of A431 cells expressing CD151-GFP or GFP alone were gently scratched with a pipette tip to produce a wound. Cell layers with a wound were photographed immediately after the incision (0 h), after 12 h, and after 24 h. Experiments were performed in duplicate with three distinct stable transfectant clones. Bars, 40 μm. (D) Migration assay of transfectants. Cell motility was evaluated by time-lapse video microscopy in triplicate assays as described in Materials and methods. Data are mean \pm SD values.

scattering of closely apposed cells in a monolayer. Determination of individual cell motility by video microscopy showed that there was no significant difference in the motility between control and CD151-overexpressing cells (Fig. 2 D), making it more likely that overexpression of CD151 retarded wound closure by strengthening cell–cell adhesion, thereby suppressing cell scattering.

CD151 is involved in actin reorganization during cell–cell adhesion

To further explore the role of CD151 at cell–cell contact sites, we examined the effect of anti-CD151 mAb on cell–cell adhesion and epithelial integrity. When plated at a high density, cells overexpressing CD151-GFP formed a typical epithelial monolayer with cobblestone morphology (Fig. 3 A). Actin filaments were organized into cortical

belts underlying cell–cell contact sites, as judged from vertical confocal images. CD151-GFP colocalized with actin filaments at cell–cell contact sites. However, pretreatment of cells with anti-CD151 mAb perturbed the integrity of the epithelial monolayer, resulting in irregular, often flatter, cell morphology. Loss of epithelial integrity was also evident from actin staining; the actin filaments were no longer uniformly localized at cell–cell contact sites, but coalesced into aggregates. CD151-GFP was also brought into aggregates by anti-CD151 pretreatment, colocalizing with actin filaments. These results raised the possibility that CD151 is linked, physically and/or functionally, to the actin cytoskeleton. A similar disturbance of epithelial integrity was also observed after pretreatment with an mAb against integrin $\alpha 3 \beta 1$, but not with an mAb against $\alpha 5 \beta 1$ (Fig. 3 A). Both integrins have been shown to be highly ex-

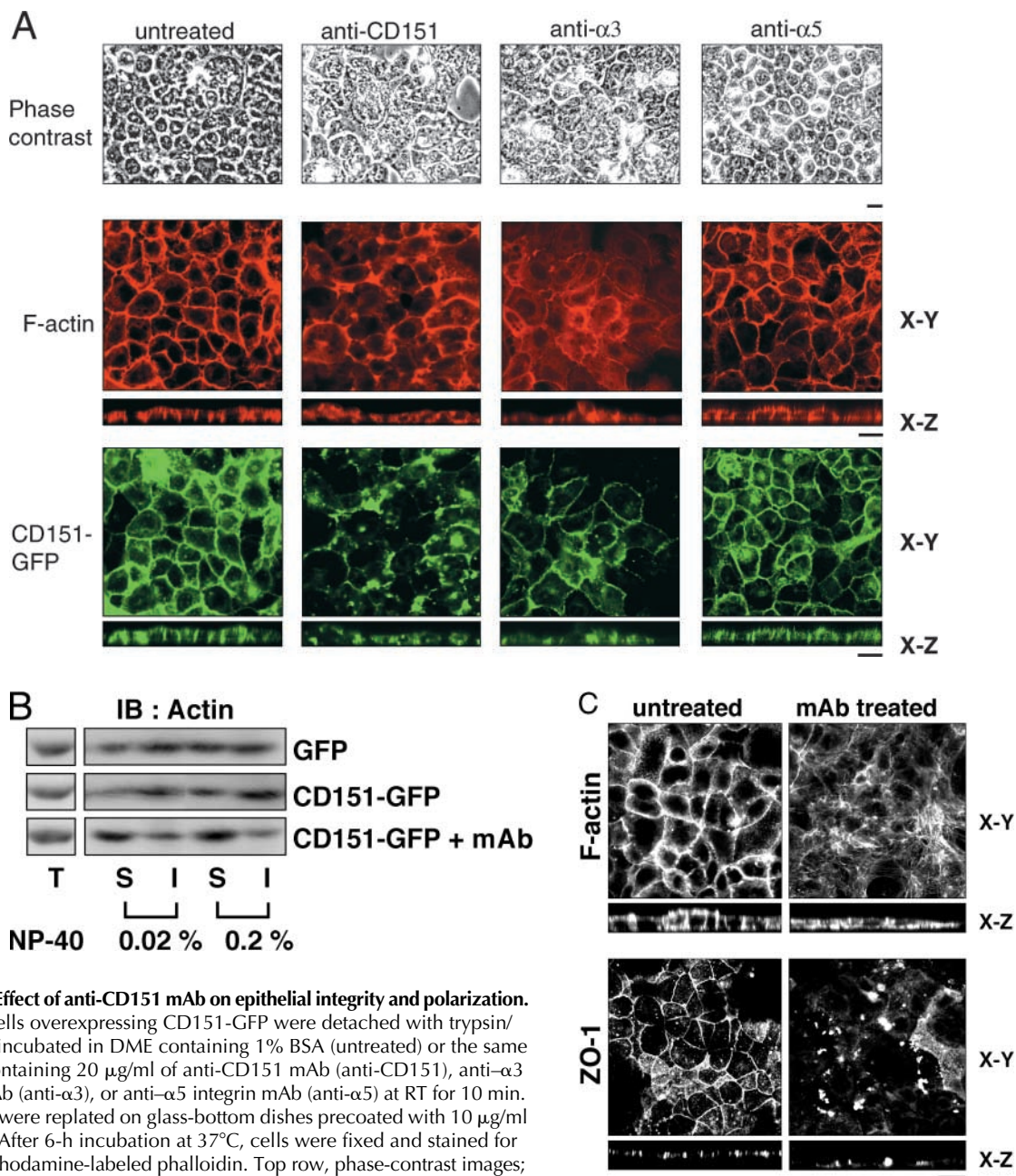


Figure 3. Effect of anti-CD151 mAb on epithelial integrity and polarization. (A) A431 cells overexpressing CD151-GFP were detached with trypsin/EDTA and incubated in DME containing 1% BSA (untreated) or the same medium containing 20 $\mu\text{g}/\text{ml}$ of anti-CD151 mAb (anti-CD151), anti- $\alpha 3$ integrin mAb (anti- $\alpha 3$), or anti- $\alpha 5$ integrin mAb (anti- $\alpha 5$) at RT for 10 min. Next, cells were replated on glass-bottom dishes precoated with 10 $\mu\text{g}/\text{ml}$ laminin-5. After 6-h incubation at 37°C, cells were fixed and stained for actin with rhodamine-labeled phalloidin. Top row, phase-contrast images; middle and bottom rows, actin filaments stained with rhodamine-labeled phalloidin (red) and GFP fluorescence (green) in the same fields. Vertical confocal images attached to each panel in the middle and bottom rows (X-Z). Bars, 10 μm . (B) A431 cells overexpressing either CD151-GFP or GFP alone were replated in the absence or presence of 20 $\mu\text{g}/\text{ml}$ anti-CD151 mAb. After 6-h incubation, monolayers were solubilized with HBS/C containing 0.02 or 0.2% NP-40. Soluble (S) and insoluble (I) fractions obtained after centrifugation of the lysates were subjected to SDS-PAGE, followed by immunoblotting with antiactin antibody. T, total lysates before centrifugation. (C) Caco-2 cells were pretreated with anti-CD151 mAb and replated as in A. The cells were stained with rhodamine-labeled phalloidin for actin filaments (top row) and anti-ZO-1 antibody (bottom row). Cells without pretreatment with anti-CD151 mAb were also stained for actin and ZO-1 as a control. X-Z, vertical confocal images. Bar, 10 μm .

pressed in A431 cells (Serru et al., 1999). Therefore, it is likely that CD151 exerts its effect on cell-cell adhesion as a complex with integrin $\alpha 3 \beta 1$. Impaired actin reorganization by anti-CD151 mAb pretreatment was further demonstrated by quantitation of the detergent-insoluble filamentous actin (Fig. 3 B). The ratio of detergent-insoluble versus detergent-soluble actin was significantly decreased upon mAb pretreatment.

The loss of epithelial integrity induced by anti-CD151 mAb pretreatment was not unique to CD151-overexpressing A431 cells. Caco-2 cells, colon carcinoma-derived cells forming a well-polarized epithelial monolayer, also lost their epithelial integrity upon pretreatment with anti-CD151 mAb, as evidenced by the loss of cortical actin filaments and appearance of stress fibers at the basal surfaces (Fig. 3 C). Cells became flatter upon mAb treatment, as shown in the

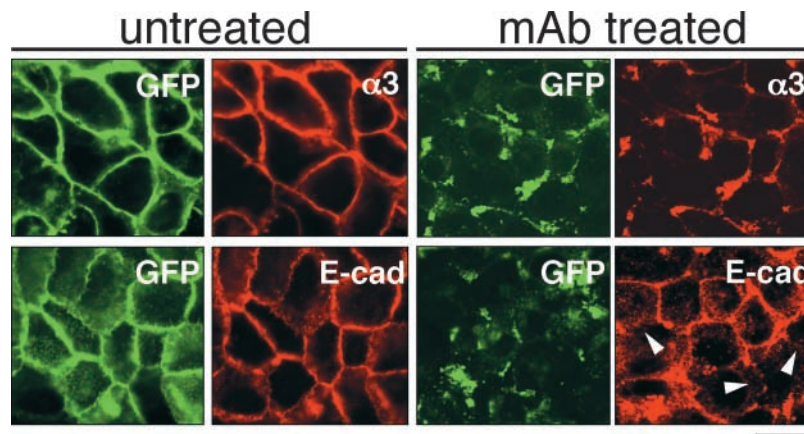


Figure 4. Effect of anti-CD151 mAb on E-cadherin distribution. A431 cells overexpressing CD151-GFP were pretreated with 20 $\mu\text{g/ml}$ anti-CD151 mAb in suspension for 10 min at RT and replated on glass-bottom dishes precoated with 10 $\mu\text{g/ml}$ laminin-5. After 6 h incubation at 37°C, cells were fixed and stained with TRITC-conjugated anti- $\alpha 3$ integrin (top row, red) or anti-E-cadherin mAb (bottom row, red). GFP fluorescence in the same fields was also photographed as a reference (green). (arrowheads) E-Cadherin distribution became irregular and discontinuous in cells treated with anti-CD151 mAb. Bar, 10 μm .

vertical confocal images. The loss of epithelial integrity was further demonstrated by immunostaining of ZO-1, a marker of tight junctions. In untreated Caco-2 cells, ZO-1 was localized uniformly at intercellular junctions, giving a dotlike staining located close to the apical surface in vertical confocal images. Upon pretreatment with anti-CD151 mAb, ZO-1 was brought into irregular aggregates located near the basal surface, indicating that tight junctions between apposed cells were completely disrupted.

Cadherin-mediated cell–cell adhesion is central to the maintenance of epithelial integrity. To ascertain whether cadherin-mediated cell–cell adhesion was disturbed by pretreatment with anti-CD151 mAb, we examined the distribution of E-cadherin in mAb-pretreated A431 cells overexpressing CD151-GFP. Although a significant fraction of E-cadherin remained localized at cell–cell contact sites, it was evident that E-cadherin distribution became irregular and discontinuous compared with untreated cells (Fig. 4). In contrast, integrin $\alpha 3\beta 1$, known to tightly associate with CD151 and localize at cell–cell contacts, was coalesced into aggregates, colocalizing with the aggregates of CD151-GFP. Given that cortical actin filaments were severely disrupted in cells pretreated with mAb (Fig. 3 A), it seems unlikely that E-cadherin at cell–cell contact sites was fully functional because strong E-cadherin–mediated cell–cell adhesion requires α -catenin–dependent anchorage of E-cadherin to cortical actin filaments.

Basal engagement of CD151 induces prominent filopodium formation

The disruption of cortical actin filaments by pretreatment with anti-CD151 mAb raised the possibility that the mAb engagement of CD151 could transduce a signal triggering reorganization of the actin cytoskeleton. To explore this possibility, A431 cells were plated at a low density on substrates coated with anti-CD151 mAb. Unexpectedly, the cells not only spread on the anti-CD151–coated substrates but extended numerous filopodia around the cell periphery (Fig. 5). The filopodial extension on the anti-CD151–coated sub-

strates was not unique to A431 cells, but was observed with other epithelial cell lines such as Caco-2, HeLa S3, and 293 (unpublished data). In contrast, no clear filopodial extension

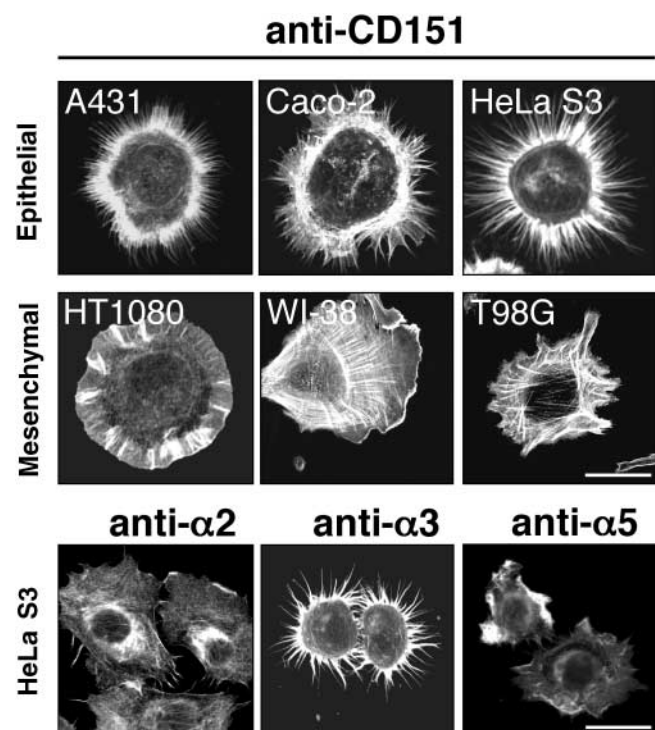


Figure 5. Basal engagement of CD151 induces filopodial extension in epithelial cells. Cells were detached with trypsin/EDTA and replated on coverslips coated with anti-CD151 mAb (20 $\mu\text{g/ml}$; top and middle rows) or mAbs against $\alpha 2$, $\alpha 3$, or $\alpha 5$ integrin chain (20 $\mu\text{g/ml}$; bottom row) at a low density in DME containing 1% BSA. Cells were incubated on the coverslips for 1 h at 37°C, fixed, permeabilized, and stained with rhodamine-labeled phalloidin. Three different types of epithelial cells (top row, A431, Caco-2, and HeLa S3) and mesenchymal cells (middle row, HT1080, WI-38, and T98G) were examined. The bottom row shows HeLa S3 cells incubated on coverslips coated with anti- $\alpha 2$, $\alpha 3$, or $\alpha 5$ integrin mAbs. Bars, 10 μm .

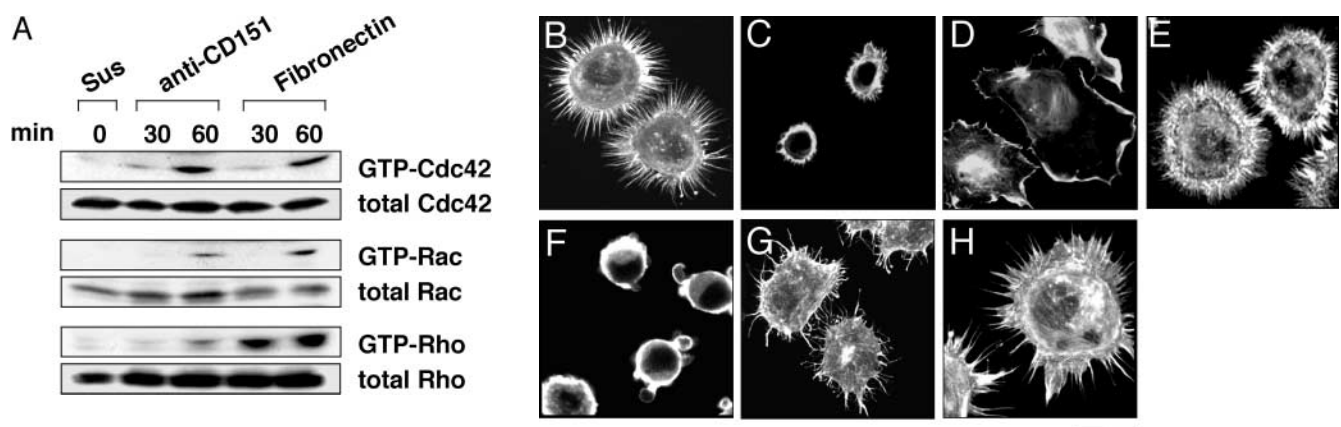


Figure 6. Involvement of Cdc42 and PKC in filopodial extension induced by basal engagement of CD151. (A) A431 cells overexpressing CD151-GFP were detached with trypsin/EDTA, kept in suspension in DME containing 1% BSA for 90 min at 37°C, and replated on 15-cm culture dishes coated with 20 μ g/ml anti-CD151 mAb or 10 μ g/ml fibronectin. Cells were allowed to adhere to the substrates for 30 or 60 min, and then lysed in lysis buffer containing 1% NP-40. GTP-loaded forms of Cdc42, Rac, and Rho were recovered by precipitation with GST-CRIB (for GTP-loaded Cdc42 and Rac) or GST-RB (for GTP-loaded Rho), followed by immunoblotting with specific antibodies against Cdc42, Rac, or Rho. Total lysates were also immunoblotted with these specific antibodies as a reference. Sus, cells kept in suspension without replating. (B–D) HeLa S3 cells were transfected with dn-Cdc42 (C) or dn-Rho (D) together with pHAd262Puro. After selection with puromycin for 48 h, transfected cells were replated on coverslips coated with 20 μ g/ml anti-CD151 mAb and incubated for 1 h at 37°C. Cells were fixed and stained with rhodamine-labeled phalloidin. (B) Untransfected cells. (E–H) HeLa S3 cells were treated in suspension for 20 min with 10 μ M Y27632 (E), 100 μ M calphostin C (F), 30 μ M rottlerin (G), or 100 μ M LY294002 (H), and replated on coverslips coated with 20 μ g/ml anti-CD151 mAb. After 1-h incubation at 37°C, cells were fixed and stained with rhodamine-labeled phalloidin. The results shown are representative of three independent experiments. Bar, 10 μ m.

was observed on the mAb-coated substrates with nonepithelial cell lines such as HT1080 (fibrosarcoma cells), WI-38 (embryonic lung fibroblasts), and T98G (glioma cells). Similar filopodial extension was also observed on substrates coated with anti- α 3 integrin mAb, but not those coated with anti- α 2 or α 5 integrin, although the cells spread well on these substrates. These results indicate that the CD151–integrin α 3 β 1 complex transduces signals that reorganize the actin cytoskeleton, particularly those inducing filopodial extension.

Cdc42 and PKC are involved in filopodial extension induced by basal engagement of CD151

Reorganization of the actin cytoskeleton has been shown to be regulated by the Rho family of small GTPases, among which Cdc42 is responsible for filopodial extension (Nobes and Hall, 1995). Pull-down assays for GTP-loaded Cdc42, Rac, and Rho in CD151-overexpressing cells demonstrated that Cdc42 was strongly activated in cells adhering to anti-CD151-coated substrates, although none of them gave significant levels of the GTP-bound forms in cells kept in suspension (Fig. 6 A) or those attached to substrates coated with poly-L-lysine (not depicted). Cells spread on substrates coated with anti- α 5 integrin mAb did not show any significant activation of Cdc42, compared with cells on anti-CD151-coated substrates (unpublished data). Such preferential activation of Cdc42 was not observed in cells adhering to fibronectin, in which Rho, but not Cdc42 or Rac, was dominantly activated. To further explore the role of Cdc42 in filopodial extension upon basal engagement of CD151, HeLa S3 cells were transfected with dominant-negative (dn) Cdc42 and replated on substrates coated with anti-CD151 mAb. HeLa S3 cells were chosen as the recipient cells because of their high susceptibility to filopodial extension by basal engagement of CD151 (Fig. 5 and Fig. 6 B). As

expected, cells transfected with dn-Cdc42 could neither spread nor extend filopodia on the mAb-coated substrates (Fig. 6 C). The cells also failed to extend filopodia upon transfection of dn-Rho; however, they remained spread with more extended lamellipodia (Fig. 6 D). The failure of filopodial extension upon transfection of dn-Rho could be due to either the active involvement of Rho in filopodial extension on anti-CD151-coated substrates or the stimulation of Rac, not Cdc42, resulting from the blockade of Rho activity (Rottner et al., 1999). To further examine the role of Rho, cells were plated on the anti-CD151-coated substrates in the presence of Y27632, an inhibitor of ROCK kinase. Y27632 only partially inhibited the filopodial extension and failed to reduce cell spreading on the mAb-coated substrates (Fig. 6 E), supporting the conclusion that basal engagement of CD151 triggers filopodial extension via preferential activation of Cdc42, but not Rho.

Although it remains unknown how CD151 transduces signals triggering activation of Cdc42, tetraspanins including CD151 have been shown to associate with cPKC (Zhang et al., 2001). To examine whether cPKC is involved, we treated cells with calphostin C, an inhibitor of cPKC, and replated them on anti-CD151-coated substrates. Calphostin C completely inhibited filopodial extension as well as cell spreading on anti-CD151 mAb-coated substrates (Fig. 6 F). Selective activation of Cdc42 on the mAb-coated substrates was also inhibited by calphostin C treatment (unpublished data). In contrast, rottlerin, an inhibitor of novel PKC, and LY294002, a phosphatidylinositol 3-kinase inhibitor, only marginally inhibited filopodial extension (Fig. 6, G and H). Together, these results indicate that basal engagement of CD151 activates Cdc42 in a cPKC-dependent manner, thereby leading to filopodial extension on anti-CD151-coated substrates.

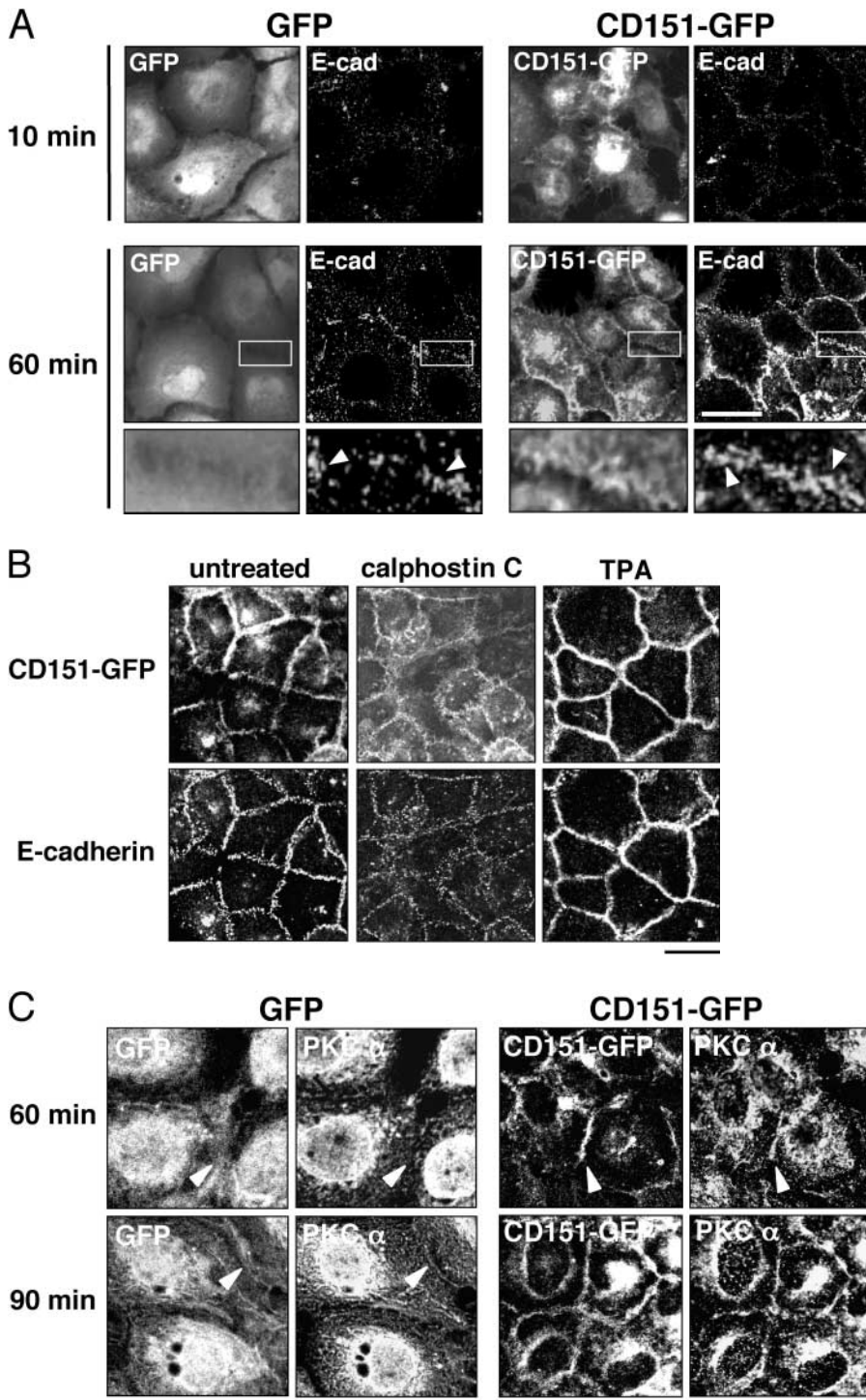


Figure 7. Effects of CD151 overexpression on calcium-stimulated E-cadherin puncta formation. (A) A431 cells overexpressing CD151-GFP or GFP alone were treated with 4 mM EGTA in the presence of 10 mM Hepes for 30 min at 37°C, and then Ca^{2+} ion concentration was restored to 1.8 mM to initiate calcium-stimulated E-cadherin-dependent cell–cell adhesion. Cells were fixed at 10 or 60 min after calcium restoration and stained with anti-E-cadherin mAb. (bottom row) A magnified view of the boxed regions. (arrowheads) E-Cadherin puncta. CD151-GFP partially colocalized with E-cadherin puncta. (B) Cells were treated with 100 μM calphostin C (middle) or 100 nM TPA (right) at calcium restoration and incubated for 90 min at 37°C. Top and bottom panels show distributions of CD151-GFP and E-cadherin, respectively. (C) A431 cells overexpressing CD151-GFP or GFP alone were fixed 60 or 90 min after calcium restoration and stained with anti-PKC α antibody. (arrowheads) Cell–cell contact sites. Bars, 10 μm .

Overexpression of CD151 promotes E-cadherin puncta formation through PKC-dependent signaling pathway

Given enhanced cell–cell adhesion and cell polarization by CD151 overexpression, the prominent filopodial extension by basal engagement of CD151 raised the possibility that CD151 might be involved in the initial stage of cell–cell adhesion through regulating filopodia-based adhesion zipper formation. To explore this possibility, we examined the effect of CD151 overexpression on the formation of E-cadherin puncta, an indication of the initial stage of E-cad-

herin-mediated cell–cell adhesion (Adams et al., 1998), by the calcium switch experiment. E-Cadherin puncta were not detectable at 10 min after calcium restoration, but became evident after 60 min (Fig. 7 A). The number and size of the E-cadherin puncta were significantly greater in CD151-overexpressing cells than control GFP-transfected cells, indicating that the initial stage of E-cadherin-mediated cell–cell adhesion was accelerated upon overexpression of CD151. It was noted that CD151-GFP was also brought into punctate aggregates, partially overlapping the E-cadherin puncta.

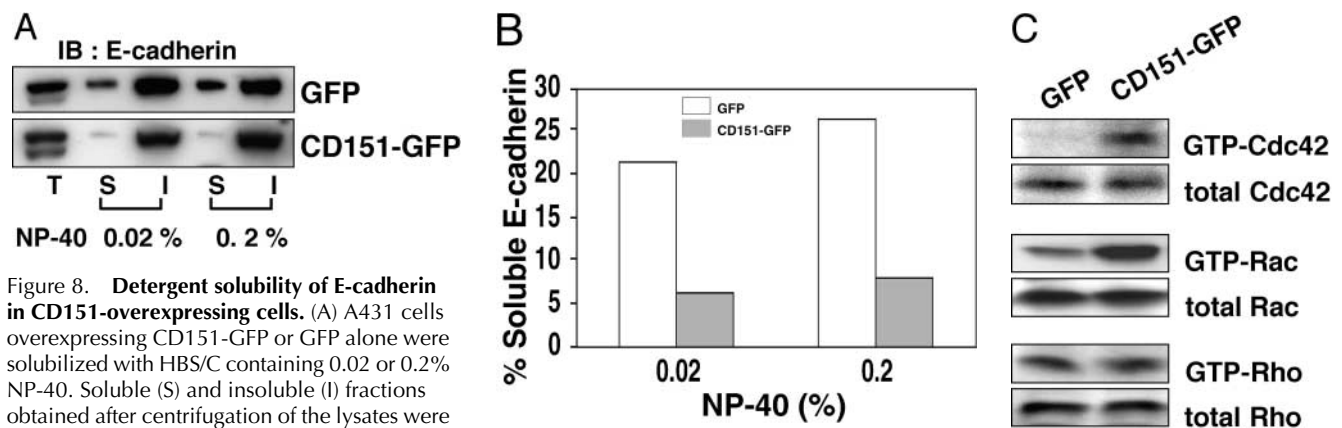


Figure 8. Detergent solubility of E-cadherin in CD151-overexpressing cells. (A) A431 cells overexpressing CD151-GFP or GFP alone were solubilized with HBS/C containing 0.02 or 0.2% NP-40. Soluble (S) and insoluble (I) fractions obtained after centrifugation of the lysates were subjected to SDS-PAGE, followed by immunoblotting with anti-E-cadherin mAb. T, total lysates before centrifugation. (B) The recovery of E-cadherin in the soluble fractions was quantified by densitometry and expressed as the percentage of the total E-cadherin (i.e., soluble plus insoluble fractions). (C) Levels of GTP-loaded Cdc42, Rac, and Rho in CD151-overexpressing cells. Subconfluent A431 cells overexpressing either CD151-GFP or GFP alone were lysed in lysis buffer containing 1% NP-40; and the lysates were subjected to pull-down assays of GTP-loaded Cdc42, Rac, and Rho, as described in Materials and methods.

Because the filopodial extension by basal engagement of CD151 was shown to be cPKC dependent, we examined whether formation of E-cadherin puncta was also cPKC dependent. Treatment of cells with calphostin C strongly inhibited the assembly of E-cadherin puncta upon calcium restoration, whereas treatment with TPA, an activator of cPKC, promoted the E-cadherin puncta formation and their subsequent coalescence into a beltlike structure (Fig. 7 B). Recruitment of CD151-GFP to cell-cell contact sites was also inhibited by calphostin C, but promoted by TPA. These results indicate that cPKC is involved in not only the assembly of E-cadherin puncta but also in the recruitment of CD151 to cell-cell contact sites.

Among known cPKCs, PKC α has been shown to be the major cPKC isoform in A431 cells (Szekeres et al., 2000). Because activation of PKC α has been shown to be associated with its translocation to plasma membrane (Vallentin et al., 2001), we examined the localization of PKC α in control and CD151-overexpressing A431 cells. Although PKC α only accumulated to a marginal extent at cell-cell contact sites in control cells, a clear accumulation of PKC α was observed with CD151-overexpressing cells (Fig. 7 C). The enhanced accumulation of PKC α at cell-cell contacts was not due to the increased expression of PKC α because the level of PKC α expression remained unchanged in CD151-overexpressing cells (unpublished data). A significant fraction of CD151-GFP was found to colocalize with PKC α at cell-cell contact sites, consistent with a previous paper that PKC α coprecipitated with CD151 (Zhang et al., 2001).

CD151 overexpression promotes E-cadherin anchorage to cytoskeletal matrix

Throughout E-cadherin-mediated cell-cell adhesion, anchorage of E-cadherin to actin filaments is a critical, rate-limiting event securing the mechanical strength of intercellular adhesion. Given the possible role of CD151 in regulating actin cytoskeleton, it is conceivable that the anchorage of E-cadherin to actin cytoskeleton is enhanced in cells overexpressing CD151. To explore this possibility, cells

were lysed with NP-40 and the amounts of E-cadherin in detergent-soluble (cytoplasmic) and -insoluble (cytoskeleton associated) fractions were determined by immunoblotting. In control cells, the majority of the E-cadherin was recovered in the insoluble fractions, leaving 20–25% in the soluble fractions, depending on the detergent concentration used (Fig. 8, A and B). However, in CD151-overexpressing cells, most of the E-cadherin was recovered in the insoluble fractions with <10% remaining in the soluble fractions, supporting the possibility that CD151 overexpression promotes the anchorage of E-cadherin to actin cytoskeletal matrix.

To further explore the effect of CD151 overexpression on actin cytoskeleton, we examined the levels of GTP-loaded forms of Rho family GTPases in control and CD151-overexpressing cells. Although the level of GTP-loaded Rho remained unaffected upon CD151 overexpression, the levels of GTP-loaded Cdc42 and Rac were significantly elevated in CD151-overexpressing cells (Fig. 8 C). Given the role of Rac activation in the assembly of cortical actin filaments (Takaishi et al., 1997; Jou and Nelson, 1998), these results are consistent with enhanced E-cadherin anchorage to actin cytoskeleton in CD151-overexpressing cells.

E-cadherin is not a prerequisite for CD151-mediated actin reorganization

Although E-cadherin serves as one of the regulators of actin dynamics during epithelial cell-cell adhesion and polarization, we finally addressed the possibility that homophilic interaction of E-cadherin might be involved in the filopodial extension induced by basal engagement of CD151. Therefore, we used mouse L cells lacking cadherin activity and their E-cadherin transfectants (designated EL cells). When L and EL cells were transfected with CD151-GFP and plated on the substrates coated with anti-CD151 mAb, both cells extended numerous filopodia regardless of whether E-cadherin was present or absent (Fig. 9). These results indicate that basal engagement of CD151 by itself can trigger the Cdc42-dependent filopodial extension without collaboration with E-cadherin.

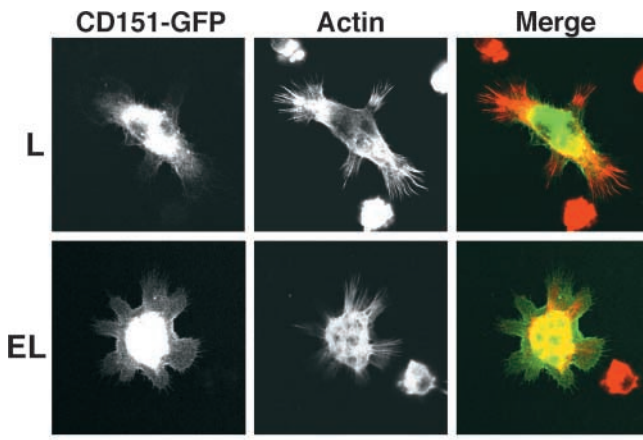


Figure 9. Actin reorganization in mouse L cells upon basal engagement of CD151. Mouse L cells (top row) and E-cadherin–transfected EL cells (bottom row) were transiently transfected with CD151-GFP and replated on coverslips coated with 20 $\mu\text{g/ml}$ anti-CD151 mAb at a low density. Cells were incubated on the coverslips for 3 h at 37°C, fixed, permeabilized, and stained with rhodamine-labeled phalloidin for actin. Bar, 10 μm .

Discussion

The formation of epithelial adherens junctions is a cadherin-mediated process associated with reorganization of the actin cytoskeleton (Braga, 2000; Fukata and Kaibuchi, 2001). Recently, a model of filopodia-based cell–cell adhesion, termed the “adhesion zipper” model, has been proposed based on the observations of epithelial adhesion during embryogenesis of worms and flies (Raich et al., 1999; Jacinto et al., 2000), and of mouse keratinocyte adhesion (Vasioukhin et al., 2000). In this model, two neighboring cells send out filopodia that, upon contact, slide along each other and project into the opposing cell’s membrane, thereby forming a double row of E-cadherin puncta. Consistent with this model are earlier observations that E-cadherin coalesces into punctate aggregates (i.e., E-cadherin puncta) in the initial stage of E-cadherin–mediated cell–cell adhesion, followed by maturation of the primordial puncta into beltlike adherens junctions (Yonemura et al., 1995; Adams et al., 1998). Based on this model, our data indicate that CD151 can transduce signals triggering actin reorganization by directly or indirectly activating Cdc42, thereby accelerating filopodia-based adhesion zipper formation. This view is supported by two lines of evidence: induction of filopodial extension by basal engagement of CD151, and accelerated E-cadherin puncta formation in CD151-overexpressing cells. The enhanced activation of Cdc42, along with Rac in CD151-overexpressing cells, further supports this possibility. It should be noted that filopodial induction by basal engagement of CD151 did not require the presence of E-cadherin on the cell surface because cadherin-deficient L cells were as equally active as E-cadherin–transfected EL cells in filopodial extension upon basal engagement of CD151. These results indicate that CD151 can transduce signals triggering filopodial extension without collaboration with E-cadherin. However, do not exclude the possibility that E-cadherin is also involved in Cdc42-dependent filopodial extension in epithelial cells be-

cause calcium-stimulated E-cadherin homophilic interaction has been shown to activate Cdc42 at cell–cell contact sites (Kim et al., 2000).

Another line of evidence for the role of CD151 in actin dynamics is the observation that treatment of cells with anti-CD151 mAb impaired not only epithelial polarization but also the assembly of cortical actin belts. Rac has been implicated in the actin reorganization into cortical belts because assembly of cortical actin filaments was potentiated by the dominant-active form of Rac (Takaishi et al., 1997; Jou and Nelson, 1998). Enhanced activation of Rac in CD151-overexpressing cells indicates that CD151 is involved in actin assembly into cortical belts through Rac activation, although the underlying mechanism remains to be defined. Given the accumulating evidence that homophilic interaction of E-cadherin activates Rac at cell–cell contact sites (Nakagawa et al., 2001; Noren et al., 2001; Betson et al., 2002), accelerated E-cadherin puncta formation in CD151-overexpressing cells may be responsible for the enhanced Rac activation in these cells. It is generally accepted that strong cadherin-mediated cell–cell adhesion requires anchorage of cadherins to cortical actin filaments via catenins. Because E-cadherin anchorage to detergent-insoluble actin cytoskeletal matrix was enhanced in CD151-overexpressing cells, it is conceivable that CD151 overexpression promotes E-cadherin–mediated cell–cell adhesion by potentiation of not only filopodia-based adhesion zipper formation at an initial stage but also E-cadherin anchorage to cortical actin filaments through enhanced Rac activation at later stages.

Our data show that cPKC is involved in the CD151-mediated actin cytoskeletal dynamics. Thus, calphostin C, a cPKC inhibitor, blocked filopodial extension induced by substrate-adsorbed anti-CD151 mAb as well as enhanced E-cadherin puncta formation in CD151-overexpressing cells. Furthermore, translocation of PKC α , the major cPKC isoform in A431 cells (Szekeres et al., 2000), to cell–cell contact sites was significantly promoted in CD151-overexpressing cells. These results are consistent with a recent paper that CD151 and some other tetraspanins coprecipitate with cPKC (Zhang et al., 2001). In support of the physical association of CD151 with cPKC, we found that a significant fraction of CD151 colocalized with PKC α during the calcium-stimulated epithelial cell–cell adhesion. Together, these results indicate that CD151 directly or indirectly associates with PKC α , thereby regulating actin reorganization during filopodia-based cell–cell adhesion and subsequent assembly of cortical actin filaments. Involvement of cPKC in filopodial extension has also been demonstrated with epithelial and neuronal cells (Beckmann et al., 1995; Cheng et al., 2000).

It remains unclear what activates the CD151-dependent signaling pathway that, in turn, leads to the activation of Cdc42 and Rac during epithelial cell–cell adhesion. One possible scenario is that an initial E-cadherin homophilic interaction induces local clustering of CD151, which in turn activates Cdc42 via cPKC, thereby promoting filopodia-based E-cadherin puncta formation. Thus, there is a positive feedback loop between the E-cadherin homophilic interaction and the CD151-dependent actin reorganization. In

fact, calcium switch experiments showed that E-cadherin puncta formation was associated with the clustering of CD151 into punctate aggregates. Another scenario for the mechanism activating the CD151 signaling pathway could be that there is an unknown counter-receptor for CD151 on the epithelial cell surface, which interacts with CD151 in trans at cell–cell contact sites. The interaction of the substrate-adsorbed mAb with CD151 on the cell surface may mimic such physiological transinteraction of CD151 with its counter-receptor, thereby triggering the signaling pathway downstream of CD151. Indeed, there is accumulating evidence that ligand capture by substrate-immobilized antibodies mimics cellular responses induced by physiological ligand–receptor interactions (Miyamoto et al., 1995; Fang et al., 1999). Although no counter-receptor for CD151 has ever been identified, CD9 was recently identified as the receptor for the pregnancy-specific glycoprotein 17 (Waterhouse et al., 2002).

CD151 forms a stable complex with integrin $\alpha 3 \beta 1$ and localizes at cell–cell contact sites. Although integrin $\alpha 3 \beta 1$ is the primary adhesion receptor for laminin-5 and laminin-10/11, and it plays a central role in adhesion of epithelial cells to basement membranes, its preferential localization at cell–cell contact sites implies that it may also be involved in cell–cell adhesion. In support of this possibility, integrin $\alpha 3 \beta 1$ has been shown to interact with integrin $\alpha 2 \beta 1$ at cell–cell contact sites (Symington et al., 1993). The role of integrin $\alpha 3 \beta 1$ in epithelial cell–cell adhesion was also addressed by Weitzman et al. (1995), who examined the intercellular adhesion of integrin $\alpha 3$ –transfected cells but obtained no clear evidence supporting the role of integrin $\alpha 3 \beta 1$ in cell–cell adhesion. Recently, Wang et al. (1999) reported that the assembly of cortical actin filaments was severely impaired in integrin $\alpha 3 \beta 1$ –deficient epithelial cells. The anchorage of the E-cadherin–catenin complex to the actin cytoskeleton was also significantly impaired in these cells. Given the stable association of integrin $\alpha 3 \beta 1$ with CD151 and the role of CD151 in E-cadherin–mediated cell–cell adhesion, it is tempting to speculate that integrin $\alpha 3 \beta 1$ also serves as a modulator of E-cadherin–mediated cell–cell adhesion and actin reorganization through its tight association with CD151. Although the mechanisms operating downstream of CD151 still remain to be defined, studies on the role of the integrin $\alpha 3 \beta 1$ –CD151 complex in actin cytoskeletal organization in epithelial cells should shed light on the delineation of the mechanisms underlying epithelial cell–cell adhesion and polarization.

Materials and methods

Cells and cell cultures

A431, HeLa S3 (human cervix adenocarcinoma cells), Caco-2, WI-38, mouse L cells, and E-cadherin–transfected EL cells (Ozawa et al., 1989) were maintained in DME supplemented with 10% FBS. HT1080, T98G, and MKN45 (human gastric carcinoma cells) were maintained in RPMI 1640 supplemented with 10% FBS.

Antibodies and reagents

The mAb against E-cadherin (ECCD-2) was obtained from TAKARA Shuzo. The mAb against Rac was obtained from Transduction Laboratories. Polyclonal antibodies against Cdc42, Rho, ZO-1, and PKC α were obtained

from Santa Cruz Biotechnology, Inc. The mAb against $\alpha 2$ integrin was obtained from Chemicon. Polyclonal antibody against actin was obtained from Sigma-Aldrich. The mAb 3G8 against $\alpha 3$ integrin was prepared as described previously (Kikkawa et al., 2000). The mAb 8C3 against CD151 was isolated along with the mAb 3G8 as another mAb against $\alpha 3$ integrin, but was later found to recognize CD151 tightly associated with integrin $\alpha 3 \beta 1$ (Stern et al., 2000; Yanez-Mo et al., 2001). The mAb 4F5 against $\alpha 5$ integrin was produced by immunizing mice with purified integrin $\alpha 5 \beta 1$ as described previously (Manabe et al., 1997). The mAb against HA was purchased from BAbCo. TRITC-conjugated anti- $\alpha 3$ integrin mAb was prepared as described by Goding (1976). Rhodamine-labeled phalloidin was obtained from Molecular Probes Inc.; calphostin C was obtained from Wako; rottlerin was obtained from Calbiochem; LY294002 was obtained from Sigma-Aldrich. Y27632, a ROCK inhibitor, was a gift from A. Yoshimura (Yoshitomi Pharmaceutical, Saitama, Japan). Laminin-5 was purified from the conditioned medium of MKN45 cells by immunoaffinity chromatography as described previously (Fukushima et al., 1998). Fibronectin was purified from human plasma by gelatin affinity chromatography.

Expression plasmids

A full-length cDNA encoding human CD151 (Hasegawa et al., 1996) was inserted in frame into pEGFP-N3 (CLONTECH Laboratories, Inc.) at the HindIII and Apal sites to produce an expression vector (pEGFP-CD151) for CD151 fused with EGFP at its COOH terminus. The expression plasmids for GST-RB (a fusion protein of GST to the Rho-binding domain of Rho kinase) and GST-CRIB (a fusion protein of GST to the Cdc42/Rac-interactive-binding domain of PAK1) as well as the dn forms of HA-tagged Cdc42 and Rho were provided by K. Kaibuchi (Nagoya University Medical School, Nagoya, Japan).

DNA transfection and selection of stable transfectants

A431 cells were transfected with pEGFP-CD151 by electroporation using a Gene Pulser (Bio-Rad Laboratories). Cells were passaged at a 1:3 dilution 12 h after transfection and maintained in medium containing 1 mg/ml G418 to select stable transfectant clones. A431 cells were also transfected with pEGFP-N3 as a control. 20 μ g of expression plasmids of dn-Cdc42 and dn-Rho were cotransfected with 5 μ g of pHA262Puro, a puromycin-resistant marker, into 3×10^6 HeLa S3 cells using LipofectAMINETM Plus (GIBCO BRL). Cells were passaged at a 1:3 dilution 12 h after transfection and maintained for 48 h in medium containing 10% FBS and 1 μ g/ml puromycin.

Cell labeling and immunoprecipitation

A431 transfectants were washed twice with biotinylation buffer (0.1 M HEPES-HCl, pH 8.0, 50 mM NaCl, 1 mM PMSF, and 20 μ g/ml leupeptin) and surface labeled with 2 mg/ml sulfo-NHS-LC-biotin for 20 min at RT. Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 0.1% SDS, 0.5% deoxycholate, 1% NP-40, 150 mM NaCl, 1 mM PMSF, and 2 μ g/ml leupeptin) and immunoprecipitated with the indicated mAbs. The precipitates were eluted into sample treatment buffer and resolved by 10% SDS-PAGE under nonreducing conditions. Proteins were transferred to PVDF membranes and visualized with HRP-conjugated streptavidin.

Immunofluorescence

Cells were fixed with 3% PFA or in methanol, permeabilized with 0.1% Triton X-100 for 5 min, and incubated with either appropriate primary antibodies or rhodamine-labeled phalloidin for 1 h at RT, followed by incubation with secondary antibodies for 1 h. The secondary antibodies used were Alexa 596-labeled goat anti-rabbit IgG (Molecular Probes) or Cy3-labeled goat anti-rat IgG (Jackson ImmunoResearch Laboratories). After three washes with PBS, cells were mounted and examined with a confocal microscope (model LSM5 PASCAL; Carl Zeiss MicroImaging, Inc.). All images were imported into Adobe Photoshop as TIFFs for contrast manipulation and figure assembly.

Pretreatment of cells with mAbs

To examine the effects of mAb treatment on epithelial integrity, cells were detached with trypsin/EDTA, washed twice with DME containing 1% BSA, and resuspended in the same medium containing 20 μ g/ml of the mAbs to be examined. Cells were kept in suspension for 10 min at RT, and then plated on substrates coated with 10 μ g/ml laminin-5. The cells were incubated on the substrates for 6 h at 37°C, fixed, and stained with appropriate antibodies or rhodamine-labeled phalloidin. The stained cells were examined with a confocal microscope.

Pull-down assay of GTP-loaded Cdc42, Rac, and Rho

Pull-down assays of GTP-loaded Cdc42, Rac, and Rho were performed as described previously (Gu et al., 2001), except that cells were grown to confluence in serum-containing medium before the assay. Cells adhering to the substrates as specified were lysed in 50 mM Tris-HCl, pH 7.4, containing 1% NP-40, 100 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM PMSF. The lysates were clarified by centrifugation at 20,000 g for 15 min at 4°C, and then incubated with either 30 µg of GST-CRIB or GST-RB for 60 min at 4°C in the presence of 30 µl of glutathione-agarose beads. The precipitates were washed three times with lysis buffer and resolved in 12% SDS-polyacrylamide gels. After electrophoretic transfer to PVDF membranes, the membranes were probed with anti-Rac mAb or polyclonal antibodies against Cdc42 or Rho.

Wound closure assay

A431 transfectants were seeded on 24-well plates and grown to confluence. The medium was replaced with fresh DME containing 1% FBS 8 h before the onset of the assay. The assay was started by scratching the confluent cells with a pipette tip to make a wound ~0.3 mm wide. Wounded cells were incubated in the same medium at 37°C for 24 h to heal. Cells were photographed at 12 and 24 h after the onset of the assay using a phase-contrast microscope (model CK40; Olympus).

Cell motility assay

The cell motility of individual cells was quantified by time-lapse videomicroscopy. A431 transfectants were replated on 35-mm culture dishes at 2.5×10^5 cells/dish in DME containing 10% FBS and allowed to adhere to the substrates. 12 h after replating, the medium was replaced with DME containing 1% FBS. Cell migration was monitored using an inverted microscope (model S-25; Carl Zeiss Microimaging, Inc.) equipped with a built-in CO₂ incubator. Video images were collected at 15-min intervals using Image-Pro software (Media Cybernetics). The positions of the nuclei were tracked to quantify the cell motility. Velocities were calculated in micrometers per 8 h using the same software.

Detergent extraction of actin and E-cadherin

Detergent extraction of E-cadherin from A431 transfectants was performed as described previously (Nagafuchi and Takeichi, 1988). In brief, subconfluent cells grown on 6-cm culture dishes were collected into a 1.5-ml centrifuge tube with a rubber policeman, centrifuged at 5,000 g for 1 min, and then lysed in 200 µl HBS/C (HEPES-buffered saline containing 1 mM CaCl₂) containing 0.02 or 0.2% NP-40 on ice for 10 min with gentle pipetting. The lysates were centrifuged at 100,000 g for 30 min, and the supernatants were mixed with 100 µl of 3× SDS sample treatment buffer and used as the detergent-soluble fractions. The pellets were dissolved in 300 µl of 1× SDS sample treatment buffer and used as the detergent-insoluble fractions. The detergent-soluble and -insoluble fractions were subjected to SDS-PAGE, followed by immunoblotting with antiactin polyclonal antibody or anti-E-cadherin mAb. Densitometric quantification of the relative intensity of the bands visualized with an ECL chemiluminescence detection kit (Amersham Biosciences) was performed using NIH Image software.

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