Rac-WAVE-mediated actin reorganization is required for organization and maintenance of cell-cell adhesion

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Accepted 23 October 2006
Journal of Cell Science 120, 86-100 Published by The Company of Biologists 2007
doi:10.1242/jcs.03311

Summary

During cadherin-dependent cell-cell adhesion, the actin cytoskeleton undergoes dynamic reorganization in epithelial cells. Rho-family small GTPases, which regulate actin dynamics, play pivotal roles in cadherin-dependent cell-cell adhesion; however, the precise molecular mechanisms that underlie cell-cell adhesion formation remain unclear. Here we show that Wiskott-Aldrich syndrome protein family verprolin-homologous protein (WAVE)-mediated reorganization of actin, downstream of Rac plays an important role in normal development of cadherin-dependent cell-cell adhesions in MDCK cells. Rac-induced development of cadherin-dependent adhesions required WAVE2-dependent actin reorganization. The process of cell-cell adhesion is divided into three steps: formation of new cell-cell contacts, stabilization of these new contacts and junction maturation. WAVE1 and WAVE2 were expressed in MDCK cells. The functions of WAVE1 and WAVE2 were redundant in this system but WAVE2 appeared to play a more significant role. During the first step, WAVE2-dependent lamellipodial protrusions facilitated formation of cell-cell contacts. During the second step, WAVE2 recruited actin filaments to new cell-cell contacts and stabilized newly formed cadherin clusters. During the third step, WAVE2-dependent actin reorganization was required for organization and maintenance of mature cell-cell adhesions. Thus, Rac-WAVE-dependent actin reorganization is not only involved in formation of cell-cell adhesions but is also required for their maintenance.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/1/86/DC1

Key words: Wave, Rac, Arp2/3, Cell-cell adhesion, Actin

Introduction

During epithelial cell-cell adhesion, the actin cytoskeleton undergoes significant reorganization. This process has been studied in various epithelial cells (Adams et al., 1998; Adams et al., 1996; Vaezi et al., 2002; Vasioukhin et al., 2000). At sites of cell-cell contact, the arrangement of actin filaments changes in response to changes in cadherin-dependent adhesions. In MDCK cells, before cell-cell contacts form, lamellipodial structures are visible and facilitate formation of cell-cell adhesions. When cells adhere with each other, cadherin clusters appear, and thin actin bundles are formed and associate with these clusters. As cell-cell contacts lengthen, new actin filaments are recruited along the cadherin-dependent cell-cell adhesions. During adhesion maturation, further accumulation of actin filaments is observed.

There are two populations of actin – junctional actin and peripheral thin bundles – that show different dynamics and play distinct roles during cell-cell adhesion formation (Zhang et al., 2005). Junctional actin is more dynamic than that in peripheral thin bundles and stabilizes cadherin-dependent cell-cell contacts. Thin bundles are required for development of epithelial cuboidal morphology.

The significance of the actin cytoskeleton in cell-cell adhesion has been established by experiments with the actin-disruptive agent cytochalasin D (Adams et al., 1998; Chu et al., 2004; Vasioukhin et al., 2000). Treatment of cells with cytochalasin D blocked formation of cell-cell adhesion in primary keratinocytes and decreased the strength of cell-cell adhesions in MDCK cells. However, it has been reported that the cadherin-catenin complex does not bind actin filaments directly at cell-cell contact sites (Drees et al., 2005; Yamada et al., 2005). The role of the actin cytoskeleton in the formation of cell-cell adhesions is not fully understood.

Recently, it was reported that two actin nucleators, the Arp2/3 complex and formin-1, are involved in actin reorganization at cell-cell contact sites (Bershadsky, 2004; Helwani et al., 2004; Kobielak et al., 2004; Kovacs et al., 2002; Verma et al., 2004). Whereas the Arp2/3 complex forms a branched actin meshwork, formin-1 forms an unbranched actin structure. These nucleators are involved in the formation of these distinct actin structures at sites of cell-cell adhesion; however, the relationship between them remains unknown. Thus, the mechanisms underlying actin reorganization during cell-cell adhesion are not yet fully understood.

The Rho family small GTPases, including Rho, Rac and Cdc42, regulate reorganization of the actin cytoskeleton and are involved in cadherin-dependent cell-cell adhesion (Betson et al., 2002; Braga et al., 2000; Braga et al., 1999; Braga et al., 1997; Braga and Yap, 2005; Fukata et al., 1999; Jou and Nelson, 1998; Jou et al., 1998; Kodama et al., 1999; Kuroda et al., 1997; Kuroda et al., 1998; Takaishi et al., 1997). The involvement of Rac in this process has been well characterized. Rac is recruited to sites of cadherin-dependent cell-cell adhesion and then activated in a phosphatidylinositol-3-kinase-

dependent manner (Ehrlich et al., 2002; Nakagawa et al., 2001). Such activation of Rac is also caused by nectins and is mediated by Rac-GDP/GTP exchange factors (Sakisaka and Takai, 2004). Expression of a constitutively active mutant of Rac (RacCA) caused accumulation of E-cadherin and actin filaments at cell-cell contact sites (Takaishi et al., 1997). By contrast, when a dominant-negative form of Rac (RacDN) was expressed, normal cadherin-dependent development of cell-cell adhesions was inhibited, and the strength of the cell-cell adhesions was decreased (Chu et al., 2004; Ehrlich et al., 2002). Although IQGAP1, a downstream effector of Rac, is known to be involved in Rac-mediated development of cell-cell adhesions, the precise mechanism by which Rac regulates cell-cell adhesion and actin reorganization remains unknown (Noritake et al., 2004).

In mammals, the Wiskott-Aldrich syndrome protein (WASP) family consists of five members, WASP, N-WASP and WASP family verprolin homologous proteins (WAVEs) (Takenawa and Miki, 2001). WASP family proteins induce Arp2/3-dependent actin polymerization and are involved in actin reorganization downstream of Rho family small GTPases. WASP and N-WASP function downstream of Cdc42 and are involved in filopodia formation, whereas WAVEs are essential for development of Rac-mediated membrane protrusions such as lamellipodia and membrane ruffles. Whereas WASP and N-WASP associate directly with the active form of Cdc42, the interactions among WAVEs and Rac are indirect. In the case of WAVE2, the intermediary protein linking Rac and WAVE2 is IRSp53, a substrate for the insulin receptor with an unknown function (Miki and Takenawa, 2002; Miki et al., 2000). Activated Rac binds to the N-terminus of IRSp53, and the C-terminal Src-homology-3 domain of IRSp53 binds to WAVE2 to form a trimolecular complex. However, this intermediary does not bind WAVE1 or WAVE3. Purified WAVE1 is constitutively active; however, it is proposed that WAVE1 is kept inactive in vivo through its association with four proteins: Nap125, PIR121, Abi2 and HSPC300 (Eden et al., 2002). This complex cannot stimulate actin polymerization in vitro; however, addition of purified, active Rac relieves this inhibition. However, other groups have reported that this complex is stable in vivo and can activate Arp2/3 complex in vitro (Stradal et al., 2004). The functions of this WAVE-regulatory complex thus remain controversial (Blagg et al., 2003; Ibarra et al., 2006; Innocenti et al., 2004; Kunda et al., 2003; Leng et al., 2005; Rogers et al., 2003; Steffen et al., 2004; Suetsugu et al., 2006).

In the present study, we show that Rac-WAVE-mediated actin reorganization is required for organization and maintenance of cell-cell adhesions. We propose a novel mechanism by which Rac regulates cell-cell adhesion and describe the relationship between cadherin-dependent adhesions and actin filaments.

Results

The role of actin reorganization at the site of cell-cell contact in confluent cell layers

To examine the role of actin dynamics in mature cell-cell adhesions, confluent cell layers were treated with cytochalasin D, which binds the barbed ends of actin filaments and inhibits actin polymerization. Cytochalasin D treatment caused gradual disruption of actin structures, which recovered after washout of the drug (Fig. 1A). These findings indicate that actin structures are not stable but are instead dynamically reorganized at cell-cell contact sites. With the collapse of actin structures, cadherin-dependent cell-cell adhesions also became disorganized (Fig. 1A, supplementary material Fig. S1). In cell layers treated with cytochalasin D, cell-cell adhesion did not disappear but accumulation of adhesion proteins and the height at the lateral domain of cell-cell adhesions decreased (Fig. 1B,C). We quantified the height of lateral domains of cell-cell adhesions with vertical sections of the cytochalasin-D-treated cells. The treatment with cytochalasin D for 120 minutes decreased the length of cell-cell adhesions (5.04±0.84 μm in control cells; 3.39±0.51 μm in cytochalasin-D-treated cells; $P<0.0001$). Cell-cell adhesions extended parallel to the substrate when treated with cytochalasin D (Fig. 1B), supporting the idea that cells overlapped. Because washout of cytochalasin D reversed the defects in cell-cell adhesion, continuous actin reorganization is required to maintain mature cell-cell adhesion (Fig. 1A-C). These data indicate that actin reorganization is necessary for maintenance of organized, mature cell-cell adhesions.

WAVE2 is concentrated at cell-cell contact sites in MDCK cells

It has been reported that Rac, which regulates actin reorganization, plays a pivotal role in formation of cell-cell adhesions between epithelial cells (Takaishi et al., 1997). However, the relationship between actin reorganization and cell-cell adhesions is unclear. To clarify the molecular mechanism that underlies Rac regulation of cell-cell adhesion, we investigated the role of WAVE family proteins, which are essential for Rac-induced actin reorganization, during cell-cell adhesion formation. WAVE1 and WAVE2 were expressed in MDCK cells. The level of WAVE2 expression in MDCK cells was approximately ten times higher than that in mouse embryonic fibroblasts (MEFs), whereas the level of WAVE1 expression was similar to that in MEFs (Fig. 2A). Therefore, we analyzed the function of WAVE2 first.

Localization of endogenous WAVE2 protein in MDCK cells was investigated with anti-WAVE2 antibody. In confluent cell layers, WAVE2 was preferentially concentrated at cell-cell contact sites and colocalized with E-cadherin and actin filaments through the lateral regions (Fig. 2B). However, WAVE2 did not colocalize with occludin, a tight junction protein (supplementary material Fig. S2). Chelation of extracellular Ca^{2+} with EGTA, disrupted cadherin-dependent cell-cell adhesions and changed localization of WAVE2 (Fig. 2B).

We next investigated the localization of WAVE2 at cell-cell contact sites by in situ extraction with 0.5% Triton X-100 (Hori et al., 2003). When cells were extracted before fixation, cytosolic WAVE2 staining decreased without affecting its localization at cell-cell contacts (Fig. 2C). These results suggest that junctional WAVE2 is recruited in Triton-insoluble complexes.

To examine whether WAVE2 associated with an adhesion protein complex, protein complexes containing WAVE2 were immunoprecipitated with anti-WAVE2 antibody (Fig. 2D). E-cadherin, β-catenin and afadin were coimmunoprecipitated with WAVE2 in lysates of confluent MDCK cell cultures (Fig. 2D, supplementary material Fig. S3). Formation of these complexes was reduced by treatment with cytochalasin D;

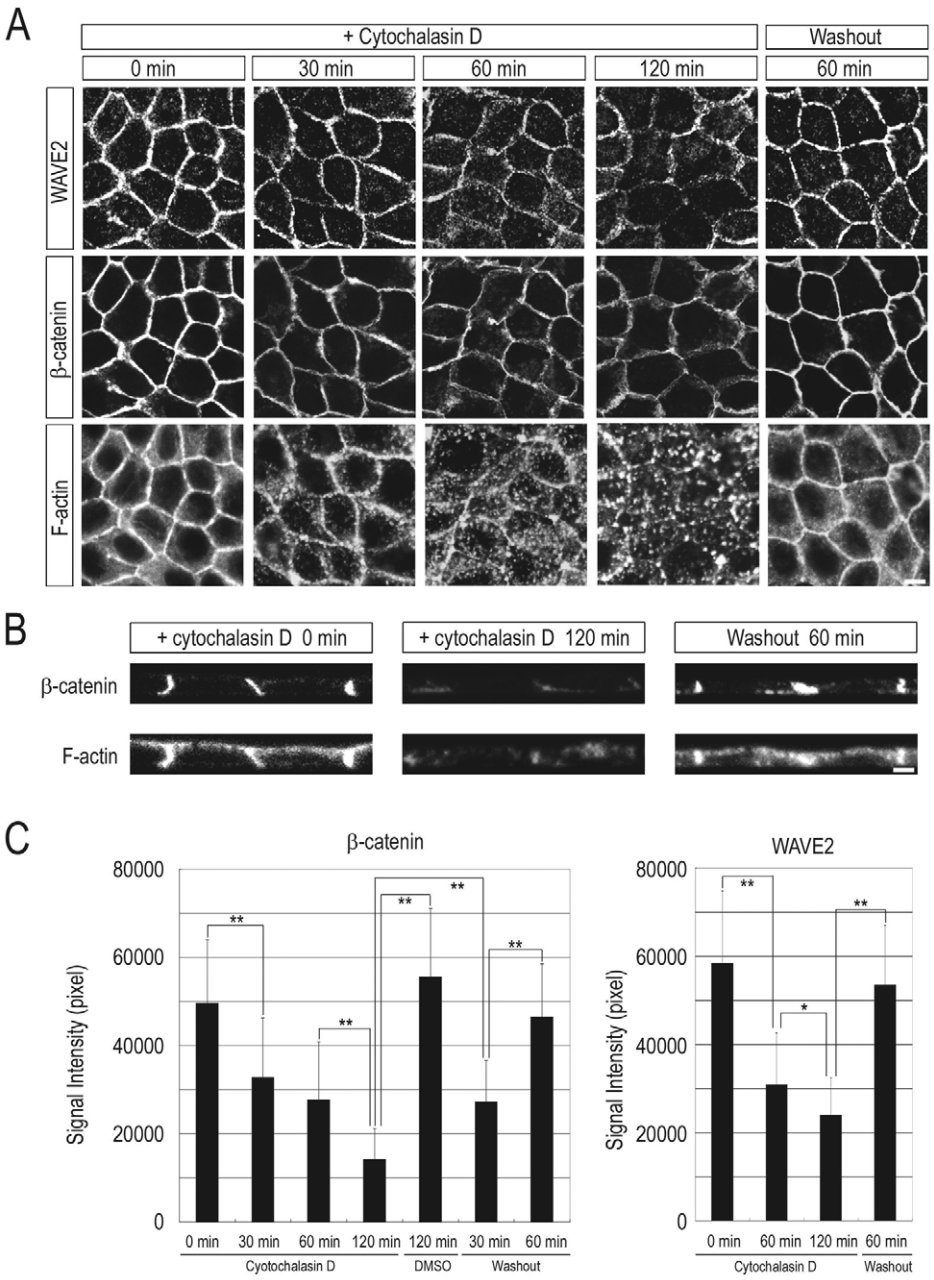

Fig. 1. Effects of cytochalasin D on cell-cell adhesion. MDCK cell layers were treated with DMSO or 10 μM cytochalasin D for the indicated times and then cytochalasin D was washed out, and cells were incubated in fresh medium for the indicated times. (A) Cell layers were fixed and stained for WAVE2, β-catenin and actin filaments (F-actin). Bar, 10 μm. (B) Vertical sections. Bar, 5 μm. (C) The signal intensities for β-catenin and WAVE2 of cross sections as shown in A were quantified with ImageJ software. All data are mean ± s.d. $^{*}P<0.05$; $^{**}P<0.0005$. Statistical significance was examined using the Student's *t*-test.

however, some complexes persisted (Fig. 2D). In cell layers treated with cytochalasin D, some WAVE2 remained at sites of cell-cell adhesion (Fig. 1A,C).

To examine whether WAVE2 localization was dependent on E-cadherin, cell-cell adhesion formation was inhibited with an anti-E-cadherin antibody, DECMA-1 (Fig. 2E). When cells were treated with DECMA-1, some cadherin-dependent cell-cell adhesions were disrupted (Fig. 2E). In the presence of DECMA-1, WAVE2 localization at cell-cell contact sites was abolished, and WAVE2 localized to the tips of lamellipodia that formed at the basal level (Fig. 2E, supplementary material Fig. S4). These data indicate that during cell-cell adhesion formation, WAVE2 localized to cell-cell contact sites and associated with the adhesion protein complex and that this localization was dependent on E-cadherin.

WAVE2 depletion by RNA interference (RNAi)

We attempted to deplete WAVE2 by transfection of a small interfering RNA (siRNA) for WAVE2. The amount of WAVE2 expression in WAVE2 siRNA-treated cells decreased to 30% of that in control siRNA (CTRL) cells (Fig. 3C,E). As the efficiency of transfection was 60-70% in MDCK cells, the remaining proteins were thought to be derived from untransfected cells. Indeed, when WAVE2 siRNA-treated cell layers were stained for WAVE2, the WAVE2 signal was not detected at 60-70% of cell-cell contacts (supplementary material Fig. S5).

In the absence of WAVE2, cell-cell adhesions remained disorganized in some areas, and accumulation of actin filaments decreased although the amounts of E-cadherin and actin did not change (Fig. 3A-C, supplementary material Fig.

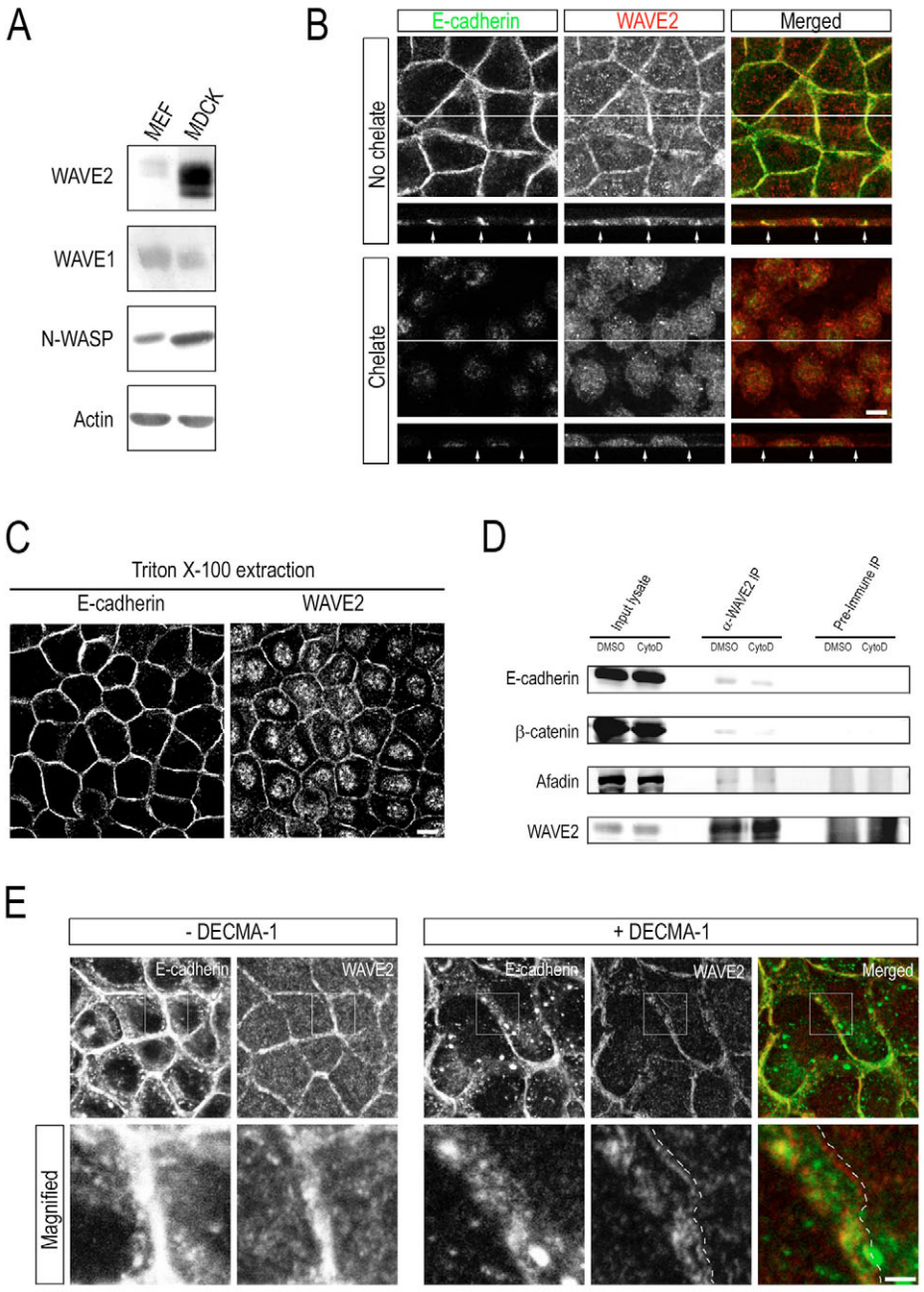

Fig. 2. Expression and localization of WAVE2 in epithelial MDCK cells. (A) Comparison of WAVE1 and WAVE2 expression in MDCK cells and mouse embryonic fibroblasts (MEFs). Cell lysates were immunoblotted with anti-WAVE1 and anti-WAVE2 antibodies. (B) Localization of endogenous WAVE2 in MDCK cells. Before (No chelate) or after (Chelate) treatment with EGTA, MDCK cell layers were fixed and then stained with anti-E-cadherin and anti-WAVE2 antibody. The regions from which vertical sections were taken are indicated by white lines. Arrows indicate cell-cell contacts. Bar, 10 μm. (C) Detergent solubility of WAVE2. MDCK cells were treated with 0.5% Triton X-100 containing buffer before fixation. Fixed cell layers were stained with anti-E-cadherin and anti-WAVE2 antibody. Scale bar, 15 μm. (D) Interaction of WAVE2 with adhesion complex proteins. Protein complexes were immunoprecipitated from lysates of DMSO- or cytochalasin D (CytoD)-treated MDCK cells with anti-WAVE2 antisera. Immunoprecipitates were immunoblotted for E-cadherin, β-catenin, afadin and WAVE2. Negative controls for immunoprecipitations were pre-immune antisera (Pre-Immune IP). (E) DECMA-1 (anti-E-cadherin antibody) inhibits recruitment of WAVE2 to cell-cell contacts. Trypsinized cells were plated on coverslips and treated with DECMA-1. After incubation for 12 hours, cells were fixed and stained for E-cadherin and WAVE2. The lower panels are magnified images of the areas indicated by white squares in each upper panel. Broken lines indicate localization of WAVE2 at the lamellipodial edge. Bars, 15 μm (upper panels); 5 μm (lower panels).

S5). Expression of WAVE2-GFP rescued the defects of cell-cell adhesions caused by WAVE2 RNAi (supplementary material Fig. S6). Thus, WAVE2 appears to be involved in cell-cell adhesions in confluent cell layers. To confirm whether this phenotype was caused by defective actin reorganization, Arp2/3-mediated actin polymerization was inhibited by depletion of Arp2. In Arp2 siRNA-treated cells, expression of Arp2 was suppressed (Fig. 3C) and accumulation of E-cadherin and actin filaments was reduced at the site of cell-cell contacts compared with that in CTRL cells (Fig. 3A,B). Thus, Arp2/3-mediated actin reorganization was required for formation of mature cell-cell adhesions. The disorganized structures in cytochalasin-D-treated cell layers resembled those in WAVE2 or Arp2 siRNA-treated cells (compare Fig. 1A with Fig. 3A).

Depletion of Arp2 by RNAi had a more severe effect on accumulation of E-cadherin and actin filaments at cell-cell contact sites than did depletion of WAVE2 (Fig. 3B). This phenotype in Arp2 siRNA-treated cells may reflect the presence of an Arp2/3 complex activator other than WAVE2 at cell-cell contact sites. WAVE1 also localized at cell-cell contact sites (described below); however, WAVE1 RNAi had no obvious effect on cell-cell adhesion (Fig. 3A,B). However, when both WAVE1 and WAVE2 expression were repressed simultaneously, defects at cell-cell contact sites were more severe than those when only WAVE2 expression was reduced (Fig. 3A,B, supplementary material Fig. S5).

When Abi1 (Rogers et al., 2003) and HSPC300 (Djakovic et al., 2006), which regulate the stability of WAVE proteins, were depleted by RNAi, expression of both WAVE1 and

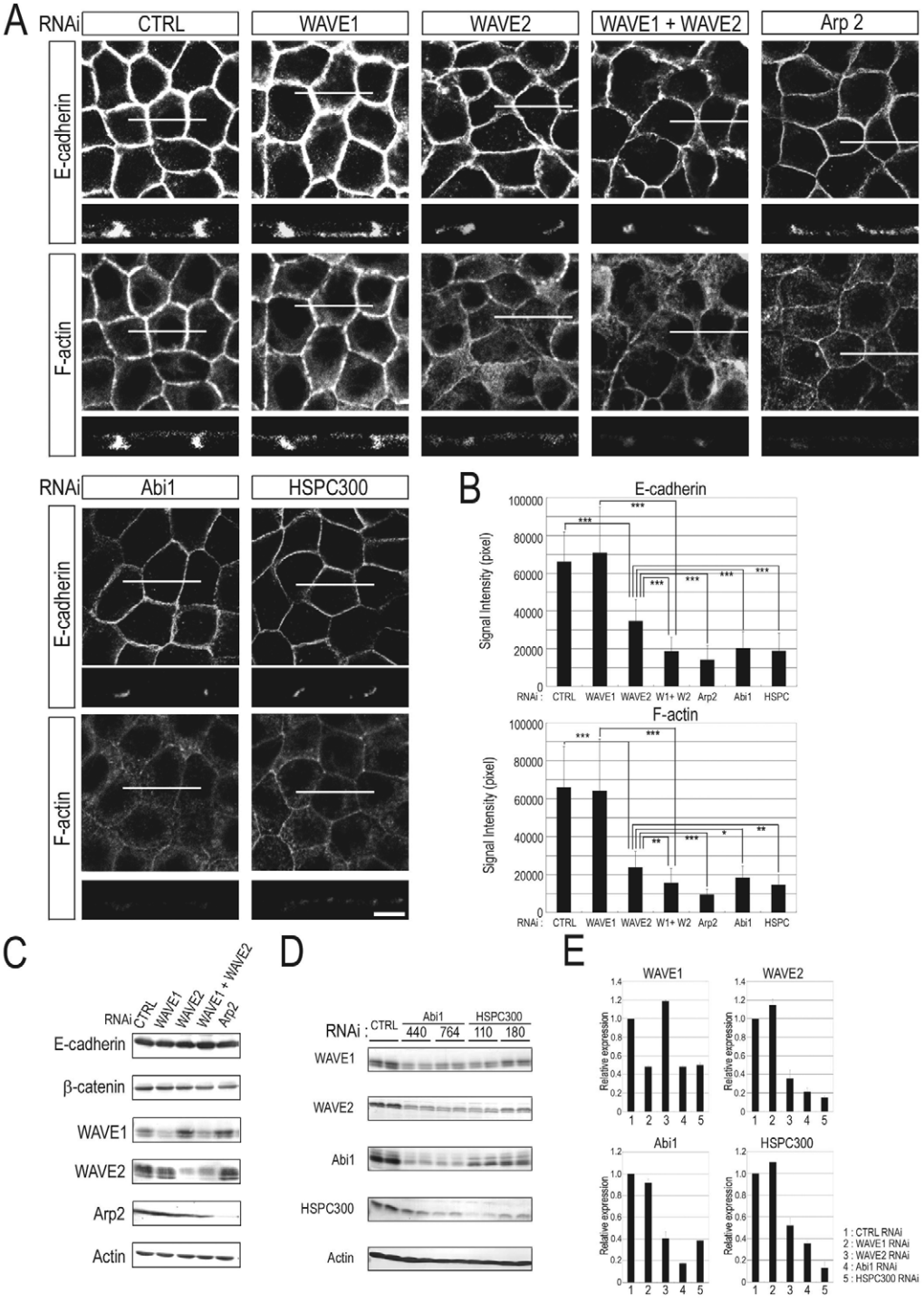

Fig. 3. WAVE1 and WAVE2 have redundant functions at cell-cell adhesions. (A) Cells treated with the indicated siRNA were fixed and stained for E-cadherin and actin filaments. Cross and vertical sections were shown. The regions from which vertical sections were taken are indicated by white lines. Bar, 15 μm (cross sections); 5 μm (vertical sections). (B) Signal intensities of E-cadherin and actin filaments in cross sections were quantified with ImageJ software. All data are mean ± s.d. **P*<0.05; ***P*<0.005; ****P*<0.0001. Statistical significance was examined using the Student's *t*-test. (C) Cells treated with siRNAs against CTRL, WAVE1, WAVE2, WAVE1 + WAVE2 and Arp2 were lysed, and WAVE was detected by immunoblotting. (D) Cells treated with siRNAs against CTRL, Abi1 (440 and 764) and HSPC300 (110 and 180) siRNAs were lysed, and WAVE was detected by immunoblotting. The results of two independent experiments are shown. (E) Signal intensities of WAVE, Abi1 and HSPC300 in D were quantified with ImageJ software. All data are mean ± s.d.

WAVE2 expression was reduced (Fig. 3D,E). Abi1 RNAi or HSPC300 RNAi also had a more significant effect on cell-cell adhesion formation than did WAVE2 RNAi (Fig. 3A,B, supplementary material Fig. S5). These results suggest that WAVE1 and WAVE2 have redundant roles in cell-cell adhesion formation and regulate cell-cell adhesion formation through Arp2/3-mediated actin reorganization.

In primary keratinocytes, VASP/Mena family proteins and formin-1, the regulators of actin reorganization other than Arp2/3 complex, were shown to be essential for cadherin-dependent cell-cell adhesion formation (Kobielak et al., 2004; Vasioukhin et al., 2000). When the function of these proteins were inhibited in MDCK cells, the dominant-negative mutant of VASP showed no effect on cell-cell adhesions but formin-1 RNAi caused defects of cadherin-dependent cell-cell adhesions (supplementary material Figs S7 and S8). Although inhibition of VASP/Mena family proteins and formin-1 function disrupted the structure of cell-cell adhesions completely in primary keratinocytes, the effect of formin-1 RNAi on cell-cell adhesions was less than that of Arp2 RNAi in MDCK cells

(supplementary material Figs S7 and S8). The importance of these regulators of actin reorganization in MDCK cells was different from that in primary keratinocytes.

WAVE2 RNAi delayed formation of new cell-cell contacts

The functions of WAVE1 and WAVE2 were redundant in this system but WAVE2 appeared to play a more significant role. Therefore, we analyzed the functions of WAVE2 during formation of cell-cell adhesions. To confirm the effect of WAVE2 RNAi on actin reorganization at the cell periphery, CTRL and WAVE2 siRNA-treated cells were plated on collagen-coated coverslips. In CTRL cells, cortical actin was reorganized and lamellipodial protrusions were formed at the cell periphery. WAVE2 was localized to at their tips of these protrusions (Fig. 4A). In WAVE2 siRNA-treated cells, cortical actin reorganization and subsequent formation of membrane protrusions were suppressed (Fig. 4A). Thus, WAVE2 is essential for actin reorganization and membrane protrusion formation in MDCK cells.

The process of cell-cell adhesion formation in epithelial cells occurs in three sequential steps. The first step is formation of new cell-cell contacts. These new contacts are stabilized and then junction maturation occurs. In all three steps, actin structures are dynamically reorganized in response to the development of cadherin-dependent adhesions (Vasioukhin and Fuchs, 2001). Therefore, we examined the involvement of WAVE2 in each step of cell-cell adhesion formation.

We first performed a Ca^{2+}-switch assay with CTRL subconfluent cell layers and examined localization of WAVE2 during the first two steps of cell-cell adhesion formation (Fig.

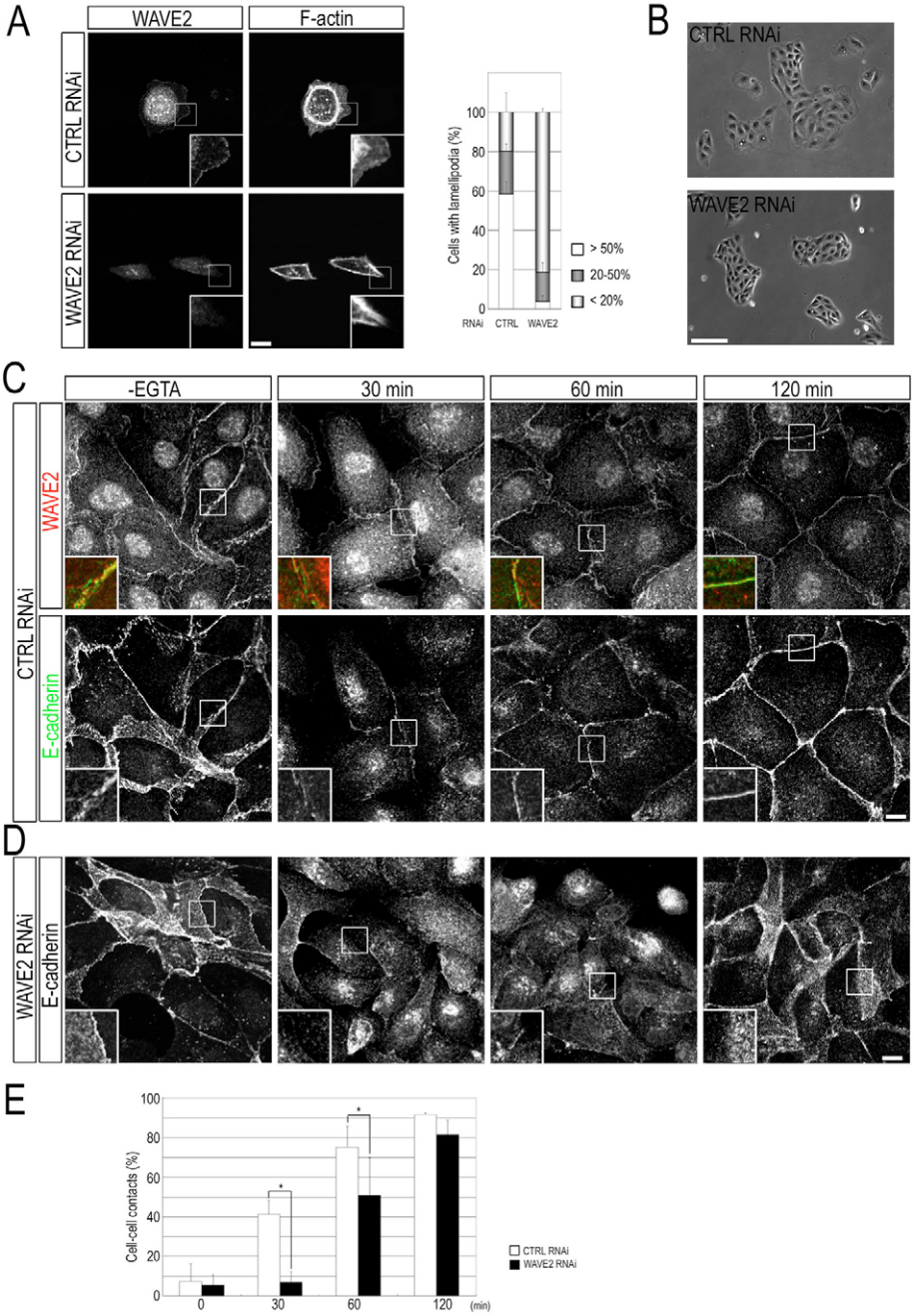

Fig. 4. Effects of WAVE2 RNAi in MDCK cells. (A) Depletion of WAVE2 in MDCK cells inhibits lamellipodium formation on collagen. CTRL or WAVE2 siRNA-treated cells were trypsinized and then plated on collagen. After 4 hours, cells were fixed and stained for WAVE2 and actin filaments (F-actin). Insets in each panel show the magnified images of the areas indicated by white squares. Bar, 10 μm. The lower graph shows the number of cells with lamellipodia. The percentage of cells with lamellipodia covering 50-100% of the cell perimeter was presented as >50%. 20-50%, 20-50% of the perimeter; <20%, 0-20% of the perimeter. Data are mean ± s.d. Three independent experiments were performed. (B) Morphologies of colonies of CTRL or WAVE2 siRNA-treated cells. Bar, 100 μm. (C,D) Ca^{2+}-switch assay with subconfluent MDCK cell layers. Subconfluent CTRL siRNA (C) or WAVE2 siRNA (D) treated cell layers were treated with 5 mM EGTA for 30 minutes. Cell layers were then treated with Ca^{2+}-containing medium for the indicated times. Cell layers were fixed and then stained for WAVE2 and E-cadherin. Merged images of WAVE2 (red) and E-cadherin (green) are shown in insets. Insets in each panel show the magnified images of the areas indicated by white squares. Scale bar, 15 μm. (E) Quantification of cell-cell contacts with E-cadherin staining. The number of cell-cell contacts with E-cadherin staining was counted during the Ca^{2+}-switch assay in C and D. Data are mean ± s.d. *P<0.05. Statistical significance was examined with the Student's t-test. Three independent experiments were performed.

4C). When MDCK cell layers were stained with anti-WAVE2 antibody, perinuclear signals were observed. However, these signals were detected even when WAVE2 siRNA-treated cells were stained (supplementary material Fig. S9). Signals in cytosol and at the cell periphery and cell-cell contacts were completely lost in these cells. Therefore, perinuclear signals were thought to be non-specific. Thirty minutes after the addition of Ca^{2+}, protrusions from the cells made contact with those of other cells and E-cadherin began to cluster at the site of contact (Fig. 4C, 30 min). WAVE2 remained at the tip of each membrane protrusion and did not colocalize with E-cadherin (Fig. 4C, 30 min, inset). This state corresponds to the first step of cell-cell adhesion formation described above. At 60 minutes, E-cadherin lined the cell-cell contact sites continuously and some WAVE2 also colocalized with E-cadherin and actin filaments at cell-cell contact sites (Fig. 4C, supplementary material Fig. S9, 60 min). This corresponds to the transition from the first to the second step of cell-cell adhesion formation. As cadherin-dependent adhesions lengthened and actin filaments lined these adhesions, WAVE2 accumulated at the cell-cell contact sites and colocalized with E-cadherin (Fig. 4C, 120 min). This state corresponds to the second step of adhesion formation: stabilization of cell-cell adhesion. Thus, localization of WAVE2 changed in response to the development of E-cadherin-dependent adhesions during the early steps of cell-cell adhesion formation.

We then performed the Ca^{2+}-switch assay in WAVE2 siRNA-treated cell layers, and the proportion of adherent cells with cadherin-dependent adhesions was compared with that in CTRL cell layers (Fig. 4D,E, supplementary material Fig. S9). In subconfluent cell layers, WAVE2 siRNA-treated cells formed more compact colonies than did CTRL cells (Fig. 4B). In subconfluent layers, cells spread and peripheral cells formed lamellipodial protrusions. In WAVE2 siRNA-treated cell layers, cells did not spread well as shown in Fig. 4A and outer lamellipodial protrusions were not observed. Furthermore, some inner cells overlapped (Fig. 4D, –EGTA). Therefore, WAVE2 siRNA-treated cells tended to form tighter colonies than CTRL cells. Thirty minutes after the addition of Ca^{2+}, localization of E-cadherin was observed at approximately 40% of cell-cell contacts in CTRL cells (Fig. 4E). However, in the absence of WAVE2, new cell-cell contacts with E-cadherin were rare 30 minutes after the addition of Ca^{2+} (Fig. 4D, 30 min; Fig. 4E). WAVE2 siRNA-treated cells did not contact each other sufficiently although their cell density was higher than that of the control cells. At 60 minutes, E-cadherin-dependent adhesions were observed in 50% of WAVE2 siRNA-treated cell-cell contacts; however, these adhesions were shorter than those in CTRL siRNA-treated cells (Fig. 4D, 60 min). At 120 minutes, the proportions of adherent cells did not differ significantly between CTRL and WAVE2 siRNA-treated cells. These data suggest that WAVE2 is needed for efficient formation of cell-cell contacts.

Role of WAVE2 in stabilization of new cell-cell contacts

In WAVE2 siRNA-treated cell layers, E-cadherin showed a diffuse localization, indicating overlap of cellular membranes (Fig. 4D, 120 min) (Vaezi et al., 2002). In CTRL cell layers, such overlap was not visible 120 minutes after addition of Ca^{2+} (Fig. 4C, 120 min) although that was often observed in long-term cultured cell layers (Fig. 4C, –EGTA). Thus, WAVE2 depletion disturbed the stabilization of cell-cell adhesions. In WAVE2 siRNA-treated cell layers, recruitment of actin filaments to new cell-cell contact sites was suppressed (supplementary material Fig. S9). These abnormalities in actin structures were thought to affect the stabilization of cadherin-dependent cell-cell adhesion. In the Ca^{2+}-switch assay shown in Fig. 4C,D, cells formed adhesions with several other cells simultaneously and it was too complicated to observe the precise dynamics of E-cadherin and actin structures at specific cell-cell contact sites during stabilization of cell-cell adhesions. Therefore, trypsinized cells were spread on collagen-coated coverslips and the process of cell-cell adhesion stabilization between two cells was observed (Fig. 5). Under these conditions, we could analyze precisely the dynamics of E-cadherin-dependent cell-cell adhesions and actin filaments during the early steps of cell-cell adhesion formation (Adams et al., 1998; Ehrlich et al., 2002). First, we observed the pre-adhesive structure but WAVE2-depletion did not affect them (supplementary material Fig. S10).

Two hours after plating, most of the CTRL cells contacted each other and E-cadherin packets lined the cell-cell contact (Fig. 5A,A′). Thick actin cables dissolved and new actin structures were formed along E-cadherin at the cell-cell contact site (Fig. 5D,D′). By 3 hours after plating, E-cadherin formed a continuous line at the cell-cell contact site and actin filaments were further accumulated (Fig. 5B,B′,E,E′). Thus, we could monitor the stabilization of cell-cell adhesion in these conditions.

At 2 hours after plating, also in the absence of WAVE2, reorganization of the circumferential actin cable was observed (Fig. 5J,J′). However, at the sites of new cell-cell contacts, recruitment of actin filaments and organization of E-cadherin was perturbed (Fig. 5G,G′,J,J′). E-cadherin showed a broad but discontinuous localization (compare Fig. 5A′ with 5G′). At 3 hours after plating, E-cadherin showed a diffuse localization in WAVE2 siRNA-treated cell layers (Fig. 5H,H′). In these cells, the membrane edges continued to protrude after formation of cell-cell contacts and the contact area increased (Fig. 5H″,K″, broken lines). Such overlapping area was not visible in CTRL cells (Fig. 5B″,E″). Interestingly, E-cadherin lined continuously the apical side of the same contact (Fig. 5I,I′). This abnormal localization of E-cadherin was also visible in the vertical sections (Fig. 5Q,R). In CTRL cell layers, cell-cell adhesions formed at the basal level (Fig. 5M,N). However, adhesions formed primarily on the apical level in WAVE2 siRNA-treated cell layers (Fig. 5Q,R). These results suggest that stabilization of new cell-cell contacts were perturbed in WAVE2 siRNA-treated cell layers.

During these processes, recruitment of actin filaments was not observed at sites of new cell-cell contact in WAVE2 siRNA-treated cells (Fig. 5K,K′,L,L′,T). Thus, recruitment of actin filaments to new cell-cell contact sites is dependent on WAVE2 and is required for stabilization of new cell-cell contacts.

WAVE2-mediated actin polymerization is needed for maturation of cell-cell adhesions

In subconfluent cell layers, cadherin-dependent cell-cell adhesions were not organized and E-cadherin often showed a punctate pattern (Fig. 4C, –EGTA) because the adherent cells migrated via lamellipodial protrusions (data not shown), and

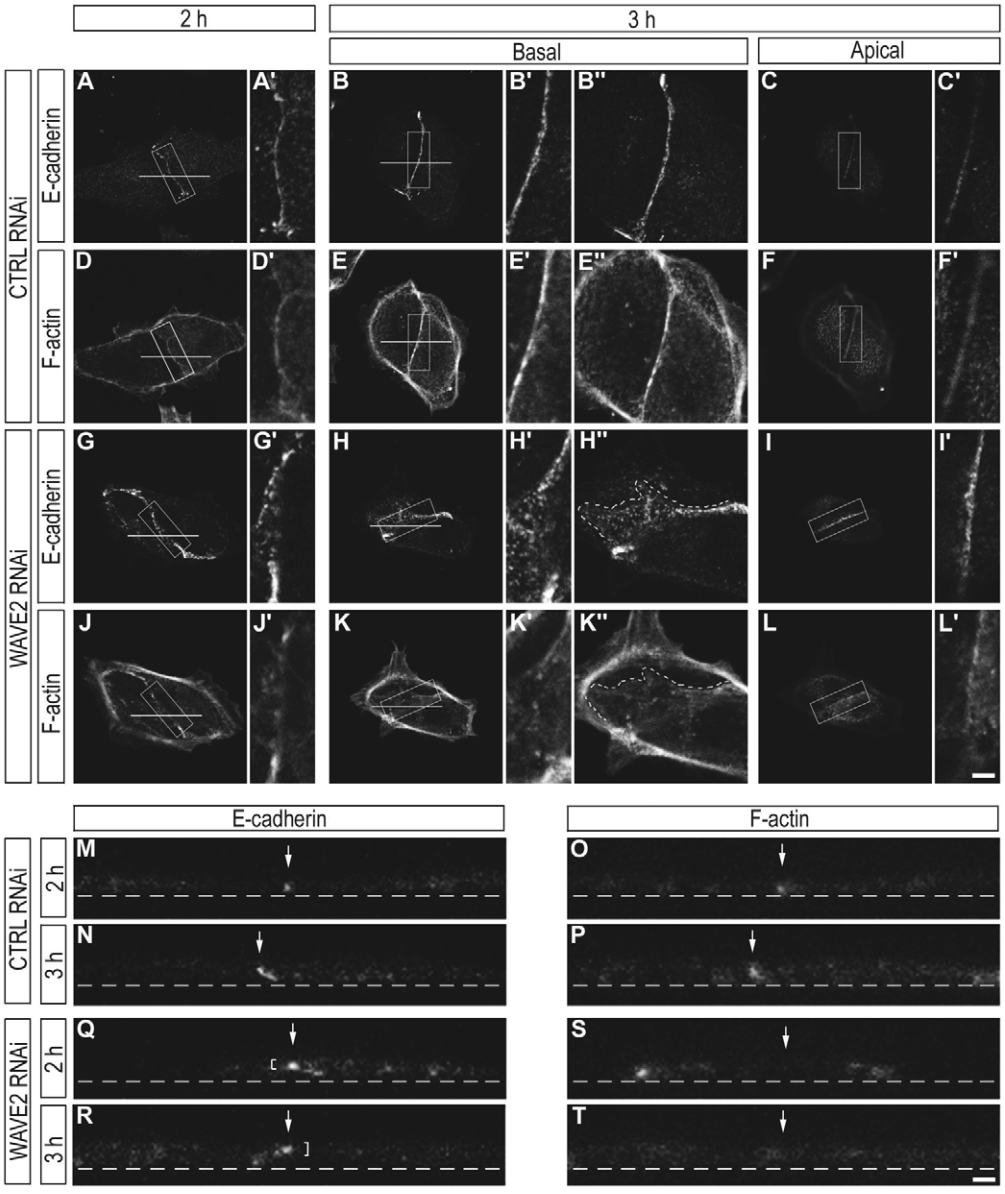

Fig. 5. Recruitment of actin filaments to new cell-cell contact sites. CTRL siRNA (A-F,M-P) or WAVE2 siRNA (G-L,Q-T) treated cells were plated on collagen-coated coverslips and then incubated for 2 or 3 hours as indicated. Cells were fixed and stained for E-cadherin and actin filaments (F-actin). (C,F,I,L) Cells in B,E,H,K photographed at the apical level. (A′-L′) Magnified images of the areas in the white squares in A-L, respectively. (B″,E″,H″,K″) Magnified images of B,E,H,K, respectively. Broken lines indicate the overlapping areas in H″ and K″. (M-T) vertical sections. The regions from which vertical sections were taken are indicated by white lines. Arrows indicate the locations of cell-cell contacts. Broken lines indicate the locations of coverslips. Brackets in Q and R indicate the abnormal localization of E-cadherin. Bars, 15 μm (A-L); 5 μm (A′-L′); 7.5 μm (B″,E″,H″,K″); 2.5 μm (M-T).

cell-cell adhesions were constantly changing. However, in confluent cell layers, cell-cell adhesions were organized continuously and linearly and showed a honeycomb-like pattern of thick lines (Fig. 6A, –EGTA). This represented the third step of cell-cell adhesion formation: maturation of cell-cell adhesions.

To examine further the process of maturation of cell-cell adhesions, we performed Ca^{2+}-switch assays with confluent cell layers. Thirty minutes after addition of Ca^{2+}, most of the cells had adhered and half of the cell-cell adhesions formed continuous linear structures (Fig. 6A,C, 30 min). As time progressed, the number of organized cell-cell adhesions increased, and these adhesions became thick (Fig. 6A,C, 60 and 120 min). This experimental condition enabled us to monitor maturation of cell-cell adhesions. In WAVE2 siRNA-treated cell layers, most of the cells adhered to each other 30 minutes after addition of Ca^{2+} (Fig. 6B, 30 min); therefore the cells were close enough together that we ignored the effect of WAVE2 RNAi on migration for cell-cell contact as was observed in subconfluent cell layers. However, the proportion of disorganized cell-cell contacts in WAVE2 siRNA-treated cell layers was twice that of CTRL cells (Fig. 6C, 30 min). Even at 60 and 120 minutes, almost half of the cell-cell contacts remained disorganized, and mature adhesions were rare (Fig. 6B,C, supplementary material Fig. S11). Even when the cells were incubated for 24 hours, maturation did not proceed further (Fig. 6B,C, –EGTA). Additionally, assembly of occludin, a tight junction protein, was also delayed at the cell-cell contact sites in WAVE2 siRNA-treated cell layers (supplementary material Fig. S2). Thus, WAVE2 depletion not only delayed but also suppressed maturation of cell-cell adhesions. In the vertical sections of WAVE2 siRNA-treated cell layers, disorganized cell-cell adhesions extended parallel to the substrate compared with the more organized adhesions observed in CTRL cells (Fig. 6D,E, 60 and 120 min, brackets).

During these processes, mature adhesions were supported by

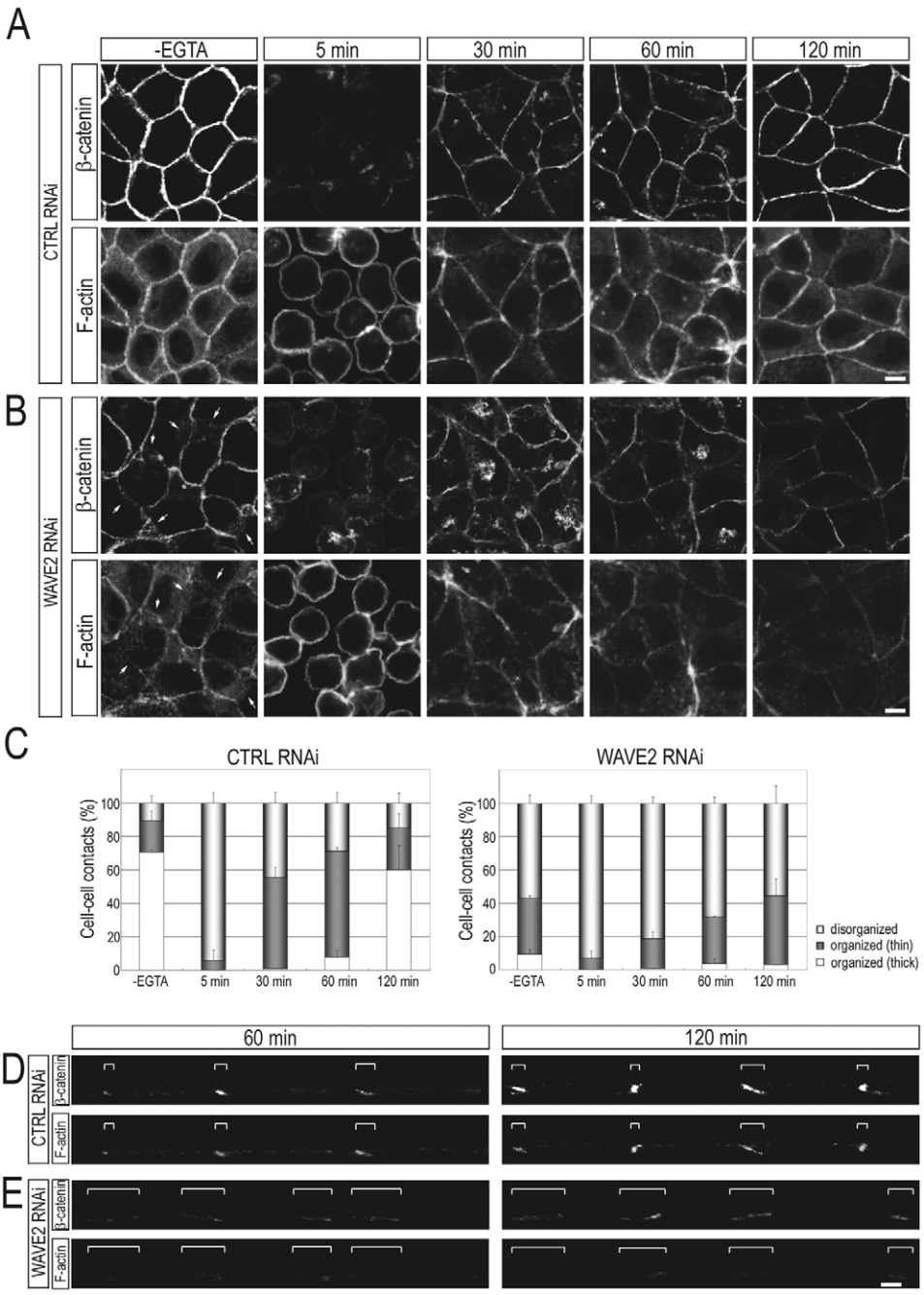

Fig. 6. Maturation of cell-cell adhesions. (A,B) Ca^{2+}-switch assay was performed with CTRL siRNA (A) or WAVE2 siRNA (B) treated confluent cell layers. Confluent cell layers were treated with 4 mM EGTA and then incubated with Ca^{2+}-containing medium for the indicated times. Cell layers were fixed and stained for β-catenin and actin filaments (F-actin). Arrows indicate disorganized cell-cell adhesions. Bar, 10 μm. (C) Cell-cell contacts were classified into three categories on the basis of the β-catenin staining pattern. Categories were thick, strong signals (thick); thin, weak signals (thin); and broad or no signal (disorganized). All data are mean ± s.d. Three independent experiments were performed. (D,E) Vertical sections of CTRL siRNAi (D) or WAVE2 siRNA (E) treated cells in the Ca^{2+}-switch assays. Brackets indicate the width of cell-cell adhesions. Bar, 7.5 μm.

actin filaments in CTRL cell layers (Fig. 6A,D). By contrast, accumulation of actin filaments was suppressed in WAVE2 siRNA-treated cell layers (Fig. 6B,E). Thus, WAVE2-mediated actin reorganization appears to be necessary for maturation of cell-cell adhesions. Indeed, the treatment of cytochalasin D also inhibited maturation of cell-cell adhesions (supplementary material Fig. S12). Additionally, expression of a dominant-negative form of WAVE2, which could not induce Arp2/3-mediated actin polymerization (Suetsugu et al., 1999), disturbed organization of cell-cell adhesions (supplementary material Fig. S13). These results suggest that WAVE2-mediated actin organization is required for maturation of cell adhesions.

Rac-induced development of cell-cell adhesions is mediated by WAVE2-dependent actin reorganization

Rac is thought to be involved in the development of cell-cell adhesions in MDCK cells. It has been reported that stable expression of constitutively active Rac (RacCA) and dominant-negative Rac (RacDN) regulated development of cadherin-dependent cell-cell adhesions positively and negatively, respectively (Takaishi et al., 1997). The Rac-WAVE2 pathway is important for formation of lamellipodial protrusions in fibroblasts (Suetsugu et al., 2003). In MDCK cells, transient expression of RacCA induced accumulation of actin filaments at cell-cell contact sites, but lamellipodial protrusions formed instead of cell-cell adhesions when cells were treated with

DECMA-1, which inhibits cadherin-dependent formation of adhesions (Fig. 7A).

To determine whether WAVE2 acts downstream of Rac during cell-cell adhesion formation, WAVE2 RNAi experiments were performed with RacCA-expressing cells. Transient expression of RacCA induced development of cadherin-dependent cell-cell adhesions and accumulation of actin filaments at cell-cell contact sites in both CTRL and WAVE2 siRNA-treated cell layers (Fig. 7B,C, arrowheads). However, β-catenin and actin filaments levels in WAVE2 siRNA-treated cell layers were lower than those in CTRL cells (Fig. 7B,C). Arp2 RNAi attenuated RacCA-induced development of cell-cell adhesions (Fig. 7B,C). Thus, the WAVE2-Arp2/3 pathway is a major mediator of actin reorganization at cell-cell contacts downstream of Rac and is needed for RacCA-induced development of cell-cell adhesions.

Rac signaling is important for WAVE2 localization at sites of cell-cell contact

In RacCA-expressing cells, further accumulation of WAVE2 was observed at sites of cell-cell contact (Fig. 8A). Conversely, transient expression of RacDN decreased localization of actin filaments and WAVE2 at cell-cell contact sites (Fig. 8A). When GFP-tagged Rac (GFP-Rac) was expressed, it formed clusters containing E-cadherin and actin filaments at sites other than cell-cell contacts, WAVE2 was also recruited to these clusters (Fig. 8B). It is possible that the localization of WAVE2 at the cell-cell contacts is regulated by Rac signaling.

It has been reported that Tiam1, the GDP-GTP exchanging factor (GEF) of Rac, is required for epithelial cell-cell adhesion (Malliri et al., 2004). To confirm the importance of Rac signaling in WAVE2 localization, Tiam1 was depleted by RNAi. Tiam1 siRNA treatment inhibited development of cell-cell adhesions (Fig. 8C, vertical section). In the absence of Tiam1, localization of WAVE2 at cell-cell contact sites was decreased at the junctions although it was normal at the basal levels (Fig. 8C, arrows). These results suggest that Rac signaling is important for recruitment of WAVE2 to cell-cell contacts.

Recruitment of WAVEs to cell-cell contacts is mediated through the N-terminal WHD

We examined which part of WAVE2 is needed for localization at cell-cell adhesions. GFP-tagged constructs of various

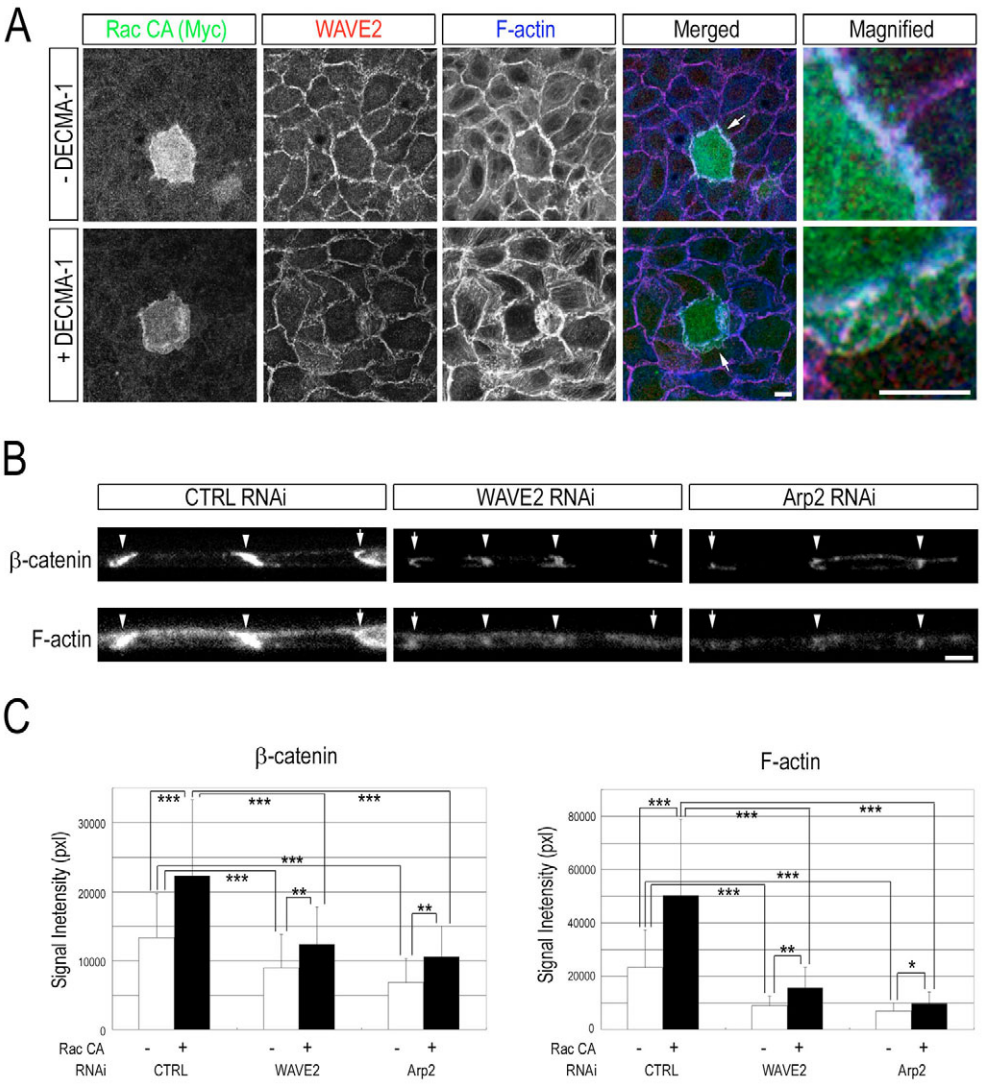

Fig. 7. WAVE2 functions downstream of Rac cell-cell contact sites. (A) DECMA-1 inhibits the RacCA-induced development of cell-cell adhesions. Cells expressing Myc-tagged RacCA were trypsinized and then re-plated in media containing DECMA-1. Cells were fixed and stained for Myc, WAVE2, and actin filaments. Arrows indicate the areas magnified the right-hand panels. Bars, 15 μm. (B) Effects of WAVE2 RNAi on RacCA-induced development of cell-cell adhesion. Myc-tagged RacCA was transiently expressed in CTRL siRNA and WAVE2 siRNA-treated cells, and its effects on cell-cell adhesions were examined. Cells were fixed and stained for β-catenin and actin filaments. Arrowheads and arrows indicate the cell-cell contacts in Myc-tagged RacCA expressing or non-expressing cells, respectively. Bar, 7.5 μm. (C) Signal intensities of β-catenin and actin filaments were quantified in vertical sections with ImageJ software. All data are mean ± s.d. *P<0.05; **P<0.005; ***P<0.0001. Statistical significance was examined using the Student's t-test.

portions of WAVE2 were transiently expressed and their localizations were examined. Both full-length WAVE2-GFP and WAVE homology domain (WHD)-GFP were localized at cell-cell contact sites (Fig. 9A). However, WAVE2 constructs lacking the N-terminal WHD (ΔWHD) were diffusely distributed and were not concentrated at cell-cell contacts. Thus, the WHD is needed for localization of WAVE2 at cell-cell contacts. It has been reported that the WHD is recruited to the edges of lamellipodial protrusions and that its localization is influenced by direct binding to Abi1 (Leng et al., 2005). Therefore, we investigated the relationship between localization of WHD at cell-cell contacts and binding of WHD to Abi1. The N-terminal region of WHD (WHDΔC, amino acids 1-129) was needed for both normal localization and binding to Abi1 (Fig. 9B, left panels). Binding of WHD to Abi1 is thought to occur through the coiled-coil structures of both proteins (Fig. 9B, right panels). Therefore, we examined the localization of leucine-zipper motif (LZM) mutant of WHD. As expected, the LZM mutant of WHD did not localize at cell-cell contacts (Fig. 9A). Thus, localization of WHD appears to require formation of a complex with Abi1. Indeed, Abi1 and WAVE2 colocalized at cell-cell contact sites (data not shown). Furthermore, in the absence of Abi1, WHD-GFP was distributed throughout the cytoplasm and was not localized at cell-cell contact sites (Fig. 9C) although the amount of WHD-GFP was similar to that in CTRL cells (data not shown). As well as Abi1, HSPC300 interacted with WHD and was needed for localization of WHD at cell-cell contacts (Fig. 9B,C).

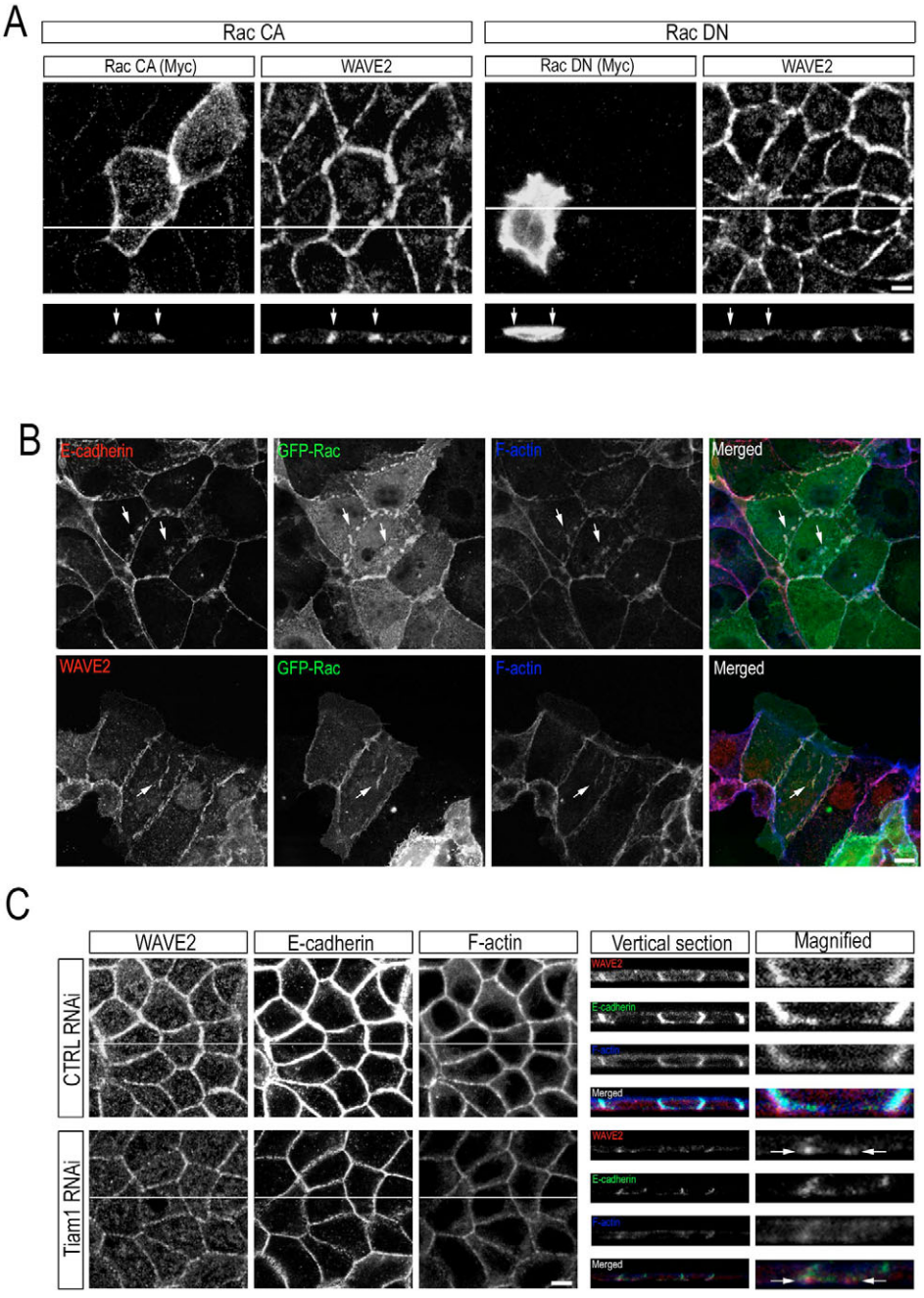

Fig. 8. Rac signaling is important for WAVE2 localization at cell-cell contacts. (A) Myc-tagged constitutively active (CA) and dominant-negative (DN) Rac were transiently expressed, and the localization of WAVE2 was examined by immunostaining. The regions from which sections were taken are indicated by white lines. Arrows indicate the positions of cell-cell contacts. Bar, 7.5 μm. (B) In MDCK cells transiently expressing GFP-Rac, the Rac-GFP formed clusters at sites other than cell-cell contacts (arrows). These clusters contained E-cadherin, actin filaments, and WAVE2. Bar, 15 μm. (C) CTRL or Tiam1 siRNA-treated cells were fixed and stained for WAVE2, E-cadherin, and actin filaments (F-actin). The regions from which vertical sections were taken are indicated by white lines. Arrows indicate localization of WAVE2. Bar, 10 μm.

Taken together, these data indicate that WAVE2 is recruited to cell-cell contact sites containing Rac-GTP through formation of a complex with Abi1 and HSPC300.

Discussion

During cell-cell adhesion formation, the actin cytoskeleton undergoes dynamic reorganization at sites of cell-cell contact. However, the role and mechanism of regulation of the actin cytoskeleton in cell-cell adhesion formation is not clear. In the present study, we found that actin rearrangement at new cell-cell contact sites is regulated by the Rac-WAVE-Arp2/3 pathway and plays a crucial role in organization and maintenance of cell-cell adhesions. The Rac-WAVE-Arp2/3 pathway is a crucial regulator of the cortical actin reorganization necessary for protrusion of membranes at the leading edges of migrating cells (Takenawa and Miki, 2001). This is the first report concerning the role of this pathway at sites of cell-cell contact.

Actin structures at cell-cell contact sites are thought to consist of bundled actin filaments. Recently, it was reported that there are two actin populations in cell-cell contacts between human keratinocytes (Zhang et al., 2005). The more stable actin population, termed thin bundles, is formed by reorganization of pre-existing actin filaments. The more

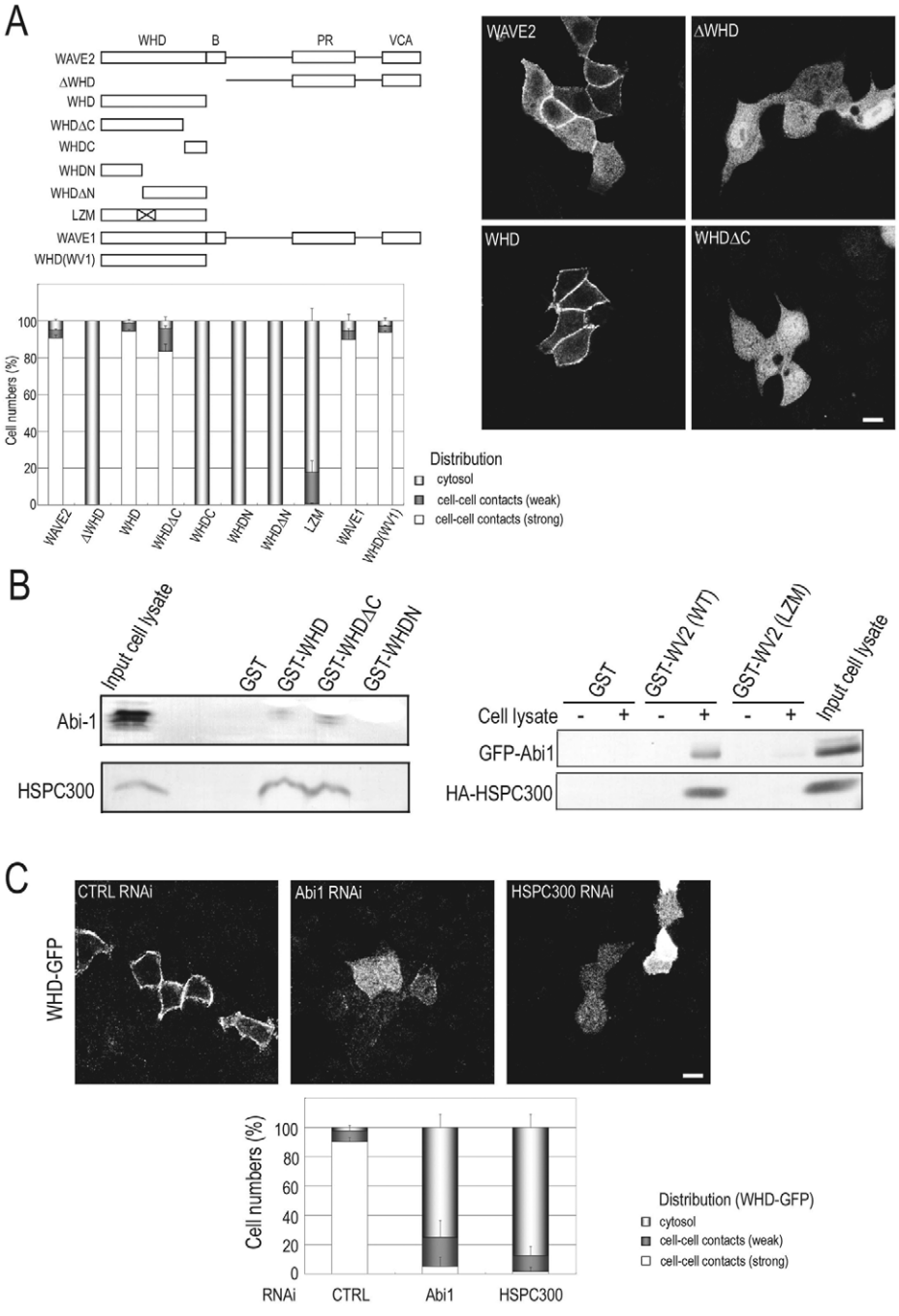

Fig. 9. WAVE2 is recruited to cell-cell contacts through its N-terminus. (A) GFP-tagged WAVE2 constructs were expressed and their localization was examined and quantified in MDCK cells. All data are mean ± s.d. Three independent experiments were performed. WHD, WAVE-homology domain; B, basic domain; PR, proline-rich domain; VCA, verprolin-homology, cofilin-homology and acidic domain. Bar, 15 μm. (B) Pull-down assay. (Left panels) MDCK cell lysates were incubated with GST-tagged truncated WAVE2 proteins and immunoblotted with anti-Abi1 and anti-HSPC300 antibodies. (Right panels) MDCK cells in which GFP-tagged Abi1 or HA-tagged HSPC300 were expressed were lysed and then incubated with GST-tagged full-length WAVE2 proteins and immunoblotted with anti-GFP and anti-HA antibodies, respectively. (C) WHD-GFP was transiently expressed in CTRL, Abi1 or HSPC300 siRNA-treated cells. Expression levels of WHD-GFP were similar in these cells (data not shown). Cells were fixed and the localization of WHD-GFP was examined and quantified. These data indicate that localization of WHD was dependent on Abi1 and HSPC300. Bar, 30 μm. All data are mean ± s.d. of three independent experiments.

dynamic population, termed junctional actin, is formed by de novo actin polymerization. Because depletion of WAVE2 inhibited recruitment of actin structures to new cell-cell contact sites but did not affect the reorganization of circumferential actin cable structures, it appears that actin structures mediated by the WAVE-Arp2/3 pathway comprise primarily junctional actin. These results suggest that reorganization of the two actin populations is regulated by distinct mechanisms. We propose three roles of WAVE-mediated actin reorganization during cell-cell adhesion formation (summarized in Fig. 10).

The first role is to protrude the membrane at the cell periphery to fill gaps among adhering cells and to promote efficient contact formation. Such membrane protrusion is thought to be similar to that observed at the leading edges of migrating cells that produces the cell locomotive force. Membrane protrusions were observed not only at new cell-cell contact sites but also in subconfluent cell layers (Fig. 4C, –EGTA). In subconfluent cell layers, adherent cells extend lamellipodia and increase the area of cell-cell contact (Fig. 4C, –EGTA). Thus, WAVE2-mediated protrusion of membranes increases the efficiency of formation of cell-cell adhesions.

The second role of WAVE-mediated actin reorganization is in the stabilization of cell-cell adhesions. When cells begin to contact, discrete clusters of E-cadherin are observed and are supported by thin actin bundles (Adams et al., 1998; Adams et al., 1996). This actin bundle formation is not necessary for WAVE-Arp2/3-mediated formation of branched actin structures. It appears that these structures are composed of formin-mediated unbranched actin filaments as seen in primary keratinocytes and/or N-WASP-Arp2/3 mediated filopodia-like actin structures (Kobielak et al., 2004; Miki et al., 1998a). However, the sequential reorganization of actin at new cell-cell contact sites requires the WAVE-Arp2/3 pathway (Fig. 5). Thus, various actin-regulating molecules are involved in this process and are regulated both spatially and temporally.

The third role of WAVE-mediated actin reorganization is maturation and maintenance of cell-cell adhesions. During maturation of cell-cell adhesions, accumulation of E-cadherin appears to be proportional to that of actin filaments, and continuous actin polymerization is required for maintenance of mature cell-cell adhesions. How does actin polymerization contribute to maturation of cell-cell adhesions? Electron microscopy revealed that tight cell-cell contacts were present only at the apical regions of lateral membranes and that cells make loose contacts at other areas (Takaishi et al., 1997). Membrane protrusions were observed ubiquitously in the areas

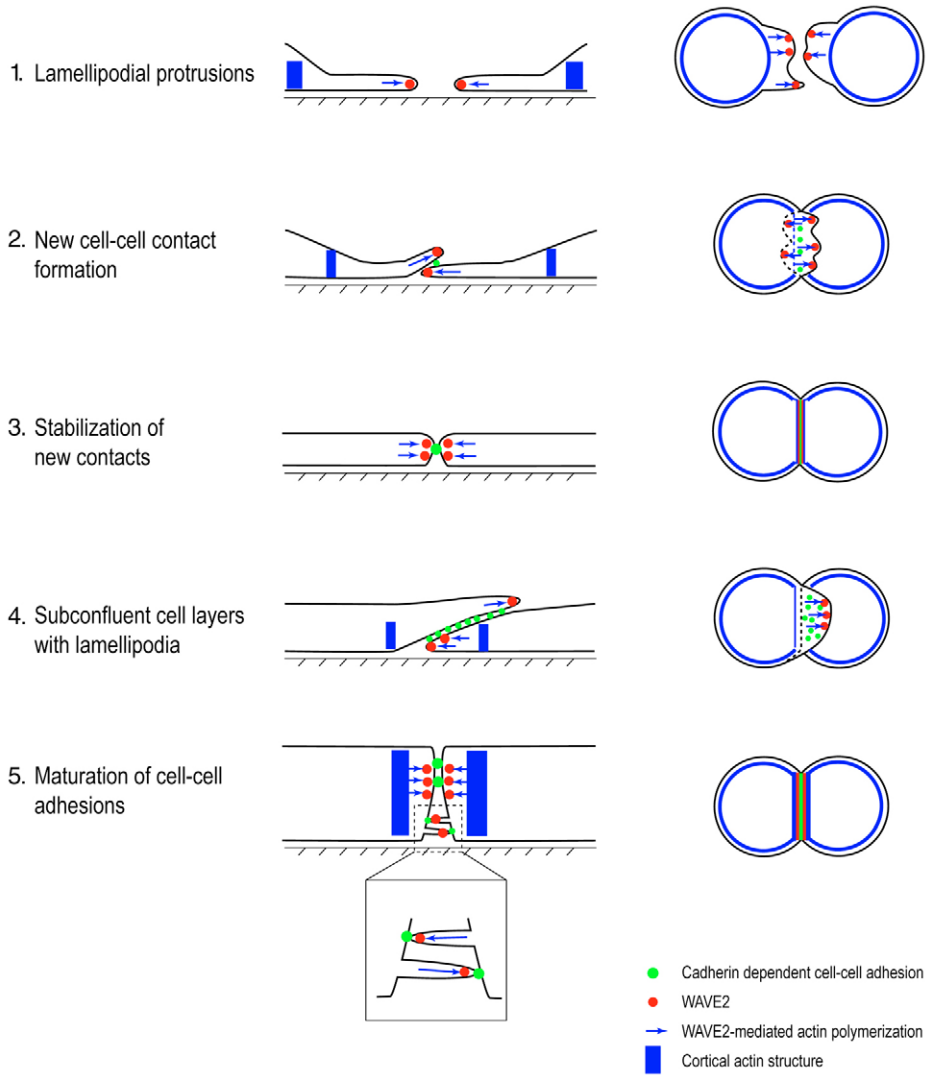

Fig. 10. A model for the function of WAVE2 during cell-cell adhesion formation in MDCK cells. (1) Non-adherent cells form WAVE2-dependent lamellipodial protrusions at the cell periphery. Such membrane protrusions facilitate efficient formation of new cell-cell contacts. (2) Clusters of cadherin appear at the sites of cell-cell contact. After cells contact each other, lamellipodial activity continues to expand the cell-cell contact and fill any gaps between the cells. (3) As cadherin-dependent cell-cell adhesions develop, WAVE2 is recruited and makes new actin structures along the adhesion, thereby stabilizing the adhesions. (4) In subconfluent cell layers, lamellipodial protrusions are often observed at sites of cell-cell contact and increase the area of cell-cell contact. (5) In confluent cell layers, membrane protrusions are not observed by light microscopy and WAVE2 is concentrated at cell-cell adhesions. At the basal part of the lateral membrane, WAVE2-mediated actin assembly pushes the membrane and promotes formation of new cell-cell contacts.

of weak contact. It is possible that WAVE2-mediated actin structures are involved in such membrane protrusions as well as in lamellipodia. Protruded membranes contact the facing membrane and form new cell-cell adhesion, resulting in further accumulation of cadherin at cell-cell adhesions.

It was recently reported that Rac was dispensable for initiation of cell-cell adhesion but disturbed its later development (Chu et al., 2004). The requirement of Rac for cell-cell adhesion corresponds to that of WAVE2 studied in this study. Electron microscopy revealed that cells expressing RacCA have tighter cell-cell contacts than do control cells (Takaishi et al., 1997). This phenotype may indicate that RacCA induces WAVE2-mediated actin reorganization, resulting in intense membrane protrusions at loose contacts. Because RacCA-induced development of cadherin-dependent adhesions was not suppressed completely by depletion of WAVE2 or Arp2, Rac must regulate cell-cell adhesion formation through other mechanisms.

Materials and Methods

Cell culture and strains

A MDCK cell line stably expressing FLAG-tagged WAVE2DN (Suetsugu et al., 1999) was established as described previously (Yamaguchi et al., 2002). The MDCK Tet-Off cell line (Clontech, Mountain View, CA), which expresses a tetracycline-controlled transactivator, was used to generate stable cell lines. MDCK Tet-Off cells were cotransfected with pTK-Hyg and pTRE2, which encodes Flag-tagged WAVE2DN, with Lipofectamine2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Single colonies were isolated after 2 weeks of hygromycin selection (300 μg/ml). All cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS).

Plasmids

To generate GFP fusion proteins, cDNAs encoding full-length, ΔWHD (residues 171-498), WHD (residues 1-170), WHDΔC (residues 1-129), WHDC (residues 130-170), WHDN (residues 1-66), WHDΔN (residues 67-170) and LZM (residues 1-170) of WAVE2 and full-length and WHD (residues 1-170) of WAVE1 were cloned into pEGFP (Clontech, Mountain View, CA). We prepared constructs of LZM in which the six residues of WHD [residues 54 (L), 61 (F), 68 (L), 75 (L), 82 (L), and 89 (V)] were replaced with alanines by site-directed mutagenesis. To generate VASP-GFP fusion proteins, cDNAs encoding the tetramerization domain (residues 277-380) of VASP were cloned into pEGFP (Clontech, Mountain View, CA) as described previously (Vasioukhin, 2000).

Antibodies

Polyclonal anti-WAVE1, anti-WAVE2, anti-N-WASP and anti-Arp2 antibodies were prepared as described previously (Yamazaki et al., 2005; Yamazaki et al., 2003). Polyclonal anti-HSPC300 antibody was generated in rabbits immunized with the His-tagged full-length HSPC300 expressed in *E. coli*. Monoclonal anti-Abi1 antibody was purchased from MBL (Woburn, MA). Monoclonal anti-E-cadherin antibody (ECCD2) was purchased from Takara (Kyoto, Japan). Rat monoclonal anti-E-cadherin antibody (DECMA-1) was from Abcam (Cambridge, UK). Monoclonal anti-β-catenin antibody was from BD Biosciences (San Jose, CA). Monoclonal anti-afadin antibody was from Hycut Biotechnology (Uden, the Netherlands). Polyclonal anti-Tiam1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-actin antibody was from Chemicon (Temecula, CA). Monoclonal and polyclonal anti-Myc antibodies were from Santa Cruz. Alexa Fluor 647-conjugated phalloidin was from Molecular Probes (Eugene, OR). Monoclonal anti-occludin antibody was purchased from Zymed (San Francisco, CA). Monoclonal anti-FLAG antibody was purchased from Sigma (St Louis, MO) and polyclonal anti-GFP antibody was from MBL (Woburn, MA).

RT-PCR analysis

Total RNA was isolated from MDCK cells and reverse transcription was performed as described previously (Yamazaki et al., 2003). Primers for PCR analysis were 5′-ACAGTAGGATATTTTGGGATGAAGC-3′ and 5′-TTATCCTTTAAATTCAGA-CACACTC-3′ (*formin-1*) and 5′-TTCCGTGTCCCCACCCCCAATGTAT-3′ and 5′-TTACTCCTTGGAGGCCATGTAGACC-3′ (*Gapdh*).

RNA interference (RNAi)

All small interfering RNAs (siRNAs) were Stealth siRNA purchased from Invitrogen (Carlsbad, CA). Targeting sense sequence for *WAVE2* is 5′-AUAGGUU-UCCAAGACAGGUACUGGG-3′ (348). Targeting sense sequence for *WAVE1* is 5′-AACUGGUACAGUCUCGCAUACUGGG-3′ (1385). Targeting sense sequence for *Tiam1* is 5′-UUUGAUUUCGGACUUCAGGAGCCGG-3′ (1738). Targeting sense sequence for *Arp2* is 5′-AAGCAAUUCAGCAACACCUACUCCC-3′ (967). Targeting sense sequences for *Abi1* are 5′-AUUGAUGGAAGACUCCAUUCUCC-GA-3′ (440) and 5′-UUUAUAAGGAGUAUUUCGUCCCAGC-3′ (764). We mainly used the latter sequences for depletion of Abi1. Targeting sense sequences for *HSPC300* are 5′-UAUCGAAGGAGUUGAGAAAGUCCGC-3′ (110) and 5′-AUGUACUCUAUUCUCCGCUCGAGGG-3′ (180). We mainly used the former sequences for depletion of HSPC300. Control sense sequence is 5′-AUUUCCC-GACCGCUUCUUGCAAAGG-3′. All siRNAs were transfected with Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. Transfection was performed twice at an interval of 24 hours. Transfected cells were used for various experiments 72 hours after the first transfection.

Immunoprecipitation

Cells cultured in dishes were washed with ice-cold phosphate-buffered saline (PBS) with 1 mM Na_3VO_4, scraped, and suspended in IP buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM NaF, 1% Triton X-100, 10% glycerol, 1 mM Na_3VO_4, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin). Cell lysates were incubated with ImmunoPure immobilized Protein A beads for 2 hours (Pierce, Rockford, IL). Supernatants were incubated with anti-WAVE2 antisera for 2 hours and then with new ImmunoPure immobilized Protein A beads for 2 hours. Beads were washed with IP buffer. Precipitates were separated by SDS-PAGE and analyzed by immunoblotting.

Immunostaining

Cells plated on coverslips were fixed with 3.7% formaldehyde in PBS for 10 minutes. Cells were permeabilized with 0.5% Triton X-100 in PBS for 10 minutes. Specimens were incubated with 1% bovine serum albumin in PBS for 1 hour, with primary antibodies at 1:100 dilution for 1 hour, and secondary antibody conjugated with Alexa Fluor 488 or Alexa Fluor 568 (Molecular Probes). Coverslips were mounted on glass slides and observed with a Radiance 2000 confocal laser-scanning microscope (Bio-Rad, Hercules, CA). When cells were extracted with Triton X-100 before fixation, cells were extracted with Triton X-100 buffer (50 mM NaCl, 10 mM PIPES pH 6.8, 3 mM $MgCl_2$, 0.5% Triton X-100 and 300 mM sucrose) on ice and then were fixed with 3.7% formaldehyde in PBS.

Ca^{2+}-switch assay

Two days before assays, 0.5×10^6 (subconfluent) or 2×10^6 (confluent) cells were plated in 35-mm culture dish with coverslips. Subconfluent or confluent cell layers were starved with serum-free DMEM for 1 hour and then washed with 5 mM EGTA containing DMEM or 4 mM EGTA containing PBS three times, respectively. After incubation with EGTA for 30 minutes, cells were transferred into serum-free DMEM and cultured for the indicated times.

Pull-down assay

GST-tagged WAVE2 truncated proteins were expressed in *E. coli* and purified as described previously (Miki et al., 1998b). GST-tagged full-length WAVE2 proteins were expressed in Sf9 cells and purified as described previously (Oikawa et al., 2004). MDCK cells were suspended in lysis buffer (40 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin) and incubated with each GST-tagged protein. Beads were washed five times with lysis buffer. Precipitates were separated by SDS-PAGE and analyzed by immunoblotting.

Quantification of signal intensities

Signal intensities at cell-cell contacts were measured with ImageJ software. More than 20 cell-cell contacts were used for quantification every experiment. Data were collected from three independent experiments.

We thank S. Suetsugu and H. Yamaguchi for helpful discussions. We thank O. Nakanishi for providing pTRE2 encoding Flag-tagged WAVE2. We also thank T. Higaki and S. Itoh for technical assistance.

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