

Recruitment and activation of Rac1 by the formation of E-cadherin-mediated cell-cell adhesion sites

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SUMMARY

Rac1, a member of the Rho family small GTPases, regulates E-cadherin-mediated cell-cell adhesion. However, it remains to be clarified how the localization and activation of Rac1 are regulated at sites of cell-cell contact. Here, using enhanced green fluorescence protein (EGFP)-tagged Rac1, we demonstrate that EGFP-Rac1 is colocalized with E-cadherin at sites of cell-cell contact and translocates to the cytosol during disruption of E-cadherin-mediated cell-cell adhesion by Ca^{2+} chelation. Re-establishment of cell-cell adhesion by restoration of Ca^{2+} caused EGFP-Rac1 to become relocalized, together with E-cadherin, at sites of cell-cell contact. Engagement of E-cadherin to the apical membrane by anti-E-cadherin antibody (ECCD-2) recruited EGFP-Rac1. We also investigated whether E-cadherin-mediated cell-cell adhesion induced Rac1 activation by measuring the amounts of GTP-bound Rac1 based on its specific binding to the Cdc42/Rac1 interactive binding region of p21-activated kinase. The formation of E-

cadherin-mediated cell-cell adhesion induced Rac1 activation. This activation was inhibited by treatment of cells with a neutralizing antibody (DECMA-1) against E-cadherin, or with wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase). IQGAP1, an effector of Rac1, and EGFP-Rac1 behaved in a similar manner during the formation of E-cadherin-mediated cell-cell adhesion. Rac1 activation was also confirmed by measuring the amounts of coimmunoprecipitated Rac1 with IQGAP1 during the establishment of cell-cell adhesion. Taken together, these results suggest that Rac1 is recruited at sites of E-cadherin-mediated cell-cell adhesion and then activated, possibly through PI 3-kinase.

Movies available on-line:

<http://www.biologists.com/JCS/movies/jcs2094.html>

Key words: Rac1, E-cadherin, cell-cell adhesion

INTRODUCTION

Cell-cell adhesion is dynamically rearranged and regulated in various situations including tissue development, the establishment of epithelial cell polarity, and wound healing (Adams and Nelson, 1998; Gumbiner, 2000; Takeichi, 1995). Cadherin is a well-known cell-cell adhesion molecule that mediates cell-cell adhesion by Ca^{2+} -dependent homophilic interactions. The membrane-distal region of the cadherin cytoplasmic domain binds to β -catenin, and this complex is linked to the actin cytoskeleton by α -catenin. This linkage between cadherin and the actin cytoskeleton is essential for cadherin-mediated cell-cell adhesion (Kemler, 1993). p120^{cas} (Ozawa and Kemler, 1998; Yap et al., 1998) and δ -catenin (Lu et al., 1999), members of the Armadillo/ β -catenin family, bind to the membrane-proximal region of the cadherin cytoplasmic domain, and are good candidates for regulators of cadherin-mediated cell-cell adhesion. Although several different mechanisms have been proposed for cadherin regulation, the regulatory mechanism is not yet fully understood.

The Rho family GTPases, including Rac1, Cdc42 and RhoA, regulate the reorganization of the actin cytoskeleton (Hall, 1998; Kaibuchi et al., 1999; Van Aelst and D'Souza-Schorey, 1997). Recent studies have revealed that they also participate

in the regulation of cadherin-mediated cell-cell adhesion (Braga et al., 1997; Kuroda et al., 1997; Kuroda et al., 1998; Takaishi et al., 1997). Overexpression of constitutively active Rac1 (Rac1^{V12}), a mutant that is defective in GTPase activity and is thought to exist constitutively in the GTP-bound form in cells, induces greater accumulation of E-cadherin, β -catenin and actin cytoskeleton at sites of cell-cell contact. By contrast, dominant negative Rac1 (Rac1^{N17}), a mutant that preferentially binds to GDP rather than GTP and is thought to exist constitutively in the GDP-bound form in cells, inhibits their accumulation (Takaishi et al., 1997). Tiam1, one of the GDP/GTP exchange factors (GEFs) and an activator of Rac1, is localized at sites of cell-cell contact. Overexpression of Tiam1 or Rac1^{V12} inhibits hepatocyte growth factor (HGF)-induced cell scattering by increasing E-cadherin-mediated cell-cell adhesion in Madin-Darby canine kidney (MDCK) cells (Hordijk et al., 1997). Furthermore, overexpression of Rac1^{N17} or Cdc42^{N17} in EL cells (L fibroblasts that stably express E-cadherin and adhere in an E-cadherin-dependent manner), markedly reduced E-cadherin-mediated cell-cell adhesion, as measured by the cell dissociation assay (Fukata et al., 1999a).

Recently, it has been reported that IQGAP1, an effector of Rac1/Cdc42, negatively regulates cadherin-mediated cell-cell adhesion through the dissociation of α -catenin from β -catenin,

and that activated Rac1 binds IQGAP1 and positively regulates adhesion by inhibiting the IQGAP1 function (Kuroda et al., 1998; Fukata et al., 1999a). Moreover, both IQGAP1 and Rac1 are localized mainly at sites of cell-cell contact (Hart et al., 1996; Kuroda et al., 1996). Thus, Rac1 regulates E-cadherin-mediated cell-cell adhesion through IQGAP1. However, it remains to be elucidated how the localization and activation of Rac1 are regulated at sites of cell-cell contact.

In the present study, we examined the dynamics of Rac1 localization during reorganization of cell-cell adhesion in MDCKII epithelial cells by using enhanced green fluorescence protein (EGFP)-tagged Rac1, and investigated whether E-cadherin-mediated cell-cell adhesion elicits Rac1 activation. We found that Rac1 was recruited at intercellular junctions during the establishment of cadherin-mediated adhesion and that E-cadherin-mediated homophilic interactions activated Rac1, possibly through phosphatidylinositol 3-kinase (PI 3-kinase).

MATERIALS AND METHODS

EL cells, MDCKII cells and anti-E-cadherin rat monoclonal antibody (mAb) ECCD-2 were kindly provided by A. Nagafuchi and Sh. Tsukita (Kyoto University, Kyoto, Japan). EL cells were cultured in DMEM supplemented with 10% fetal calf serum containing 0.1 mg/ml of G418 (Nagafuchi et al., 1994). MDCKII cells were cultured in DMEM supplemented with 10% calf serum. DECMA-1 (Vestweber and Kemler, 1985), a rat mAb against E-cadherin, was kindly provided by R. Kemler (Max-plank Institute für Immunbiologie, Freiburg, Germany) and M. Ozawa (Kagoshima University, Kagoshima, Japan). Anti-GFP antibody (mFX73) was kindly provided by S. Mitani (Tokyo Women's Medical University School of Medicine, Tokyo, Japan) and used for immunoprecipitation. Another anti-GFP antibody was purchased from CLONTECH Laboratories Inc. (Heidelberg, Germany) and used for immunoblotting. Anti-Rac1 mouse mAb was purchased from Upstate Biotechnology Inc. (Lake Placid, NY); and anti-RhoA mouse mAb from Cytoskeleton Inc. (Denver, CO). Anti-IQGAP1 rabbit polyclonal antibody was generated against GST-IQGAP1 (aa 1-216). The plasmid of pGEX-Rho binding domain (RBD) (Ren et al., 1999) of Rhotekin was kindly donated by M. A. Schwartz (The Scripps Research Institute, CA); and the cDNA of α PAK was donated by S. Ohno (Yokohama City University School of Medicine, Yokohama, Japan). Wortmannin, an inhibitor of PI 3-kinase, was purchased from Wako Chemical (Osaka, Japan). All materials used in the nucleic acid study were purchased from Takara Shuzo Co. Ltd (Kyoto, Japan). Other materials and chemicals were obtained from commercial sources.

Plasmid constructions

To obtain EGFP-Rac1 and EGFP-Cdc42, we subcloned the cDNA fragments of Rac1 and Cdc42 into the *Bam*HI site of EGFP-C1 (CLONTECH), respectively. To obtain EGFP-Rac1^{V12} and Rac1^{N17}, we subcloned the corresponding fragments of Rac1 mutants into *Bam*HI and *Xba*I sites, and *Bgl*II and *Eco*RI sites of EGFP-C1, respectively. A fragment harboring Cdc42/Rac1 interactive binding region (CRIB) of α PAK (aa 70-106) was generated by PCR using oligonucleotides CTGAGGATCCAAGGAGCGGCCAGAGATT-CTCT and CTGAGGATCCTACAAGCGGGCCCACTGTTCTG, digested with *Bam*HI, and inserted into the *Bam*HI site of pGEX-4T-1 (Pharmacia Biotech, NJ) to obtain pGEX-CRIB.

Preparation of GST-CRIB and GST-RBD

The GST-CRIB and GST-RBD fusion proteins were expressed and purified as described previously (Ren et al., 1999).

Detection of GTP-bound Rho family small GTPases by use of GST-CRIB or GST-RBD (GTPase activity assay)

EL cells (3×10^6 cells/10 cm dish) were seeded and cultured for 18 hours. EL cells were transfected with the desired plasmids by using LipofectAMINE (GIBCO BRL, Grand Island, NY). After a 48 hour incubation, the cells were washed twice with ice-cold Hepes-buffered saline (containing 20 mM Hepes, pH 7.4, 137 mM NaCl and 3 mM KCl), and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 1% NP-40, 150 mM NaCl, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 10 μ M (p-aminodiphenyl)-methanesulfonyl fluoride). The lysates were then centrifuged at 20,000 *g* for 7 minutes at 4°C, and the supernatant was incubated with purified GST-CRIB immobilized beads at 4°C for 1 hour. The beads were washed three times with an excess of lysis buffer, and eluted with Laemmli sample buffer. The eluates were subjected to SDS-PAGE, followed by immunoblotting with the appropriate antibodies.

Immunoprecipitation of EGFP-Rac1

Immunoprecipitation was performed as described previously (Kuroda et al., 1998). In brief, EL cells were transfected with EGFP-Rac1. After a 48 hour incubation, the lysates were incubated with protein A Sepharose beads for 1 hour at 4°C for preclearing. The supernatant was then incubated with anti-GFP antibody for 1 hour at 4°C. The immunocomplex was subjected to SDS-PAGE, followed by immunoblotting with anti-GFP and anti-IQGAP1 antibodies.

Activation of Rho family small GTPases by the formation of E-cadherin-mediated cell-cell adhesion

MDCKII cells (5×10^6 cells/10 cm dish) were seeded into dishes, cultured for 24 hours, and then the cells were serum-starved. After a 24 hour incubation, E-cadherin-mediated cell-cell contacts were disrupted by treatment with 4 mM EGTA at 37°C for 30 minutes. Thereafter, intercellular contacts were allowed to reform in the presence of normal Ca²⁺-containing medium (CaCl₂ ~1.8 mM) for 30-120 minutes at 37°C. The activation of the Rho family small GTPases was measured by the GTPase activity assay using GST-CRIB or GST-RBD fusion protein (Ren et al., 1999). To examine the effect of DECMA-1 antibody or wortmannin on Rac1 activation, we included DECMA-1 antibody or wortmannin (200 nM) in the medium.

Immunoprecipitation of IQGAP1

Immunoprecipitation was performed essentially similar to the GTPase activity assay. In brief, MDCKII cells (5×10^6 cells/10 cm dish) were seeded into dishes, cultured for 24 hours, and then the cells were serum-starved. After a 24 hour incubation, calcium switch was performed. The lysates were then centrifuged and the supernatant was incubated with purified anti-IQGAP1 antibody immobilized beads at 4°C for 1 hour. The eluates were subjected to SDS-PAGE, followed by immunoblotting with anti-IQGAP1 and anti-Rac1 antibodies.

Dynamics of Rac1 localization in living cells (time-lapse analysis)

To observe living cells, cells were seeded on glass-bottomed dishes (Matsunami glass Inc., Osaka, Japan) (5×10^5 cells/dish). At 24 hours after seeding, the cells were transfected with EGFP-Rac1 via LipofectAMINE 2000 (GIBCO BRL, Grand Island, NY). The cells were serum-starved in medium without phenol red for 24 hours and then calcium switch was performed. Images from living cells were acquired with a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with Zeiss Axiovert 100 M (Plan Apochromat 63 $\times$ /1.40 NA oil immersion objective). Optical scans were collected every 10 minutes. The focus, contrast and brightness settings were constant during the course of image acquisition. The images were arranged sequentially in a movie sequence on Adobe ImageReady (Adobe System Inc.).

Localization of E-cadherin and EGFP-Rac1 in MDCKII cells

MDCKII cells were seeded onto three 13 mm coverslips in each well of a 6-well cell culture plate (1.5×10^5 cells/well). At 24 hours after seeding, the cells were transfected with EGFP-Rac1 and then serum-starved for 24 hours. Thereafter, calcium switch was performed. The cells were fixed with 3% formaldehyde in phosphate-buffered saline (PBS) for 10 minutes at room temperature and then permeabilized with PBS containing 0.2% Triton X-100 and 2 mg/ml BSA for 15 minutes. The fixed cells were finally stained with indicated antibodies. Images were acquired with a Zeiss LSM 510 laser scanning confocal microscopy. XZ scans were generated with a 0.4 μ m motor step. The focus, contrast and brightness were set so that all pixels were in the linear range.

Recruitment of EGFP-Rac1 by engagement of E-cadherin

MDCKII cells (2.5×10^5 cells/6 cm dish containing three 13 mm coverslips) were transfected with EGFP-Rac1. Twenty-four hours after the transfection, the cells were placed on ice for 15 minutes and then incubated with ECCD-2 for 60 minutes on ice. After having been washed with ice-cold DMEM, the cells were treated with Texasred-conjugated anti-rat antibody for 60 minutes at 4°C and then washed with ice-cold DMEM. Subsequently, the cells were incubated for 60 minutes at 37°C. Finally, the cells were fixed with 3% formaldehyde in PBS (Fujimoto, 1996).

RESULTS

EGFP-Rac1 functions as Rac1

To examine the localization of Rac1 in living cells, we constructed cDNAs of wild-type, constitutively active and dominant negative Rac1 tagged with EGFP at its N-terminus (designated as EGFP-Rac1, -Rac1^{V12}, and -Rac1^{N17}, respectively). As an initial characterization, EL cells were transfected with EGFP-Rac1, -Rac1^{V12} and -Rac1^{N17} for transient expression, because the transfection efficiency was high and the membrane ruffling, the most typical phenotype of Rac1, is induced by dominant active Rac1 in EL cells, but not in MDCKII epithelial cells (Ridley et al., 1995). Immunoprecipitation by anti-GFP antibody was then performed. IQGAP1, an effector of Rac1, was coimmunoprecipitated with EGFP-Rac1^{V12}, but not with EGFP, EGFP-Rac1 nor EGFP-Rac1^{N17} (Fig. 1A). EGFP-

Rac1^{V12} induced membrane ruffling in EL cells (Fig. 1B). When EGFP-Rac1^{V12} was used to transfect into MDCKII epithelial cells to monitor its localization, EGFP-Rac1^{V12} accumulated at sites of cell-cell contact (data not shown). The localization of EGFP-Rac1^{V12} at sites of cell-cell contact was indistinguishable from that of HA- or myc-tagged Rac1^{V12} (Jou and Nelson, 1998; Kuroda et al., 1997; Takaishi et al., 1997). Moreover, the localization of EGFP-Rac1 (Fig. 2A,B) was similar to that of endogenous Rac1 (Royal et al., 2000; Michaelson et al., 2001). Thus, we concluded that EGFP-Rac1 functioned as endogenous Rac1.

Dynamics of Rac1 localization during the reorganization of E-cadherin-mediated cell-cell adhesion in living cells

Using the calcium switch model in MDCKII epithelial cells (Pece et al., 1999), we examined the dynamics of EGFP-Rac1 localization in living cells by time-lapse analysis (Fig. 2A; see movie, <http://www.biologists.com/JCS/movies/jcs2094.html>). In MDCKII cells cultured in normal Ca²⁺ levels (~1.8 mM), EGFP-Rac1 was localized at sites of cell-cell contact. By contrast, in cells treated with EGTA, a Ca²⁺ chelator, EGFP-Rac1 was translocated from sites of cell-cell contact to the cytosol and became diffusely distributed there. After the restoration of Ca²⁺, EGFP-Rac1 could again be detected at sites of cell-cell contact, and by 120 minutes after the restoration, formation of E-cadherin-mediated cell-cell adhesion sites was complete. These results suggest that EGFP-Rac1 dynamically relocates during the reorganization of E-cadherin-mediated cell-cell adhesion. We next examined the localization of both EGFP-Rac1 and E-cadherin in MDCKII cells. EGFP-Rac1 and E-cadherin were colocalized at sites of cell-cell contact in MDCKII cells cultured in normal Ca²⁺ levels (Fig. 2B). After the chelation of Ca²⁺, both EGFP-Rac1 and E-cadherin disappeared from sites of adhesion and relocated to the cytosol, then translocated to sites of adhesion by Ca²⁺ restoration (30 minutes). After 120 minutes of Ca²⁺ restoration, both EGFP-Rac1 and E-cadherin were colocalized at sites of cell-cell adhesion. Moreover, we confirmed that EGFP-Rac1 was colocalized with E-cadherin but not with ZO-1, a marker of tight junction, at lateral membrane by XZ scans (data not

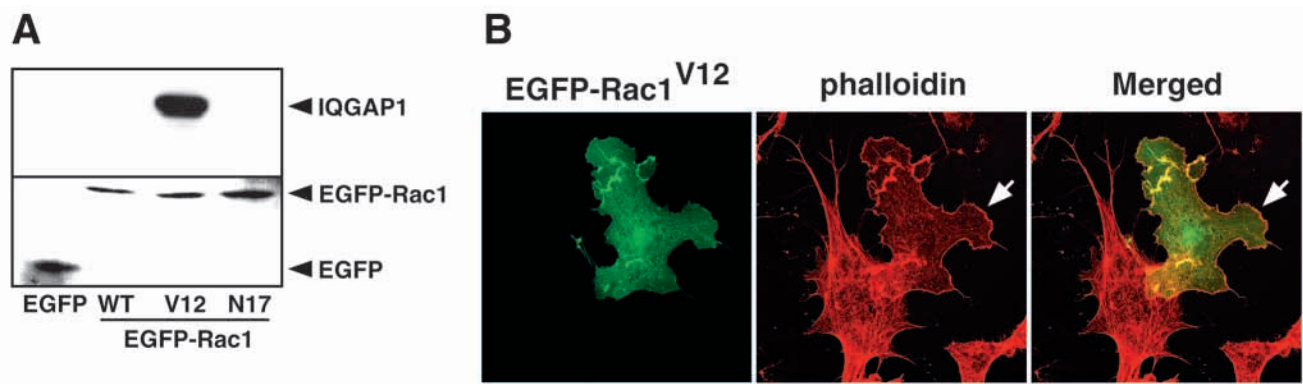


Fig. 1. Characterization of EGFP-Rac1. (A) EL cells were transfected with EGFP alone or with EGFP-Rac1 mutants for transient expression. GFP-fusion proteins were immunoprecipitated with anti-GFP antibody. The immunocomplexes were subjected to SDS-PAGE, followed by immunoblotting with anti-IQGAP1 and anti-GFP antibodies. (B) EL cells were transfected with EGFP-Rac1^{V12}. After a 48 hour incubation, the cells were fixed and then stained with tetramethylrhodamine B isothiocyanate-phalloidin. An arrow indicates the membrane ruffling. Bar, 10 μ m.

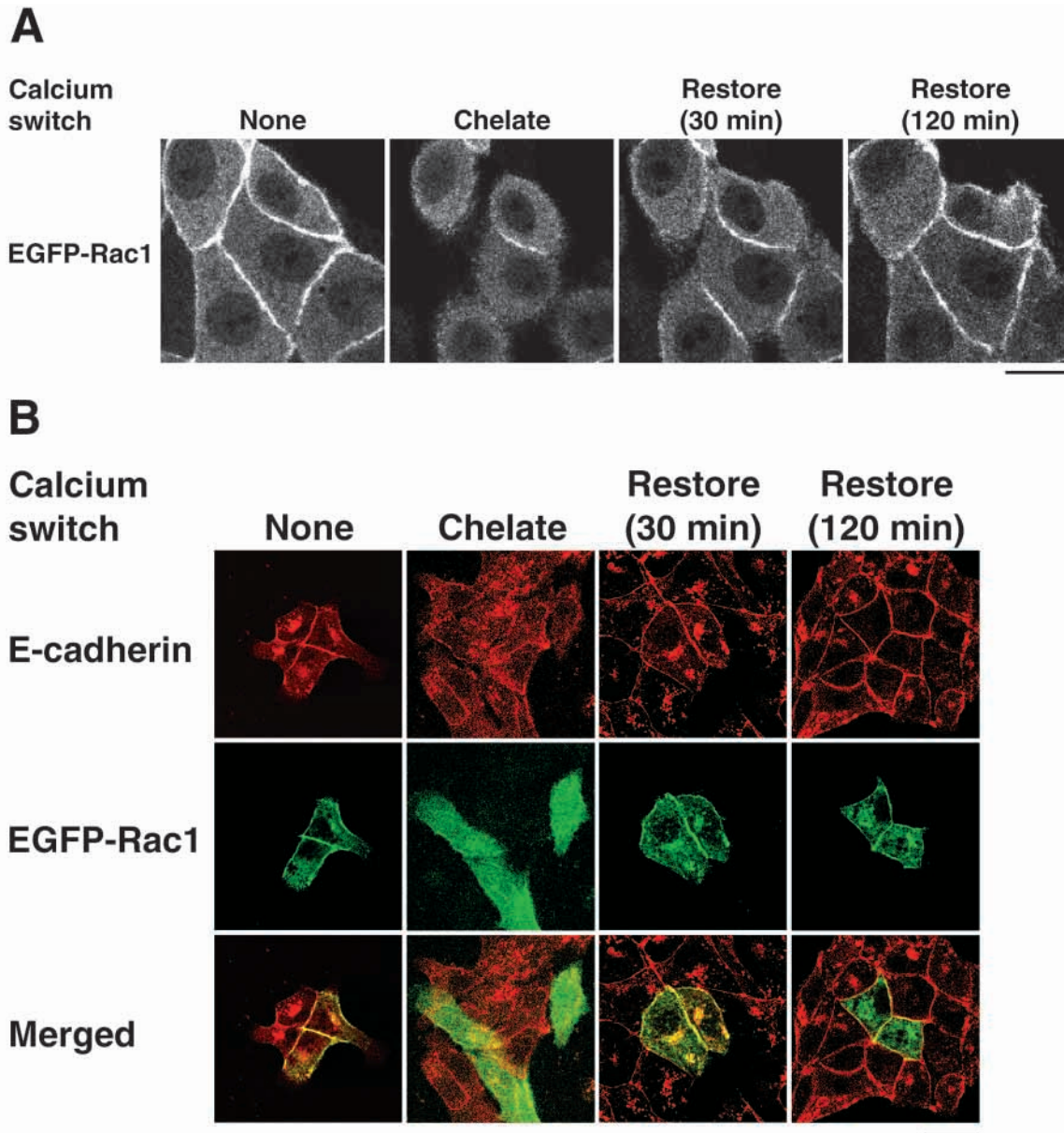


Fig. 2. Localization of EGFP-Rac1 during a calcium switch. (A) Dynamics of EGFP-Rac1 localization was analyzed by time-lapse imaging. MDCKII cells were transfected with EGFP-Rac1. The cells were treated with 4 mM EGTA for 30 minutes. Subsequently, the cells were treated with Ca^{2+} -containing medium for the indicated times (see movie, <http://www.biologists.com/JCS/movies/jcs2094.html>) (B) MDCKII cells were transfected with EGFP-Rac1 and then calcium switch was performed. The cells were stained with anti-E-cadherin antibody (ECCD-2), followed by Texasred-conjugated anti-rat antibody. All confocal images were acquired under the same conditions. Bars, 10 μm .

shown). Thus, EGFP-Rac1 and E-cadherin behaved in a similar manner during the reorganization of cell-cell adhesion sites.

DECMA-1, but not wortmannin, affects localization of EGFP-Rac1 and E-cadherin

To further investigate the relationship between EGFP-Rac1 and E-cadherin, we examined the effect of DECMA-1 (Vestweber and Kemler, 1985), a neutralizing antibody against E-cadherin, on the localization of EGFP-Rac1 and E-cadherin. Pretreatment of the cells with DECMA-1 before Ca^{2+} restoration inhibited the recruitment of EGFP-Rac1 and E-cadherin at sites of cell-cell contact (Fig. 3). Although

DECMA-1-treated cells may look less confluent, the reason is because the cells cannot adhere each other.

It has been reported that E-cadherin-mediated cell-cell adhesion induces PI 3-kinase activation in MDCKII cells followed by Akt/PKB activation (Pece et al., 1999). Moreover, the interaction of PI 3-kinase with E-cadherin (Pece et al., 1999) or β -catenin (Espada et al., 1999) has been reported. Because activated PI 3-kinase is thought to activate Rac1 (Hawkins et al., 1995; Kotani et al., 1994; Reif et al., 1996), these observations suggest the involvement of PI 3-kinase in the E-cadherin-dependent Rac1 recruitment. To address this possibility, the effect of wortmannin, an inhibitor of PI 3-

kinase, on EGFP-Rac1 and E-cadherin localization was examined. Preincubation of cells with wortmannin (200 nM) prior to Ca^{2+} restoration had no effect on the localization of EGFP-Rac1 and E-cadherin (Fig. 3). Thus, PI 3-kinase does not appear to be involved in the recruitment of EGFP-Rac1 and E-cadherin at sites of cell-cell contact.

Recruitment of Rac1 by accumulation of E-cadherin

Is Rac1 specifically recruited at the E-cadherin-based adhesion sites or simply recruited to the plasma membrane? To elucidate whether Rac1 specifically accumulates at E-cadherin-based adhesion sites, we developed an assay to force the accumulation of E-cadherin at the apical membrane using anti-E-cadherin antibody (ECCD-2) and examined whether EGFP-Rac1, EGFP-Rac1^{V12} and EGFP-Rac1^{N17} are recruited in an E-cadherin-dependent manner. The cells were first incubated with ECCD-2 and treated with Texasred-conjugated secondary antibody on ice without fixation. Then, the cells were transferred to 37°C to allow the secondary antibody to crosslink the first antibody and to cluster E-cadherin at the apical membrane as aggregates. α -catenin and β -catenin, which are anchoring molecules of E-cadherin, were recruited to the aggregates (data not shown). Under these conditions, EGFP-Rac1, EGFP-Rac1^{V12} and EGFP-Rac1^{N17} were recruited to aggregates composed of E-cadherin at the apical membrane (Fig. 4A). EGFP alone was not recruited (Fig. 4A) and hardly any EGFP-Cdc42 and EGFP-RhoA were recruited (data not shown). It was confirmed by XZ scans that E-cadherin was accumulated at the apical membrane and that EGFP-Rac1 was recruited to accumulated E-cadherin (Fig. 4B). There were E-cadherin clusters where EGFP-Rac1 was not visible, probably because some of the E-cadherin clusters have been endocytosed and EGFP-Rac1 is no longer associated. E-cadherin aggregates are thought to mimic E-cadherin clustering and this clustering is required for E-cadherin-mediated cell-cell adhesion (Gumbiner et al., 2000). Taken together, these results indicate that Rac1 could be recruited by E-cadherin-mediated cell-cell adhesion.

GST-CRIB specifically binds to GTP-bound Rac1/Cdc42

To examine whether Rac1 is activated during the formation of E-cadherin-mediated cell-cell adhesion, we took the advantage of the fact that Rac1 effectors interact only with GTP-bound Rac1 (Ren et al., 1999). We constructed GST-fused Cdc42/Rac1 interactive binding region (CRIB) of PAK, PAK being a Rac1/Cdc42 effector. To evaluate the specificity of GST-CRIB, we coupled GST-CRIB to glutathione beads and incubated them with lysates from EL cells expressing GFP-tagged mutants of the Rho family. The beads were washed, and the bound proteins were analyzed by immunoblotting with an anti-GFP antibody. GST-CRIB specifically bound to Rac1^{V12} and Cdc42^{V12} (Fig. 5A). No specific binding was detected with wild-type Rac1, Rac1^{N17}, wild-type Cdc42 and Cdc42^{N17} (Fig. 5A). GST-CRIB did not bind to dominant active RhoA (data not shown). Similar results were obtained using HA-tagged small GTPases instead of GFP-tagged ones (data not shown). These results indicate that this GST-CRIB has a high specificity for GTP-bound Rac1 and Cdc42. For detection of GTP-bound RhoA, GST-fused Rho-binding domain (RBD) of Rhotekin was used (Ren et al., 1999).

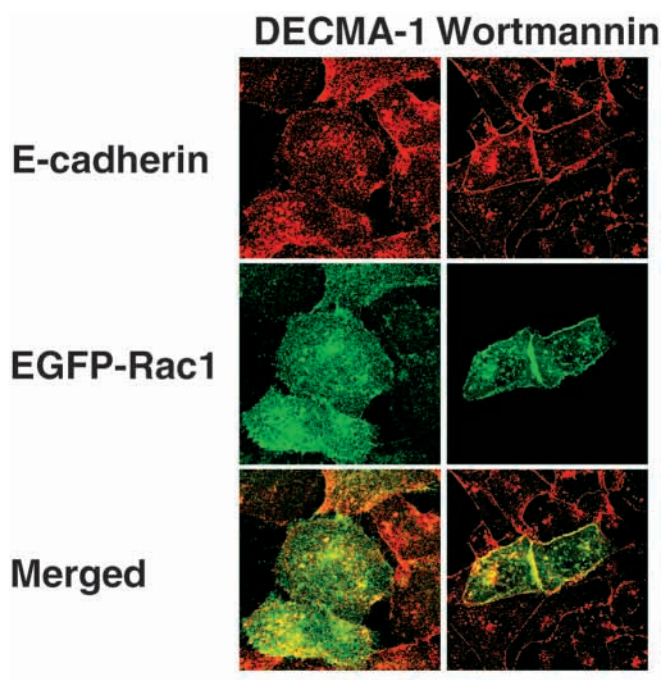


Fig. 3. DECMA-1, but not wortmannin, inhibits recruitment of Rac1 to sites of cell-cell contact. MDCKII cells transfected with EGFP-Rac1 were treated with 4 mM EGTA in the presence of either a neutralizing antibody against E-cadherin (DECMA-1) or an inhibitor of PI 3-kinase (wortmannin; 200 nM) for 30 minutes. Subsequently, the cells were treated with Ca^{2+} -containing medium in the presence of either DECMA-1 or wortmannin for 30 minutes. Then the cells were fixed and stained with ECCD-2, followed by Texasred-conjugated anti-rat antibody. The results are representative of three independent experiments. Bar, 10 μm .

E-cadherin-mediated cell-cell adhesion activates Rac1

Active Rac1 was measured by affinity precipitation using GST-CRIB during a calcium switch (see MATERIALS and METHODS). In MDCKII cells cultured in medium with normal Ca^{2+} levels, Rac1 in its active GTP-bound form could be detected; lane indicated by 'EGTA-' and 'Calcium+' (Fig. 5B). When E-cadherin-mediated cell-cell adhesion was disturbed by Ca^{2+} chelation, the level of active Rac1 decreased. The amounts of active Rac1 rapidly increased as E-cadherin-mediated cell-cell adhesion was restored. The Rac1-GTP level peaked at 30 minutes and sustained to 120 minutes after restoration (Fig. 5B). Then, the Rac1-GTP level gradually returned to the basal level (data not shown). Total Rac1 in lysates did not change during this process. No Rac1 was detected in samples incubated with GST alone. In contrast to Rac1, the level of active RhoA as measured by GST-RBD, was not affected and remained at the basal level during a calcium switch (Fig. 5B). Although we tried to detect active Cdc42 by using GST-CRIB, we could not detect it under the conditions employed (data not shown, see DISCUSSION). These results suggest that Rac1 is activated by the formation of cell-cell adhesions. The findings that Rac1 was greatly accumulated at sites of cell-cell contact and activated during formation of cell-cell adhesion sites, suggest that Rac1 was activated at sites of cell-cell contact.

DECMA-1 inhibits Rac1 activation

These results are, however, not sufficient to conclude that E-cadherin-mediated cell-cell adhesion induced Rac1 activation. Because MDCKII cells adhere to each other through not only E-cadherin but also other cell adhesion molecules, it is possible that cell adhesion molecules besides E-cadherin activate Rac1. Moreover, influx of Ca^{2+} into cells might induce Rac1 activation. To exclude the possibility that Rac1 activation was due to factors other than E-cadherin, we tested the effect of DECMA-1, a neutralizing antibody against E-cadherin. MDCKII cells were incubated with EGTA in the presence of DECMA-1, and then Ca^{2+} was restored to the medium in the presence of DECMA-1. Rac1 activation induced by Ca^{2+} restoration was inhibited by DECMA-1 antibody (Fig. 6A). This result indicates that E-cadherin homophilic interaction is responsible for the Rac1 activation. As described above, accumulating evidence suggests that PI 3-kinase is involved in E-cadherin-dependent Rac1 activation. To address this possibility, we examined the effect of wortmannin, an inhibitor of PI 3-kinase, on Rac1 activation. Preincubation of cells with wortmannin (200 nM) prior to Ca^{2+} restoration inhibited the Rac1 activation by Ca^{2+} restoration (Fig. 6B), suggesting that Rac1 was activated by the formation of E-cadherin-mediated cell-cell adhesion through PI 3-kinase. Because wortmannin inhibited E-cadherin-induced Rac1 activation but did not affect the localization of Rac1 and E-cadherin (Fig. 3), the recruitment of Rac1 to sites of E-cadherin-mediated cell-cell adhesion is probably not sufficient for Rac1 activation (see DISCUSSION).

The amounts of coimmunoprecipitated Rac1 with IQGAP1 increase by E-cadherin-mediated cell-cell adhesion

By using EGFP-Rac1 and GST-CRIB, we

found that Rac1 is recruited at sites of cell-cell contact and activated by E-cadherin-mediated cell-cell adhesion. However, we have not directly detected and visualized GTP-bound Rac1 at sites of cell-cell contact. The method to achieve this is not available at present. Instead, to support the idea that Rac1 is activated at sites of cell-cell contact, we examined the localization of effectors of Rac1, which specifically bind to GTP-bound Rac1 and might be recruited in a GTP-bound

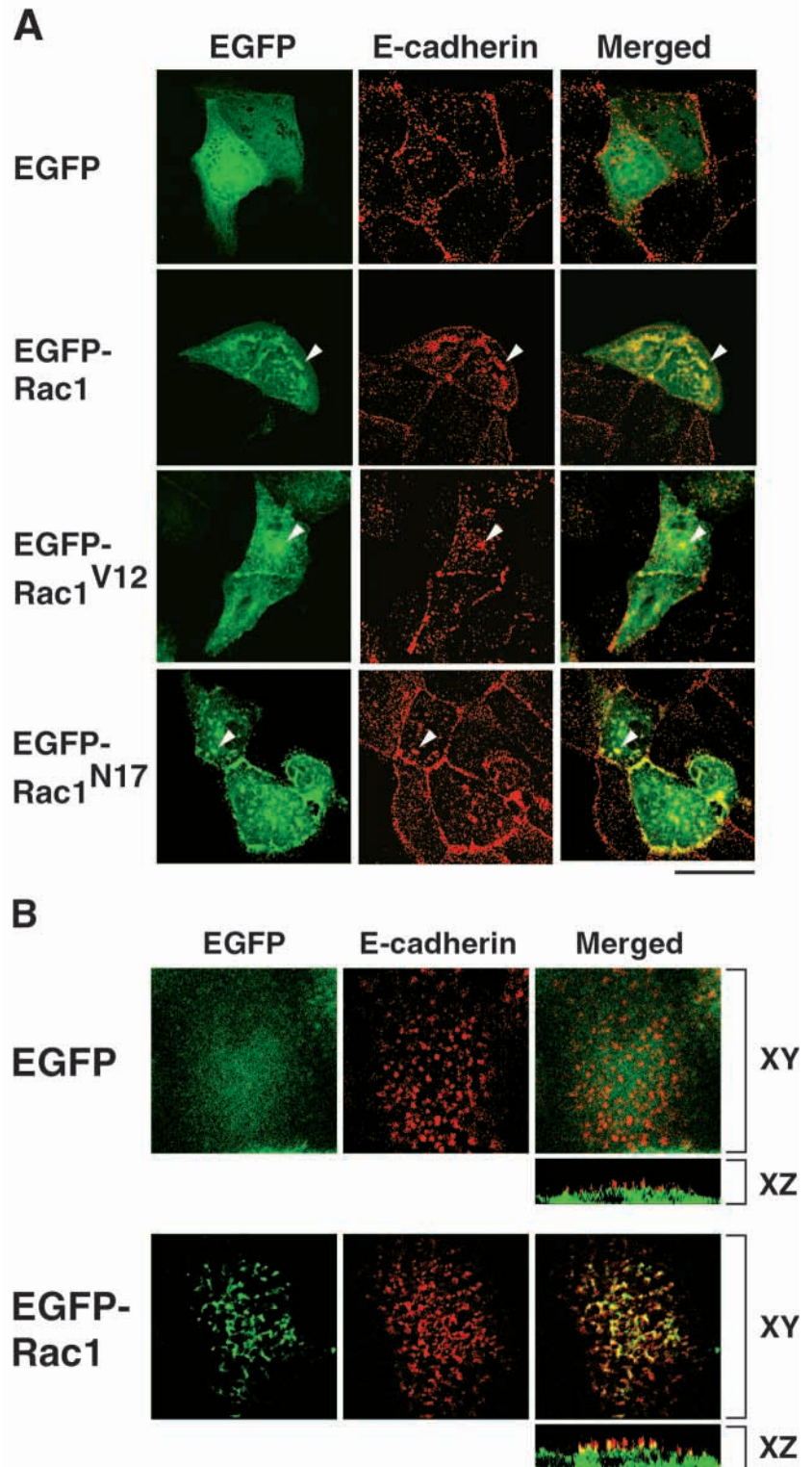


Fig. 4. Engagement of E-cadherin recruits Rac1. (A) MDCKII cells were transfected with the indicated plasmids. The cells were placed on ice for 15 minutes and then incubated with anti-E-cadherin antibody (ECCD-2) for 60 minutes on ice. After having been washed with ice-cold DMEM, the cells were treated with Texasred-conjugated anti-rat antibody for 60 minutes at 4°C and then washed with ice-cold DMEM. Subsequently the cells were incubated for 60 minutes at 37°C to promote clustering, and then fixed. Arrowheads indicate the E-cadherin clusters accumulated by ECCD-2. EGFP-Rac1, EGFP-Rac1^{V12} and EGFP-Rac1^{N17}, but not EGFP alone, were recruited at E-cadherin-clustered aggregates. All confocal images were acquired under the same conditions. Bar, 10 μm . (B) The localization of E-cadherin and EGFP-Rac1 was examined at apical membrane by XY and XZ scans. The results are representative of three independent experiments.

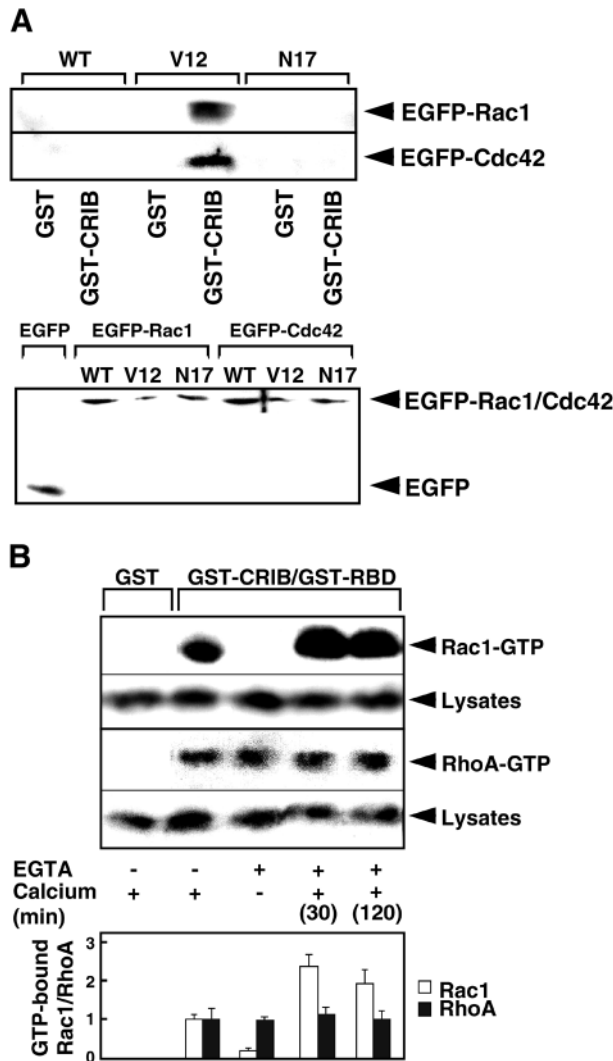


Fig. 5. E-cadherin-mediated cell-cell adhesion activates Rac1. (A) EL cells were transfected with the indicated plasmids. The cells were lysed with lysis buffer, and the lysates were incubated with GST-CRIB. The proteins bound to GST-CRIB were subjected to SDS-PAGE, followed by immunoblotting with anti-GFP antibody (upper panel). The lower panel shows the expression of EGFP, EGFP-Rac1 or EGFP-Cdc42 proteins. (B) In MDCKII cells, Rac1 activation during a calcium switch was measured as described (see MATERIALS AND METHODS). Data are means $\pm$ s.e.m. of five independent experiments. Note that the lane indicated by 'EGTA- and Calcium+' shows Rac1 activation cultured in normal Ca^{2+} levels.

Rac1-dependent manner. IQGAP1 was selected as an effector of Rac1. We previously proposed that IQGAP1 negatively regulates cadherin-mediated cell-cell adhesion (Kuroda et al., 1998) and that activated Rac1 binds IQGAP1 and positively regulates adhesion by inhibiting the IQGAP1 function (Fukata et al., 1999a). Moreover, IQGAP1 is mainly localized at sites of cell-cell contact (Hart et al., 1996; Kuroda et al., 1996). In MDCKII cells cultured in normal Ca^{2+} levels, IQGAP1, EGFP-Rac1 and E-cadherin were colocalized at sites of cell-cell contact (Fig. 7A); whereas, in cells treated with EGTA, they were translocated from sites of cell-cell contact to the cytosol. At 30 minutes after restoration of Ca^{2+} , they could again be

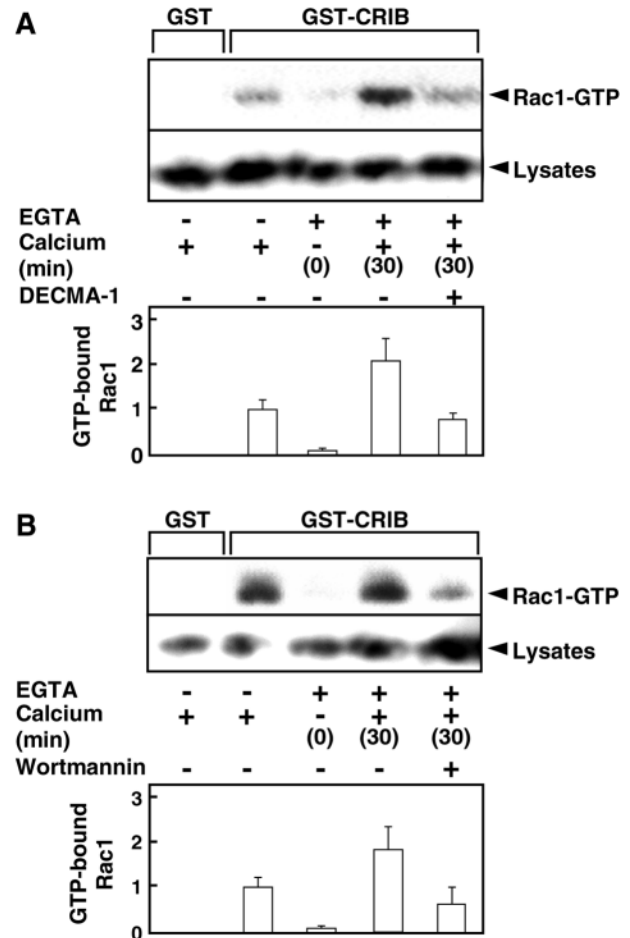


Fig. 6. E-cadherin-mediated cell-cell adhesion activates Rac1 via PI 3-kinase. (A) MDCKII cells were treated with 4 mM EGTA in the absence or presence of DECMA-1 for 30 minutes. Rac1 activation was measured as described in Fig. 5B. (B) MDCKII cells were treated with 4 mM EGTA in the absence or presence of wortmannin (200 nM) for 30 minutes. Rac1 activation was measured as described above. Data are means $\pm$ s.e.m. of five independent experiments.

detected at sites of cell-cell contact. Thus, IQGAP1 and Rac1 behaved in a similar manner during the reorganization of E-cadherin-mediated cell-cell adhesion. Next, we examined the Rac1 activation by measuring the amounts of coimmunoprecipitated Rac1 with IQGAP1 during a calcium switch. Because, IQGAP1 and Rac1 are mainly localized at sites of cell-cell contact and IQGAP1 specifically binds to GTP-bound Rac1, the amounts of coimmunoprecipitated Rac1 could at least contain the amounts of GTP-bound Rac1 at sites of cell-cell contact. In normal Ca^{2+} conditions, Rac1 was coimmunoprecipitated with IQGAP1 (Fig. 7B,C). This coimmunoprecipitated Rac1 is thought to be a basal Rac1 activation at sites of cell-cell adhesion. Subsequently, coimmunoprecipitated Rac1 with IQGAP1 decreased by Ca^{2+} chelation and increased again by Ca^{2+} restoration (Fig. 7B,C). These results were consistent with the data of GTPase activity assay using GST-CRIB and suggest that Rac1 is activated by the formation of E-cadherin-mediated cell-cell adhesion at sites of cell-cell contact.

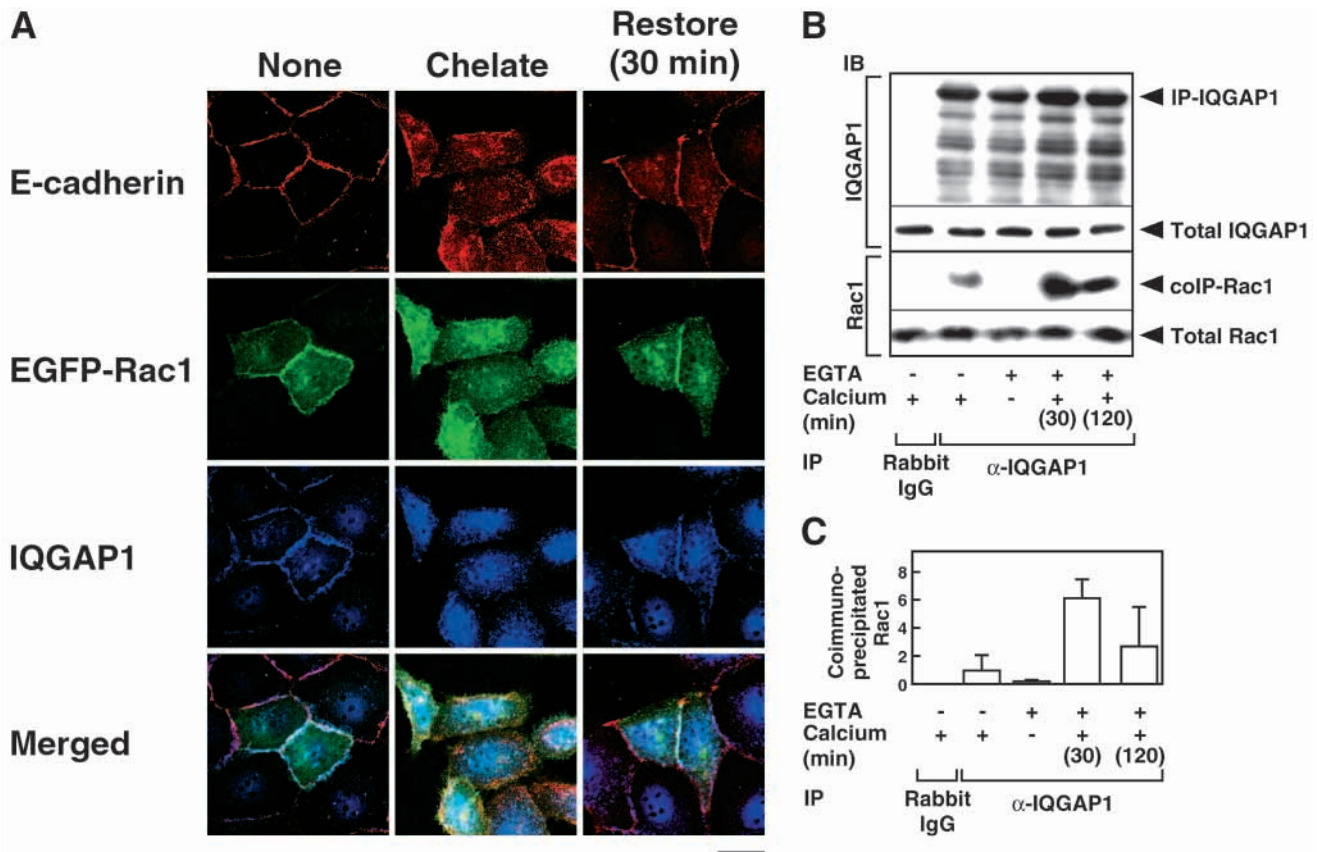


Fig. 7. Localization and interaction of Rac1 with IQGAP1 during a calcium switch. (A) Localization of E-cadherin, EGFP-Rac1 and IQGAP1 was examined by immunostaining. The cells expressing EGFP-Rac1 were doubly stained with anti-E-cadherin antibody (ECCD2) and anti-IQGAP1 antibody, followed by Texasred-conjugated anti-rat antibody and Cy5-conjugated anti-rabbit antibody, respectively. Bar, 10 μ m. (B) In MDCKII cells, Rac1 activation was examined by measuring the amounts of coimmunoprecipitated Rac1 with IQGAP1 during a calcium switch. (C) Data are means $\pm$ s.e.m. of five independent experiments.

DISCUSSION

Although the Rho family GTPases regulate cadherin-mediated cell-cell adhesion (Fukata et al., 1999b), it remains to be elucidated how their localization and activation are regulated. Here, we investigated whether and how Rac1 is recruited and activated by E-cadherin-mediated cell-cell adhesion. We found that Rac1 was recruited to sites of E-cadherin-mediated cell-cell adhesion and that E-cadherin-mediated homophilic interactions and PI 3-kinase activity were required for Rac1 activation, but not for Rac1 recruitment. Moreover, we found that IQGAP1 and Rac1 behaved in a similar manner during the formation of E-cadherin-mediated cell-cell adhesion and that Rac1 activation by E-cadherin-mediated cell-cell contact was confirmed by its binding to IQGAP1. On the basis of these observations, we speculate that there should exist at least two steps through which E-cadherin-mediated cell-cell adhesion activates Rac1: (1) Rac1 recruitment at sites of cell-cell contact; and (2) Rac1 activation by a certain GEF.

Several observations suggest that Rho, another Rho family protein, shuttles between the cytosol and specific membrane sites (Sasaki and Takai, 1998). In resting cells, Rho exists mostly in its GDP-bound form and in complexes with Rho dissociation inhibitor (Rho GDI) in the cytosol. Upon stimulation with extracellular signals such as thrombin, Rho is

likely to be dissociated from Rho GDI by Rho GDI displacement factors (GDFs), such as the ezrin/radixin/moesin (ERM) family of molecules (Takahashi et al., 1998), and specific GEFs for Rho are activated. The GDP-bound form of Rho is then converted to its GTP-bound form by GEF activation. Rho is targeted to specific membrane sites through its C-terminal modification (geranylgeranylation) and interacts with its specific effectors. GTP-bound Rho is reconverted to the GDP-bound form by its intrinsic GTPase activity and GTPase-activating proteins (GAPs). Rho GDI can then form a complex with the GDP-bound Rho, extract Rho from the membrane and move it into the cytosol. C-terminal modification of Rho plays a crucial role in anchoring Rho to the cellular membrane and in interacting with Rho GDI (Michaelson et al., 2001). Because Rho GDI interacts with Rac1, blocks the dissociation of GDP from GDP-bound Rac1 and extracts Rac1 from membranes, as in the case of Rho, the mechanism underlying the recruitment of Rac1 can be predicted to be like that of Rho. GDP-bound Rac1 is sequestered in the cytosol by Rho GDI before establishment of cadherin-mediated cell-cell adhesion. When cadherin-mediated homophilic interactions occurred, GDP-bound Rac1 dissociated from Rho GDI, possibly through the action of GDF, and targets plasma membrane by its C-terminal CAAL motif or another regulatory mechanisms. GDP-bound Rac1 is then

converted to GTP-bound Rac1 through the action of a certain GEF. Further studies are required to address this issue.

Pece et al., reported that the engagement of E-cadherins in homophilic cell-cell interaction resulted in rapid PI 3-kinase activation (Pece et al., 1999). We found that wortmannin, an inhibitor of PI 3-kinase, inhibited the Rac1 activation by establishment of E-cadherin-mediated cell-cell adhesion (Fig. 6B) but not the recruitment of Rac1 and E-cadherin at sites of cell-cell contact (Fig. 3). These data suggest that PI 3-kinase functions upstream of Rac1 in the process of E-cadherin-induced Rac1 activation. E-cadherin-mediated cell-cell adhesion might be established by two steps: (1) recruitment of E-cadherin at sites of cell-cell contact; and (2) maturation and maintenance of the adhesion (e.g. by cadherin-clustering and anchoring by actin cytoskeleton). We think the action of Rac1 activation is involved in the second step. PI 3-kinase might regulate this second activation process and be required for the maturation and maintenance of cadherin-based adhesions rather than the initial recruitment.

Tiam1 is a good candidate for a Rac1 regulator downstream of E-cadherin. Sander et al., have shown that Tiam1 functions downstream of PI 3-kinase (Sander et al., 1998). Thus, it is suggested that E-cadherin-mediated cell-cell adhesion activates Rac1 through PI 3-kinase and Tiam1. Recently, it has been shown that the hyaluronic acid-binding receptor CD44, which mediates cell-cell or cell-substratum adhesion, directly interacts with Tiam1 and that the binding of hyaluronic acid to CD44 stimulates Tiam1-catalyzed Rac1 activation (Bourguignon et al., 2000b). Ankyrin, which is a membrane-associated cytoskeletal protein that binds to many plasma membrane-associated proteins, such as band3, Na⁺/K⁺-ATPase and CD44, also binds to Tiam1; this interaction also promotes its GEF activity (Bourguignon et al., 2000a). In MDCKII cells, Tiam1 is colocalized with E-cadherin (Hordijk et al., 1997). Therefore, it will be important to examine whether Tiam1 interacts with E-cadherin, β -catenin or α -catenin. We attempted to determine whether Tiam1 was coimmunoprecipitated with E-cadherin, β -catenin or α -catenin from MDCKII cells. However, we were unable to detect the interaction of Tiam1 with E-cadherin, β -catenin or α -catenin (data not shown). Vav2, which is a GEF for Rac1, Cdc42, RhoA and RhoG, is another candidate for a Rac1 regulator downstream of E-cadherin. p120^{cas}, which binds to the membrane-proximal region of the cadherin, interacts with Vav2 in HEK293 cells (Noren et al., 2000). Overexpression of p120^{cas} activates Rac1 and Cdc42 through Vav2 in CHO cells. Further studies are necessary to elucidate whether, and if so how, Tiam1 or Vav2 are involved in the activation of Rac1 at sites of E-cadherin-mediated cell-cell adhesion.

We showed earlier by a cell dissociation assay that Cdc42 and Rac1 are required for the E-cadherin-mediated cell-cell adhesion activity (Fukata et al., 1999a). It has recently been shown that E-cadherin-mediated cell-cell adhesion induces Cdc42 activation in L cells stably expressing E-cadherin (Kim et al., 2000). However, we could not detect Cdc42 activation under the same conditions that we used to show Rac1 activation, probably owing to the fact that a smaller amount of Cdc42 than Rac1 is expressed in MDCKII cells. The intracellular concentrations of Rac1 and Cdc42 in MDCKII cells were calculated to be about 500 nM and 100 nM, respectively. In MDCKII cells, Rac1 may play a more central

role in the regulation of cadherin-mediated cell-cell adhesion than Cdc42. Modulation of the cadherin-mediated cell-cell adhesion by Rac1 or Cdc42 might depend on the cell type.

In this study, we found that E-cadherin-mediated cell-cell adhesion activated Rac1 (outside-in signaling). A previous study showed that Rac1^{V12} promoted accumulation of E-cadherin at sites of cell-cell contact, whereas dominant negative Rac1^{N17} inhibited it (inside-out signaling; Takaishi et al., 1997). As in the case of integrin-mediated cell-substratum adhesion (Schoenwaelder and Burridge, 1999), there exists bi-directional signaling pathways between E-cadherin and the Rho family GTPases.

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