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 (for a review, Fukata et al., J. Cell Sci. 112, 4491-4500, 1999). 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, I demonstrated that EGFP-Rac1 was colocalized with E-cadherin at sites of cell-cell contact and translocated to the cytosol during disruption of E-cadherin-mediated cell-cell adhesion by Ca^{2+} chelation. Reestablishment 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 wild-type, constitutively active, and dominant negative Rac1. I also investigated whether E-cadherin-mediated cell-cell adhesion induced Rac1 activation by use of an assay to measure 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 (PI3-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. Tiam1, an activator of Rac1, was localized at the sites of cell-cell contact and accumulated to the cadherin-clusters in an E-cadherin-dependent manner. Taken together, these results suggest that Rac1 is recruited at sites of E-cadherin-mediated cell-cell adhesion, irrespective of whether Rac1 binds to GTP or GDP, and is then activated, possibly through PI3-kinase and Tiam1.

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 and 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). $p120^{ctn}$ (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, are also good candidates as 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, whereas 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, which are 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., 1999b). Moreover, both IQGAP1 and Rac1 are mainly localized 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, I 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. I found that Rac1 was recruited at intercellular junctions, irrespective of whether Rac1 bound to GTP or GDP, and that E-cadherin-mediated homophilic interactions activated Rac1, possibly through phosphatidylinositol 3-kinase (PI3-kinase) and Tiam1.

MATERIALS AND METHODS

Materials and chemicals

EL cells, MDCKII cells, and anti-E-cadherin rat monoclonal antibody (ECCD-2) were kindly provided by Drs. 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 monoclonal antibody against E-cadherin, was kindly provided by Drs. R. Kemler (Max-plank Institute fur Immunbiologie, Freiburg, Germany) and M. Ozawa (Kagoshima University, Kagoshima, Japan). Anti-GFP antibody (mFX73) was kindly provided by Dr. 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 monoclonal antibody was purchased from Upstate Biotechnology Inc. (Lake Placid, NY); and anti-RhoA mouse monoclonal antibody, 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 Dr. M. A. Schwartz (The Scripps Research Institute, CA); the cDNA of αPAK, by Dr. S. Ohno (Yokohama City University School of Medicine, Yokohama, Japan); and the cDNA of Tiam1, by Dr. J. G. Collard (The Netherlands Cancer Institute, The Netherlands). Wortmannin, an

inhibitor of PI3-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, I subcloned the cDNA fragments of Rac1 and Cdc42 into the BamHI site of EGFP-C1 (CLONTECH Laboratories Inc.), respectively. To obtain EGFP-Rac1^{V12} and Rac1^{N17}, I subcloned the corresponding fragments of Rac1 mutants into BamHI and EcoRI sites of EGFP-C1. A fragment harboring Cdc42/Rac1 interactive binding region (CRIB) of αPAK (aa 70–106) was generated by PCR using oligonucleotides CTGAGGATCCAAGGAGCGGCCAGAGATTTCTCT and CTGAGGATCCTCACAAGCGGGCCCACTGTTCTG, digested with BamHI, and inserted into the BamHI site of pGEX-4T-1 (Pharmacia Biotech, NJ) to obtain pGEX-CRIB.

Preparation of GST-CRIB and GST-RBD

The expression and purification of GST-CRIB and GST-RBD fusion proteins were done as described (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 x 10^6 cells/10-cm dish) were seeded and cultured for 18 h. EL cells were transfected with the desired plasmids by

using LipofectAMINE (GIBCO BRL, Grand Island, NY). After a 48-h 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 $MgCl_2$, 1% NP-40, 150 mM NaCl, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μM (p-amidinophenyl)-methanesulfonyl fluoride). The lysates were then centrifuged at 20,000 g for 7 min at 4°C, and the supernatant was incubated with purified GST-CRIB immobilized beads at 4°C for 1 h. 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-h incubation, the lysates were incubated with protein A Sepharose beads for 1 h at 4°C for preclearing. The supernatant was then incubated with anti-GFP antibody for 1 h 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 and cultured for 24 h, and then the cells were serum-starved. After

a 24-h incubation, E-cadherin-mediated cell-cell contacts were disrupted by treatment with 4 mM EGTA at 37°C for 30 min. Thereafter, intercellular contacts were allowed to reform in the presence of normal Ca^{2+}-containing medium ($CaCl_2$ ~ 1.8 mM) for 30-120 min at 37°C. Then, 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, I included DECMA-1 antibody or wortmannin (200 nM) in the medium.

Immunoprecipitation of IQGAP1

Immunoprecipitation was performed essentially similar as GTPase activity assay. In brief, MDCKII cells (5×10^6 cells /10-cm dish) were seeded into dishes and cultured for 24 h, and then the cells were serum-starved. After a 24-h 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 h. 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 h 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 h 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 63X/1.40 NA oil immersion objective). Optical scans were collected every 10 min. 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., CA).

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 h after seeding, the cells were transfected with EGFP-Rac1 and then serum-starved for 24 h. Thereafter, calcium switch was performed. Next the cells were fixed with 3% formaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature and then permeabilized with PBS containing 0.2% Triton X-100 and 2 mg/ml BSA for 15 min. 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 μm 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 min and then incubated with ECCD-2 for 60 min on ice. After having been washed with ice-cold DMEM, the cells were treated with Texasred-conjugated anti-rat antibody for 60 min at 4°C and then washed with ice-cold DMEM. Subsequently the cells were incubated for 60 min 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, I constructed cDNAs of wild-type, constitutively active, and dominant negative Rac1 tagged with EGFP at its amino 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, since 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 neither 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 **(Fig. 4A)**. 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). Thus, I 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), I examined the dynamics of EGFP-Rac1 localization in living cells by time-lapse analysis (**Fig. 2A**). In MDCKII cells cultured in normal Ca^{2+} levels (~1.8 mM), EGFP-Rac1 was localized at sites of cell-cell contact. Whereas, in cells treated with EGTA, a Ca^{2+} chelator, EGFP-Rac1 was translocated from sites of cell-cell contact to the cytosol and became diffusely distributed there. After the restoration of Ca^{2+}, EGFP-Rac1 could again be detected at sites of cell-cell contact; and by 120 min after the restoration, formation of E-cadherin-mediated cell-cell adhesion sites was complete. These results suggest that EGFP-Rac1 dynamically relocalizes during the reorganization of E-cadherin-mediated cell-cell adhesion. Next, I 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 Ca^{2+} levels (**Fig. 2B**). After the chelation of Ca^{2+}, both EGFP-Rac1 and E-cadherin were disappeared from sites of adhesion, then translocated to sites of adhesion by Ca^{2+} restoration (30 min). After 120 min Ca^{2+} restoration, both EGFP-Rac1 and E-cadherin were colocalized at sites of cell-cell adhesion. 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, I 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 prior to 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 PI3-kinase activation in MDCKII cells followed by Akt/PKB activation (Pece et al., 1999). Moreover, the interaction of PI3-kinase with E-cadherin (Pece et al., 1999) or β-catenin (Espada et al., 1999) has been reported. Since activated PI3-kinase is thought to activate Rac1 (Hawkins et al., 1995; Kotani et al., 1994; Reif et al., 1996), these observations suggest the possible involvement of PI3-kinase in the E-cadherin-dependent Rac1 recruitment. To address this possibility, the effect of wortmannin, an inhibitor of PI3-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, PI3-kinase does not appear to be involved in the recruitment of EGFP-Rac1 and E-cadherin at sites of cell-cell contact.

EGFP-Rac1 is colocalized with E-cadherin but not with ZO-1 at sites of cell-cell contact irrespective of whether EGFP-Rac1 binds to GTP or GDP

To elucidate the mechanism underlying the recruitment of Rac1

at sites of cell-cell contact, I examined the localization of EGFP-Rac1^{V12} and EGFP-Rac1^{N17} in addition to that of EGFP-Rac1. EGFP-Rac1^{V12} and EGFP-Rac1^{N17}, but not EGFP, were also localized at sites of cell-cell contact (**Fig. 4A**). This result is consistent with previous observations that myc- or HA-tagged Rac1^{V12} and Rac1^{N17} were localized at sites of cell-cell adhesion (Jou and Nelson, 1998; Kuroda et al., 1997; Takaishi et al., 1997). Moreover, I examined the colocalization of EGFP-Rac1 with E-cadherin at sites of adhesion by XZ scans (**Fig. 4B**). E-cadherin was localized at lateral membrane and colocalized with EGFP-Rac1 (**Fig. 4B**). ZO-1, a marker of the tight junction, was localized at apical membrane, whereas EGFP-Rac1 was localized at lateral membrane. Similar results were obtained using EGFP-Rac1^{V12} and EGFP-Rac1^{N17} instead of EGFP-Rac1 (data not shown). These results indicate that EGFP-Rac1 is colocalized with E-cadherin at lateral membrane irrespective of whether EGFP-Rac1 binds to GTP or GDP.

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, I developed an assay to force the accumulation of E-cadherin at the apical membrane using anti-E-cadherin antibody (ECCD-2) and then used it to examine 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 cross-link the first antibody and to cluster E-cadherin at the apical membrane as aggregates. Alpha-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. 5A)**. EGFP alone was not recruited **(Fig. 5A)**. EGFP-Cdc42 and EGFP-RhoA hardly did (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. 5B**). 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, I took the advantage of the fact that Rac1 effectors interact only with GTP-bound Rac1 (Ren et al., 1999). I constructed GST-fused Cdc42/Rac1 interactive binding region (CRIB) of PAK, PAK being a Rac1/Cdc42 effector. To evaluate the specificity of GST-CRIB, I 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. 6A)**. No specific binding was detected with wild-type Rac1, $Rac1^{N17}$, wild-type Cdc42, and $Cdc42^{N17}$ **(Fig. 6A)**. 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).

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 having the normal Ca^{2+} levels, Rac1 in its active GTP-bound form could be detected; lane indicated by "EGTA- and Calcium+" **(Fig. 6B)**. 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 min after restoration **(Fig. 6B)** and gradually returned to the basal level. 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 Rho as measured by GST-RBD, was not affected and remained at the basal level during a calcium switch **(Fig. 6B)**. Although I tried to detect active Cdc42

by using GST-CRIB, I 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. Since MDCKII cells adhere to each other through not only E-cadherin but also other cell adhesion molecules, it is also possible that cell adhesion molecules besides E-cadherin may activate Rac1. Moreover, influx of Ca^{2+} into cells might induce Rac1 activation. To exclude the possibility that Rac1 activation was due to other factors than E-cadherin, I 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. 7A**). This result indicates that E-cadherin homophilic interaction is responsible for the Rac1 activation. As described above, accumulating evidence suggests the possible involvement of PI3-kinase in E-cadherin-dependent Rac1 activation. To address this possibility, I examined the effect of wortmannin, an inhibitor of PI3-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. 7B)**, suggesting that Rac1 was activated by the formation of E-cadherin-mediated cell-cell adhesion through PI3-kinase. Since 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, I found that Rac1 is recruited at sites of cell-cell contact and activated by E-cadherin-mediated cell-cell adhesion. However, I 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, I examined the localization of effectors of Rac1, because they specifically bind to GTP-bound Rac1 and might be recruited in a GTP-bound Rac1-dependent manner. IQGAP1 was selected as an effector of Rac1. I 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. 8A)**. Whereas, in cells treated with EGTA, they were translocated from sites of cell-cell contact to the cytosol. At 30 min after restoration of Ca^{2+}, they could again be 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, I examined the Rac1 activation by measuring the amounts of coimmunoprecipitated Rac1 with IQGAP1 during a calcium switch. Since, IQGAP1 is localized at sites of cell-cell contact and specifically binds to GTP-bound Rac1, the amounts of coimmunoprecipitated Rac1 could reflect the amounts of GTP-bound Rac1 at sites of cell-cell contact. In normal Ca^{2+} conditions, Rac1 was coimmunoprecipitated with IQGAP1 **(Fig. 8B and 8C)**. This coimmunoprecipitated Rac1 is thought to be a basal Rac1 activation at sites of cell-cell adhesion. Then, coimmunoprecipitated Rac1 with IQGAP1 decreased by Ca^{2+} chelation and increased again by Ca^{2+} restoration **(Fig. 8B and 8C)**. These results were consistent with the data of GTPase activity assay using GST-CRIB and suggest that Rac1 could be activated by the formation of E-cadherin-mediated cell-cell adhesion at sites of cell-cell contact.

Localization of Tiam1, a GEF for Rac1, at the sites of cell-cell contact

Next question is by which Rac1 is activated downstream of E-cadherin. It has been known that Rho family GTPases are activated

by GDP/GTP exchange factors (GEFs). Tiam1, one of the 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). Sander et al. have shown that Tiam1 functions downstream of PI3-kinase (Sander et al., 1998). Thus, it is suggested that E-cadherin-mediated cell-cell adhesion activates Rac1 through PI3-kinase and Tiam1. Tiam1 has two PH domains (N-terminus PHn and C-terminus PHc) and a PDZ domain (**Fig. 9A**). It has been reported that N-terminus PHnCCEX region is sufficient for membrane localization of Tiam1 (Stam et al., 1997). At first, I examined whether Tiam1 contributes to cadherin-mediated cell-cell adhesion. Tiam1-PHnCCEX region was transfected into L and EL cells and then immunostaining was performed. L cells are mouse fibroblasts and do not express endogenous E-cadherin. EL cells are L cells stably expressing E-cadherin and exert E-cadherin-dependent adhesive activity. Tiam1-PHnCCEX accumulated at the sites of cell-cell contact in EL cells, but not in L cells (**Fig. 9B**). These observations suggest that Tiam1-PHnCCEX accumulates at the sites of cell-cell contact in an E-cadherin-dependent manner. To confirm these results, I examined whether Tiam1-PHnCCEX is recruited at apical membrane by engagement of E-cadherin using ECCD2. Tiam1-PHnCCEX was transfected into MDCKII cells and then E-cadherin was clustered at apical membrane by ECCD2. Tiam1-PHnCCEX was recruited to the aggregates composed

of E-cadherin at apical membrane (**Fig. 9C**). Tiam1-C1199 was also accumulated at E-cadherin aggregates (data not shown). Taken together, these results indicate that Tiam1 could be recruited by E-cadherin-mediated cell-cell adhesion.

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, I investigated whether and how Rac1 is recruited and activated by E-cadherin-mediated cell-cell adhesion. I found that Rac1 was recruited to sites of E-cadherin-mediated cell-cell adhesion, irrespective of whether Rac1 bound to GTP or GDP, and that E-cadherin-mediated homophilic interactions and PI3-kinase activity were required for Rac1 activation, but not for Rac1 recruitment. Moreover, I 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. I also found that Tiam1 was localized at the sites of cell-cell contact in an E-cadherin-dependent manner. On the basis of these observations, I 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 (Tiam1) (**Fig. 10**).

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. Then, by GEF activation the GDP-bound form of Rho is converted to its GTP-bound form. Rho is targeted to specific membrane sites through its carboxyl-terminal modification (geranylgeranylation) and then 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 and extract Rho from the membrane and move it into the cytosol. Carboxyl-terminal modification of Rho plays a crucial role in anchoring Rho to the cellular membrane and in interacting with Rho GDI. Since Rho GDI also 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. However, I found that GDP-bound Rac1 was still localized at sites of cell-cell contact (**Fig. 4A**) and that the localization of Rac1 at sites of cell-cell contact required the carboxyl-terminal sequence of Rac1 (CAAL motif), irrespective of whether or not Rac1 bound GTP or GDP (data not shown). Taken together, it is tempting to speculate that GDP-bound Rac1 or GDP-bound Rac1/Rho GDI complex is recruited to the membrane upon stimulation with E-cadherin-mediated homophilic interactions, and then converted to the GTP-bound form 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 PI3-kinase activation (Pece et al., 1999). I found that wortmannin, an inhibitor of PI3-kinase, inhibited the Rac1 activation by establishment of E-cadherin-mediated cell-cell adhesion (**Fig. 7B**) but not the recruitment of Rac1 and E-cadherin at sites of cell-cell contact (**Fig. 3**). These data suggest that PI3-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 then 2) maturation and maintenance of the adhesion (e.g. by cadherin-clustering and anchoring by actin cytoskeleton). I think the action of Rac1 activation is involved in the 2nd step. PI3-kinase might regulate this 2nd 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 PI3-kinase (Sander et al., 1998). I found that Tiam1-PHnCCEX accumulates at the sites of cell-cell contact possibly in an E-cadherin-dependent manner (**Fig. 9**). Thus, it is suggested that E-cadherin-mediated cell-cell adhesion activates Rac1 through PI3-kinase and Tiam1. Recently, it has been shown that CD44, which is the hyaluronic acid-binding receptor and 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 and binds to many plasma membrane-associated proteins, such as band3, Na^+/K^+-ATPase, and CD44, also binds to Tiam1; and 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. I attempted to determine whether Tiam1 was coimmunoprecipitated with E-cadherin, β-catenin, or α-catenin from MDCKII cells. However, I was unable to detect the interaction of Tiam1 with E-cadherin, β-catenin, nor α-catenin (data not shown). It is possible that membrane localization of Tiam1 might be regulated by other molecules. Now, I am trying to identify novel interacting molecules of Tiam1. Vav2, which is a GEF for Rac1, Cdc42, RhoA, and RhoG, is another candidate for a Rac1 regulator downstream of E-cadherin. $p120^{ctn}$, which binds to the membrane-proximal region of the cadherin, interacts with Vav2 in HEK293 cells (Noren et al., 2000). Overexpression of $p120^{ctn}$ activates Rac1 and Cdc42 through Vav2 in CHO cells. Further studies are necessary to elucidate whether, and if so how, Tiam1 or Vav2 is involved in the activation of Rac1 at sites of E-cadherin-mediated cell-cell adhesion.

I showed earlier by a cell dissociation assay that Cdc42 as well as Rac1 is 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, I could not detect Cdc42 activation under the same conditions that I used to show Rac1 activation, probably due 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, I 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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FIGURE LEGENDS

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-h incubation, the cells were fixed and then stained with tetramethylrhodamine B isothiocyanate-phalloidin. An arrow indicates the membrane ruffling. Bar, 10 μm.

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 min. Subsequently, the cells were treated with Ca^{2+}-containing medium for the indicated times. *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 imaged under the same conditions. Bars, 10 μm.

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 PI3-kinase (wortmannin; 200 nM) for 30 min. Subsequently, the cells were treated with Ca^{2+}-containing medium in the presence of either DECMA-1 or wortmannin for 30 min. 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.

Fig. 4 Localization of EGFP-Rac1 mutants at sites of cell-cell contact. *A*, MDCKII cells were transfected with the indicated plasmids. The localization of EGFP-Rac1 was examined as described in Fig. 2B. Note that EGFP-Rac1 was localized at sites of cell-cell contact, irrespective of whether Rac1 bound to GTP ($Rac1^{V12}$) or to GDP ($Rac1^{N17}$). All confocal images were imaged under the same conditions. Bar, 10 μm. *B*, The localization of EGFP-Rac1, E-cadherin, and ZO-1 was examined by XZ scans.

Fig. 5 Engagement of E-cadherin recruits Rac1. *A*, MDCKII cells were transfected with the indicated plasmids. The cells were placed on ice for 15 min and then incubated with anti-E-cadherin antibody (ECCD-2) for 60 min on ice. After having been washed with ice-cold DMEM, the cells were treated with Texasred-conjugated anti-rat antibody for 60 min at 4°C and then washed with ice-cold DMEM. Subsequently the cells were incubated for 60 min at 37°C to promote clustering and then fixed. Arrowheads indicate the E-cadherin-clusters accumulated by ECCD-2. EGFP-Rac1, $EGFP\text{-}Rac1^{V12}$, and $EGFP\text{-}Rac1^{N17}$, but not EGFP alone, were recruited at E-

cadherin-clustered aggregates. All confocal images were imaged 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. 6 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). 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 ±S.E. of five independent experiments. Note; lane indicated by "EGTA- and Calcium+" shows Rac1 activation cultured in normal Ca^{2+} levels.

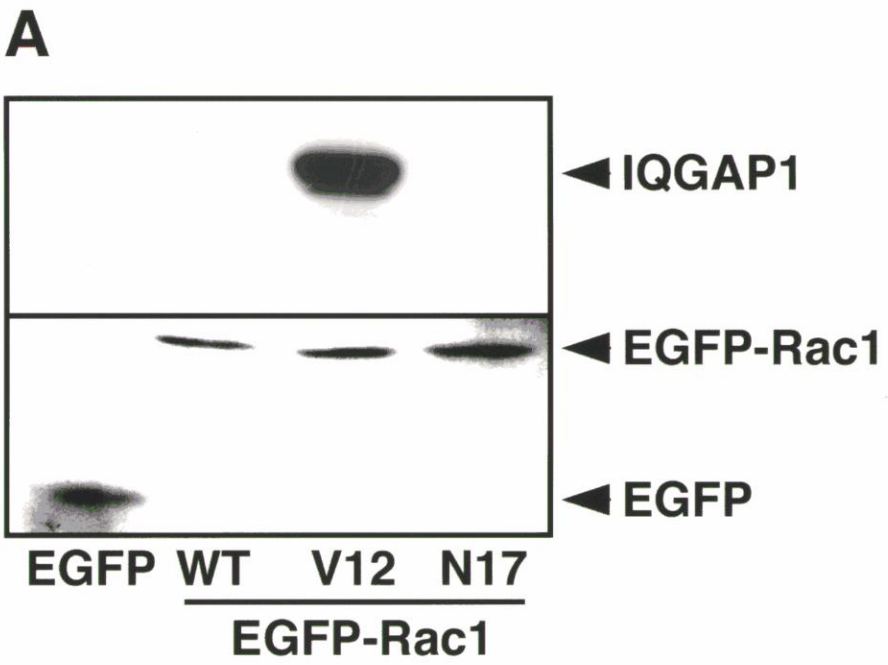
Fig. 7 E-cadherin-mediated cell-cell adhesion activates Rac1 via PI3-kinase. *A*, MDCKII cells were treated with 4 mM EGTA in the absence or presence of DECMA-1 for 30 min. Rac1 activation was measured as described in Fig. 6B. *B*, MDCKII cells were treated with 4 mM EGTA in the absence or presence of wortmannin (200 nM) for 30 min. Rac1 activation was measured as described above. Data are means ±S.E. of five independent experiments.

Fig. 8 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 μm. *B*, In MDCKII cells, Rac1 activation was examined by measuring the amounts of coimmunoprecipitated Rac1 with IQGAP1 during a calcium switch. Data are means $\pm$S.E. of five independent experiments.

Fig. 9 Localization of Tiam1 at the sites of cell-cell contact. *A*, Schematic representation of Tiam1. M, myristoylation site; PEST, PEST region; PHn and PHc, N- and C-terminal pleckstrin homology domain; CC, putative coiled coil region; EX, EX region; DHR, disc large homology region (also called as PDZ); DH, Dbl homology domain. *B*, In L and EL cells, transfected Tiam1-PHnCCEX was visualized by immunostaining. *C*, MDCKII cells were transfected with Tiam1-PHnCCEX. E-cadherin was clustered at apical membrane using ECCD2. Recruitment of Tiam1 at apical membrane was examined as described in Fig. 5. Bars, 10 μm.

Fig. 10 A proposed model. Model for the Rac1 recruitment and activation by E-cadherin-mediated cell-cell adhesion; 1) Rac1 recruitment at sites of cell-cell contact and 2) Rac1 activation by PI3-kinase and Tiam1.

Fig. 1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EGFP
EGFP WT V12 N17
EGFP-Rac1

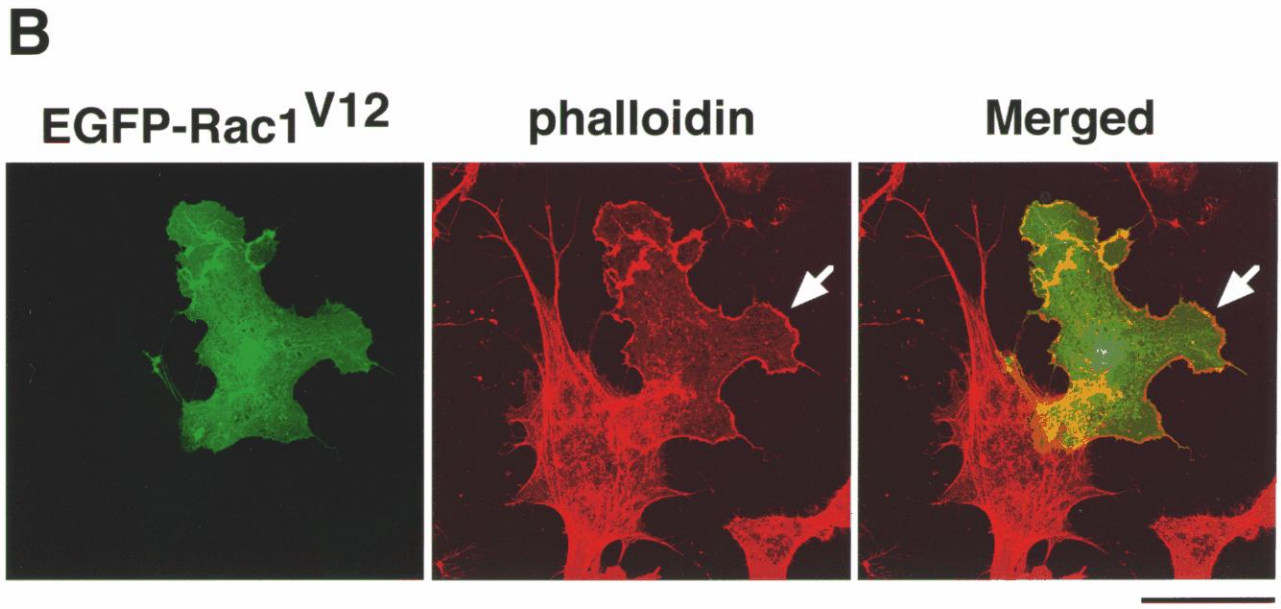
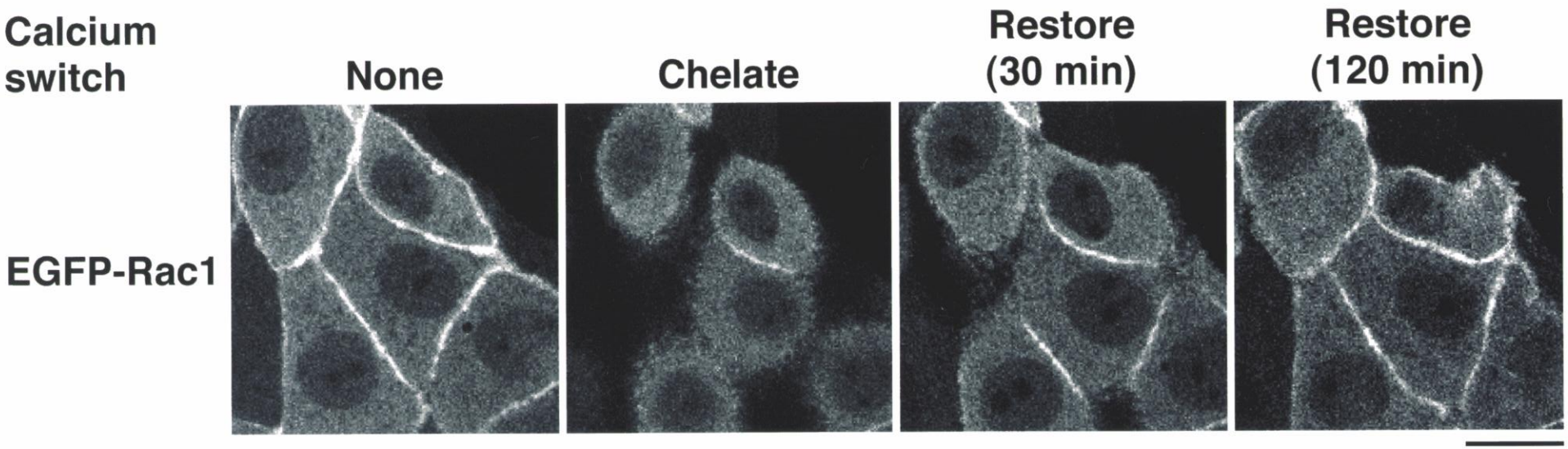

B
EGFP-Rac1V12
phalloidin
Merged

Fig. 2

A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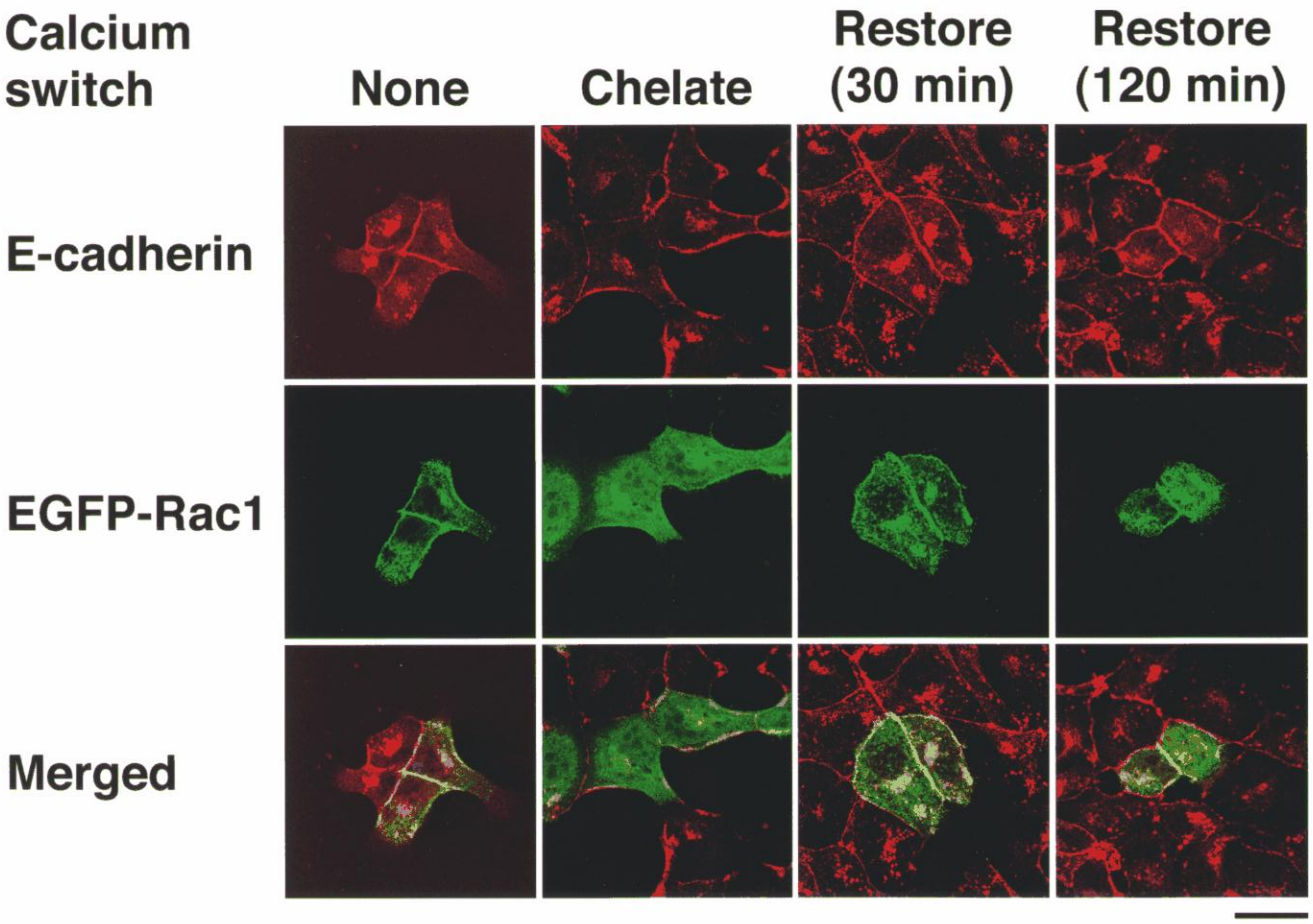
B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Chelate
Restore (30 min)
Restore (120 min)
E-cadherin
EGFP-Rac1
Merged

Fig. 3

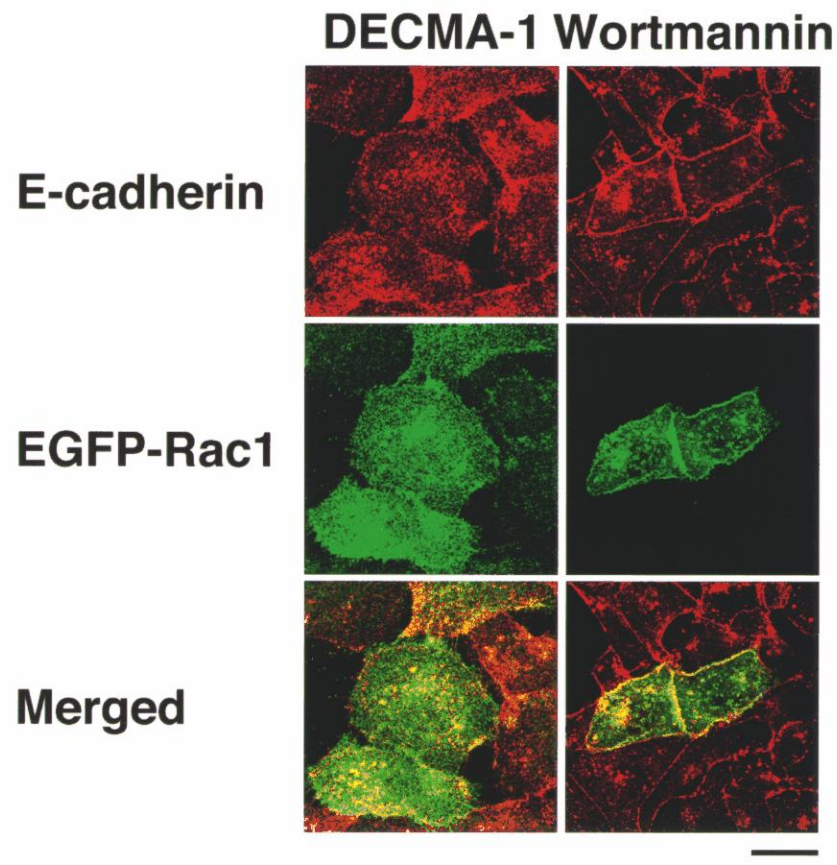
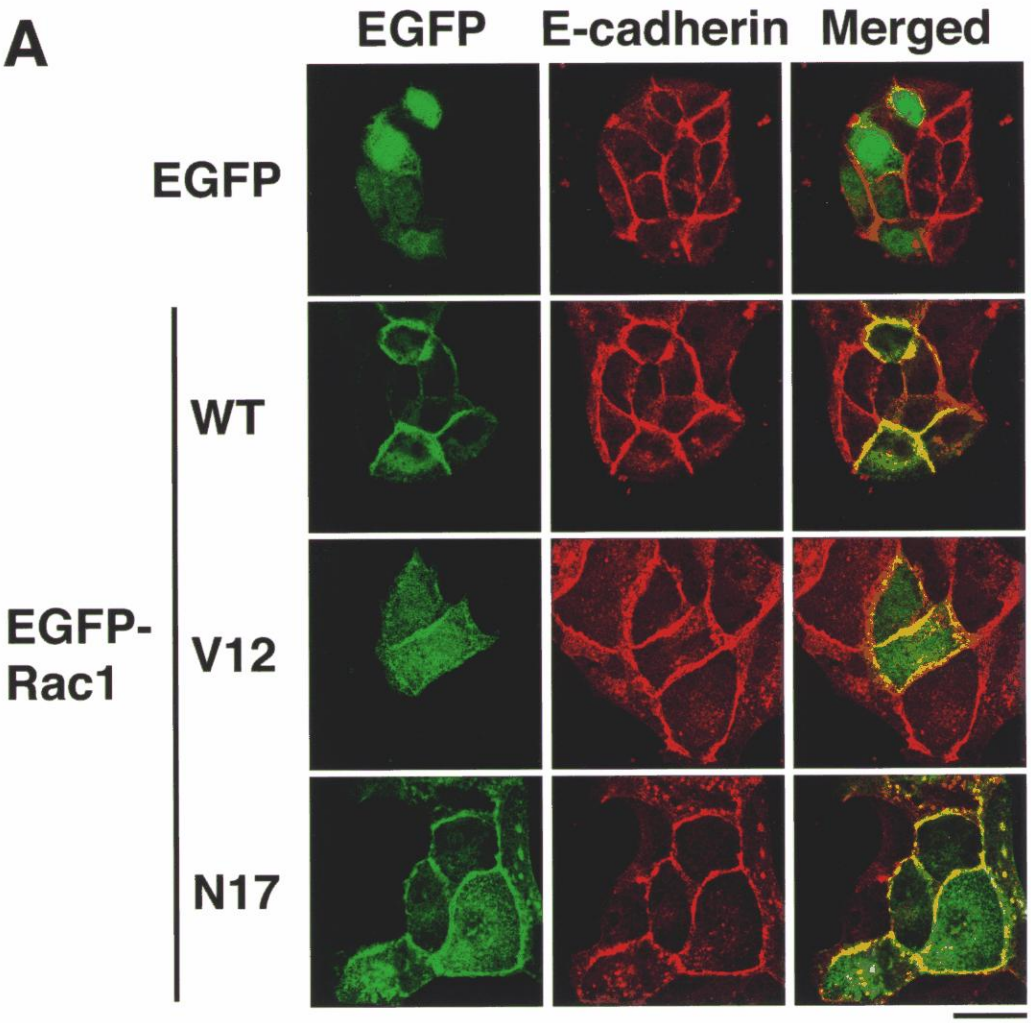


Fig. 4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E-cadherin
Merged
EGFP
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WT
V12
N17
B
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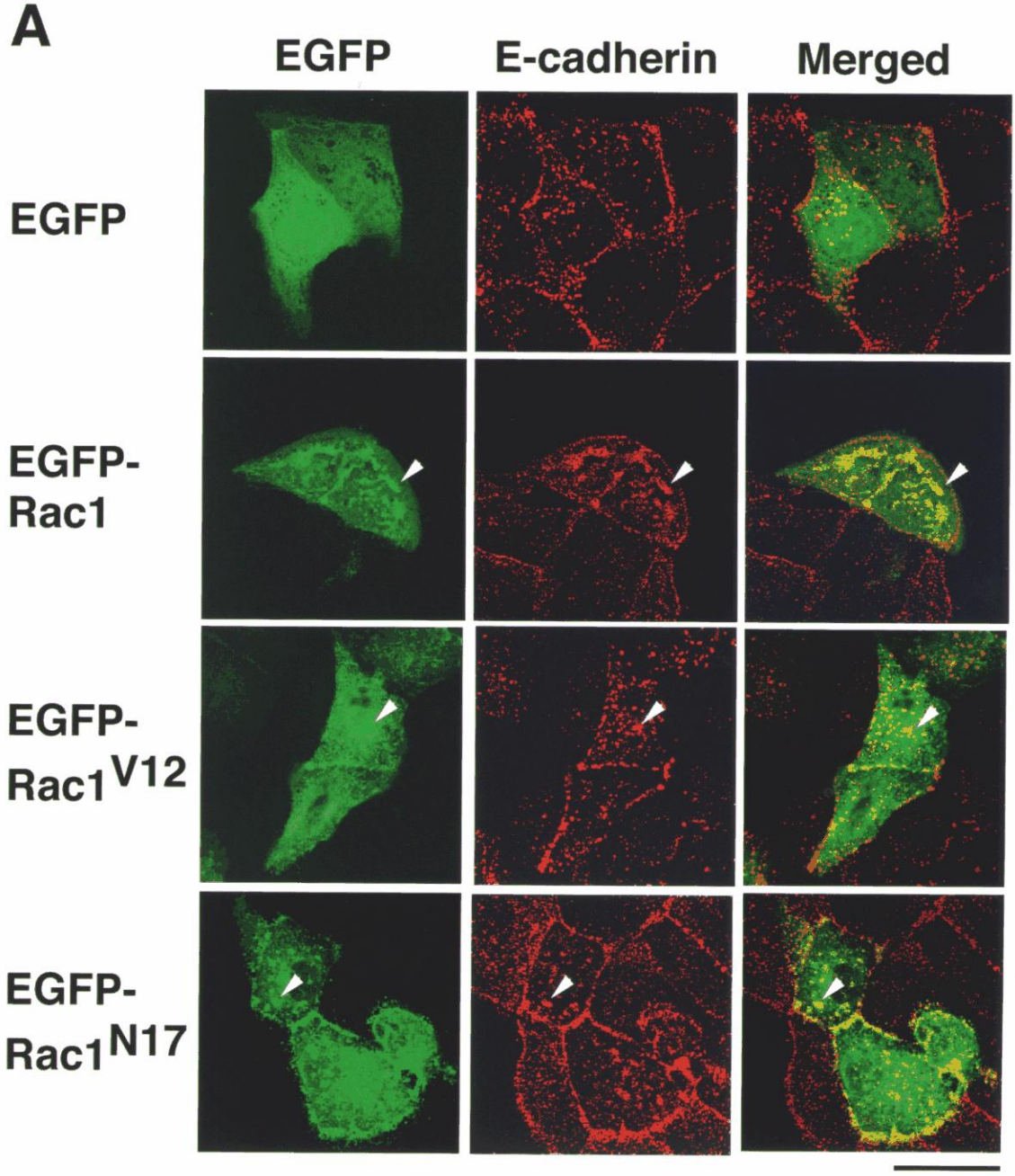
ZO-1
Merged
EGFP-Rac1
E-cadherin
Merged

Fig. 5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EGFP
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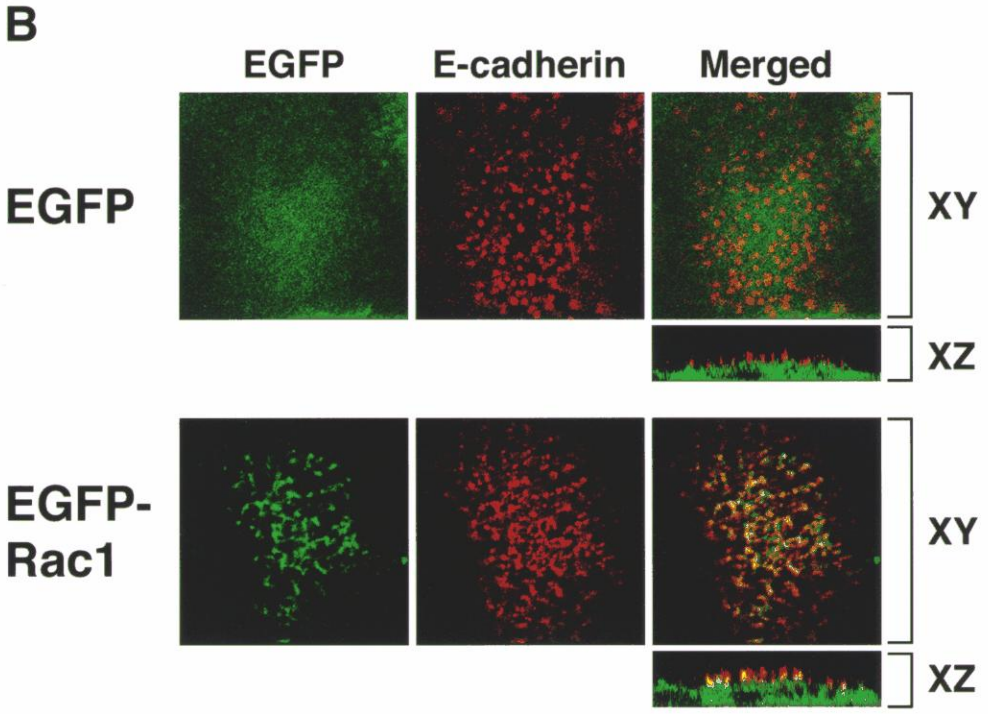
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E-cadherin
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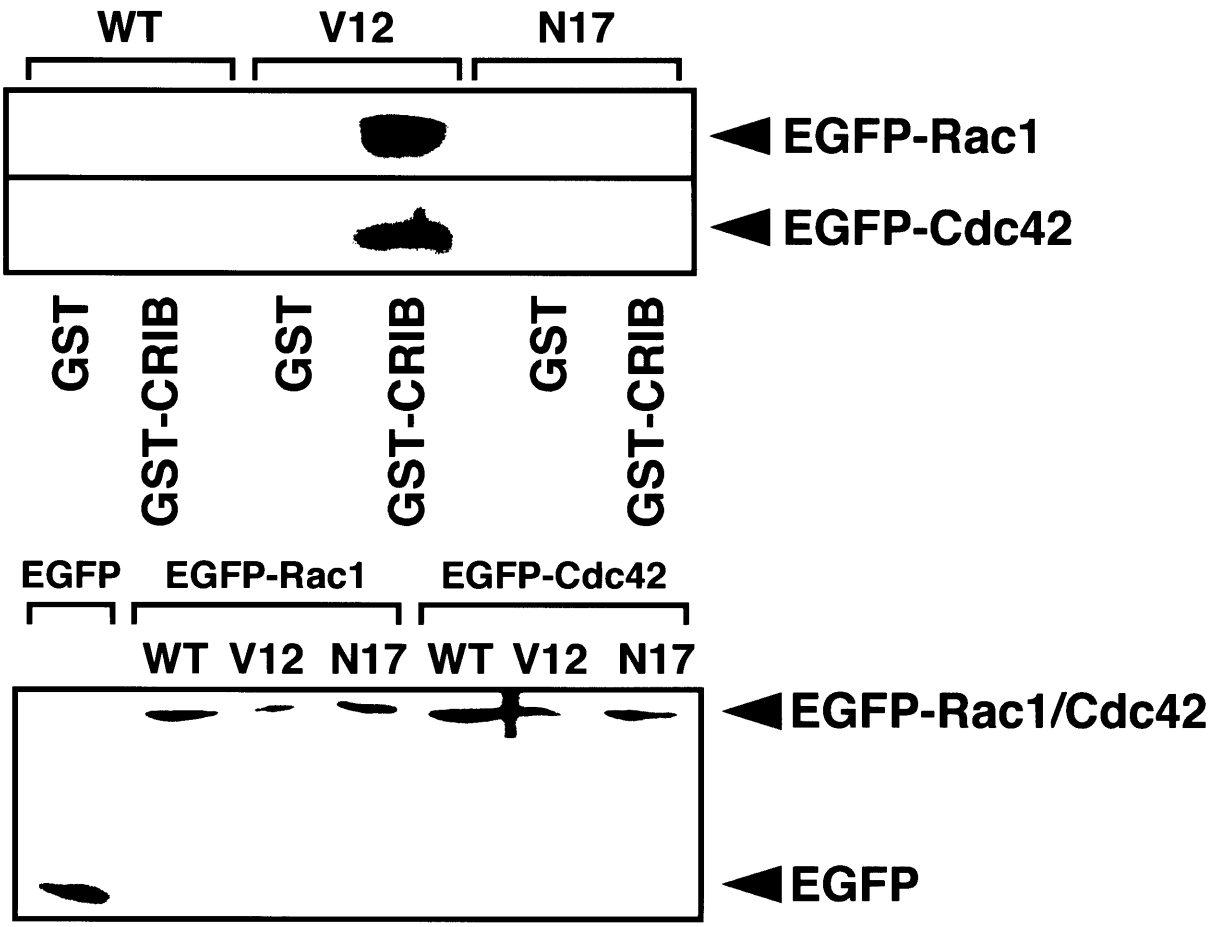
EGFP
XY
XZ
EGFP-
Rac1
XY
XZ

Fig. 6

A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V12
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GST
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EGFP-Cdc42
WT V12 N17 WT V12 N17
EGFP-Rac1/Cdc42
EGFP

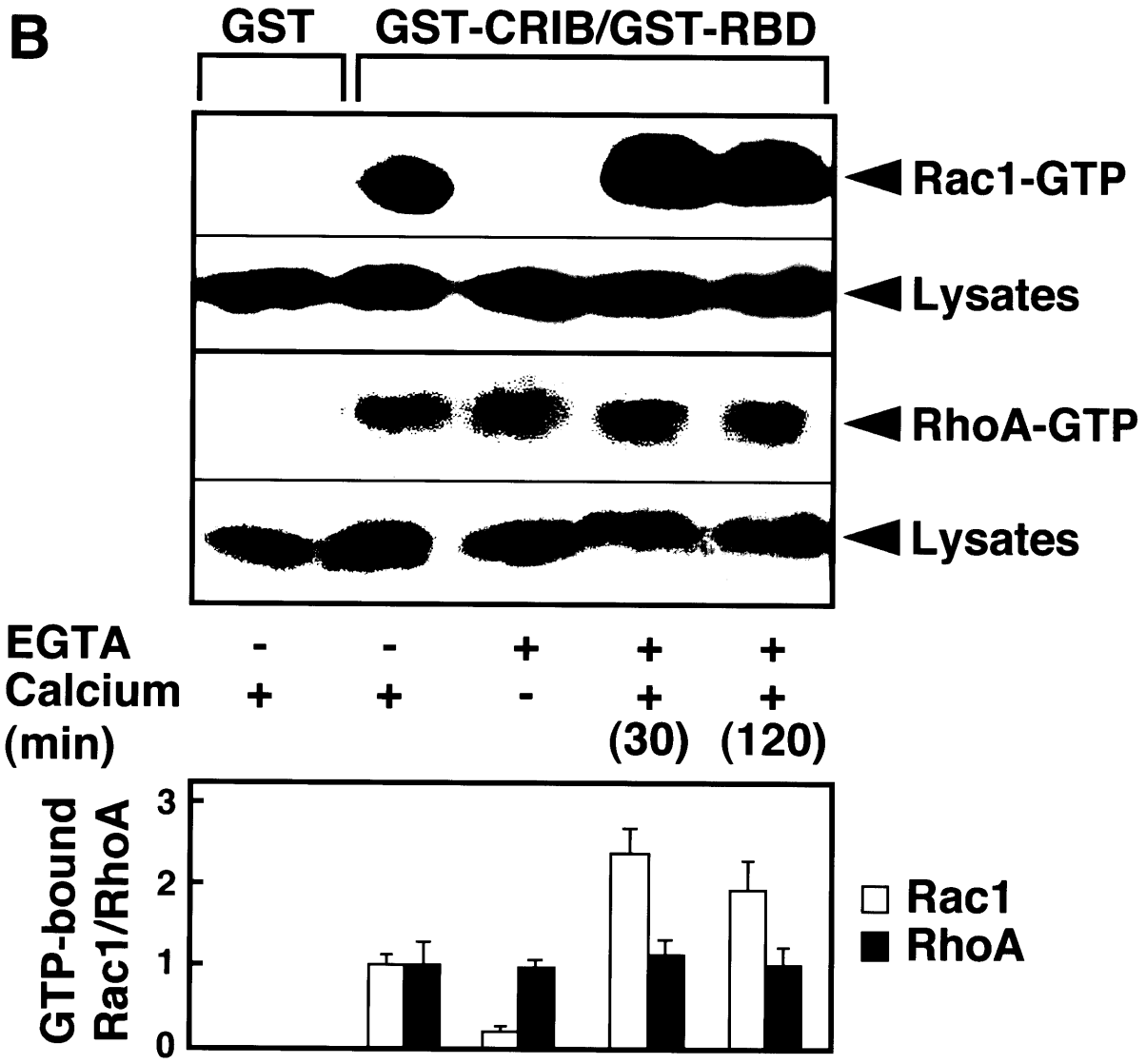
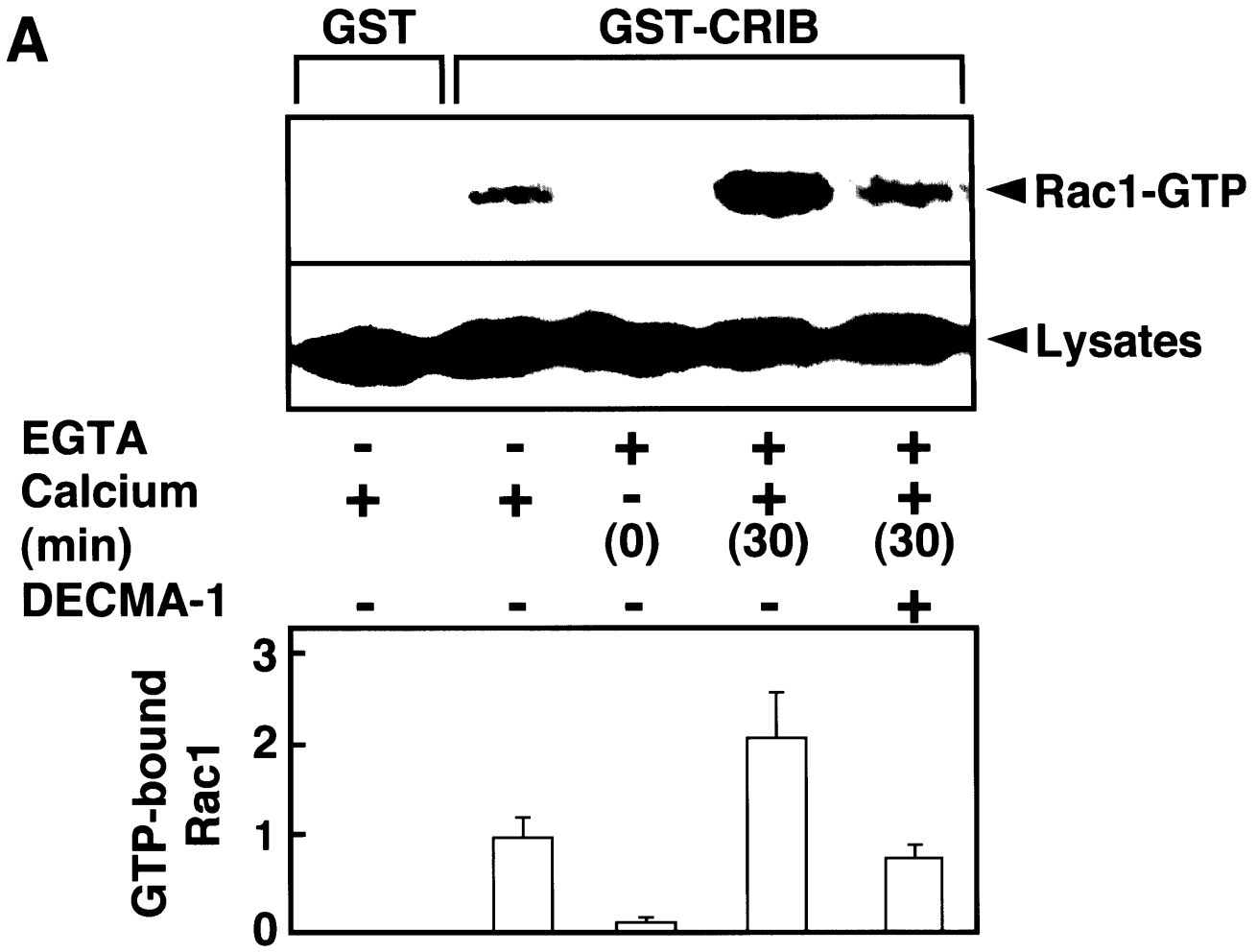

B
GST
GST-CRIB/GST-RBD
Rac1-GTP
Lysates
RhoA-GTP
Lysates
EGTA - - + + +
Calcium + + - + +
(min) (30) (120)
GTP-bound Rac1/RhoA
3
2
1
0
Rac1
RhoA

Fig. 7


A
GST
GST-CRIB
Rac1-GTP
Lysates
EGTA - - + + +
Calcium + + - + +
(min) (0) (30) (30)
DECMA-1 - - - - +
GTP-bound Rac1
3
2
1
0

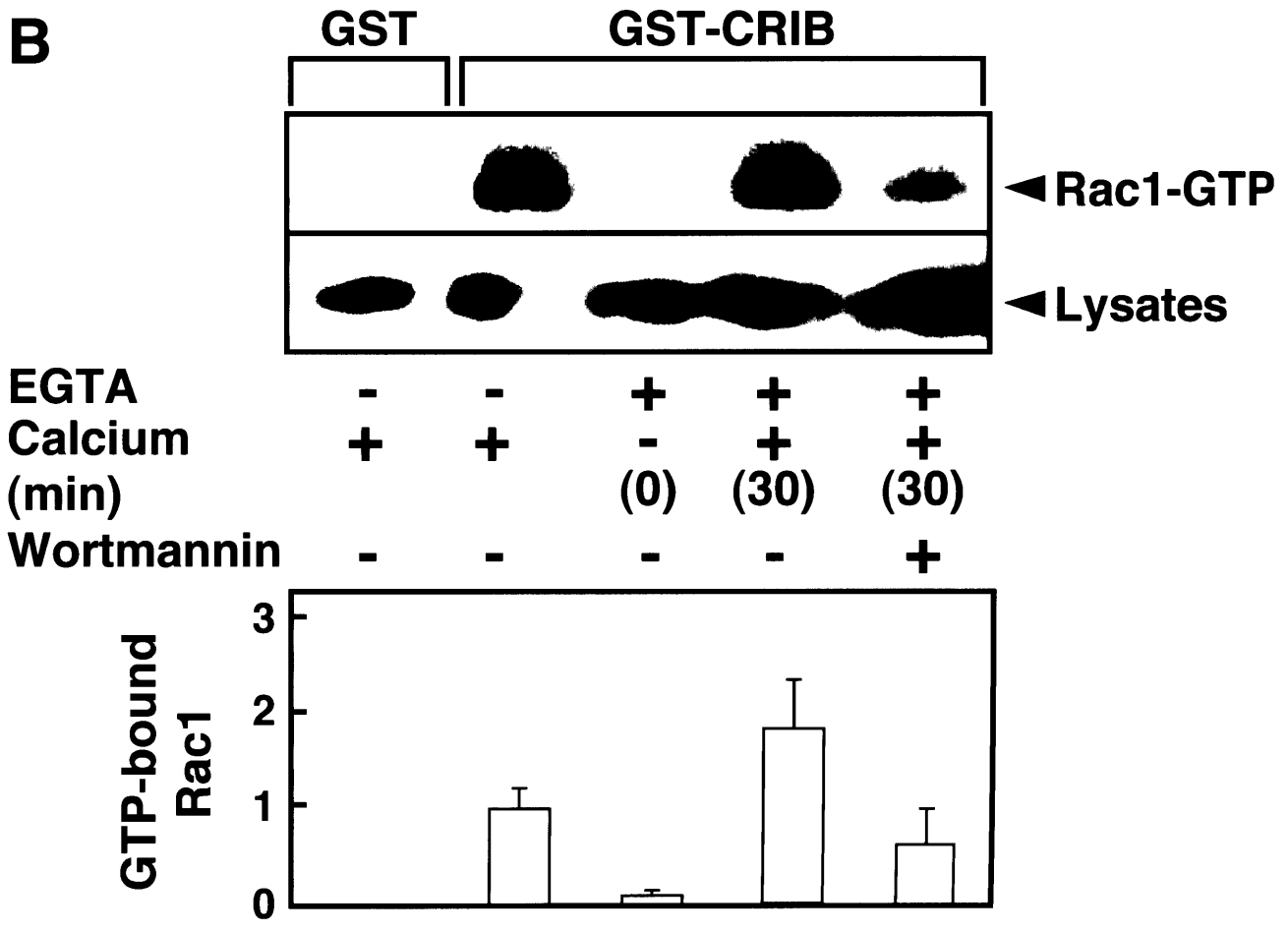
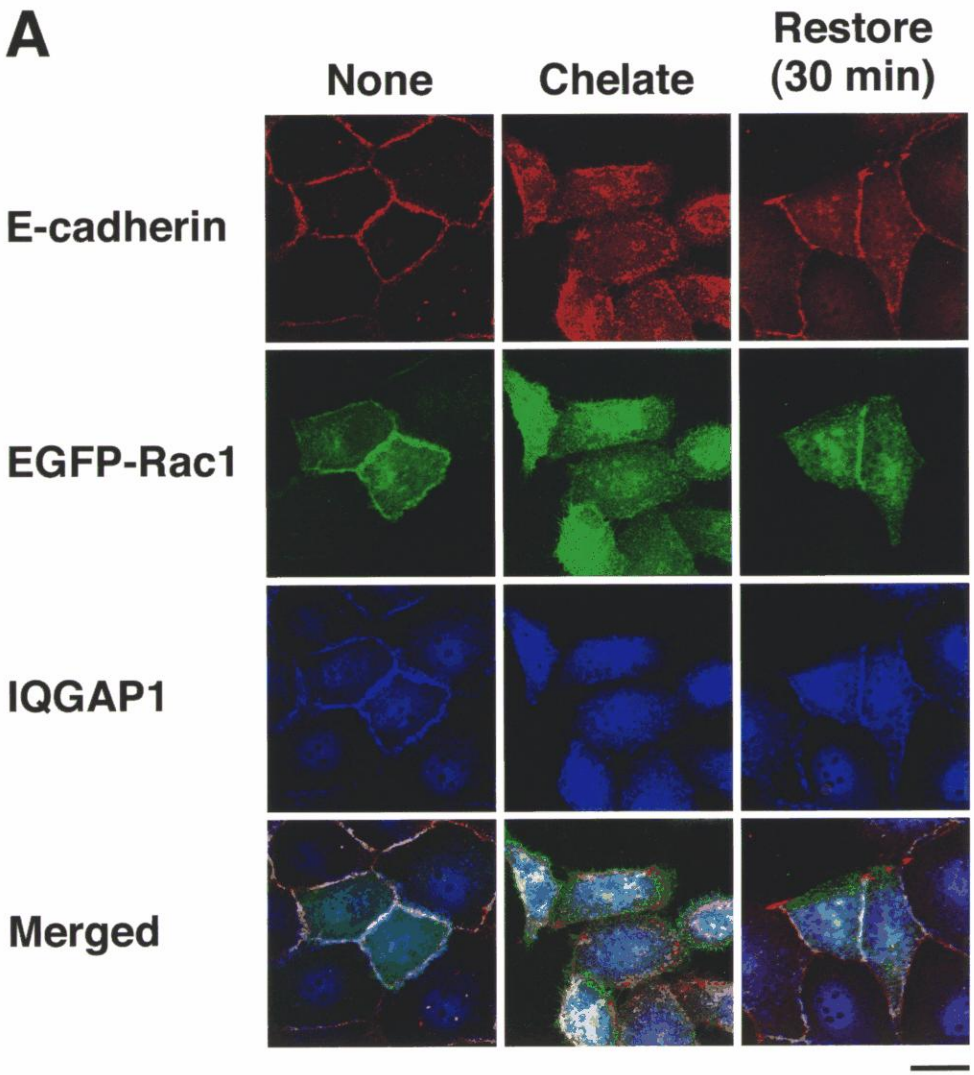

B
GST
GST-CRIB
Rac1-GTP
Lysates
EGTA - - + + +
Calcium + + - + +
(min) (0) (30) (30)
Wortmannin - - - - +
GTP-bound Rac1
3
2
1
0

Fig. 8


A
None
Chelate
Restore
(30 min)
E-cadherin
EGFP-Rac1
IQGAP1
Merged

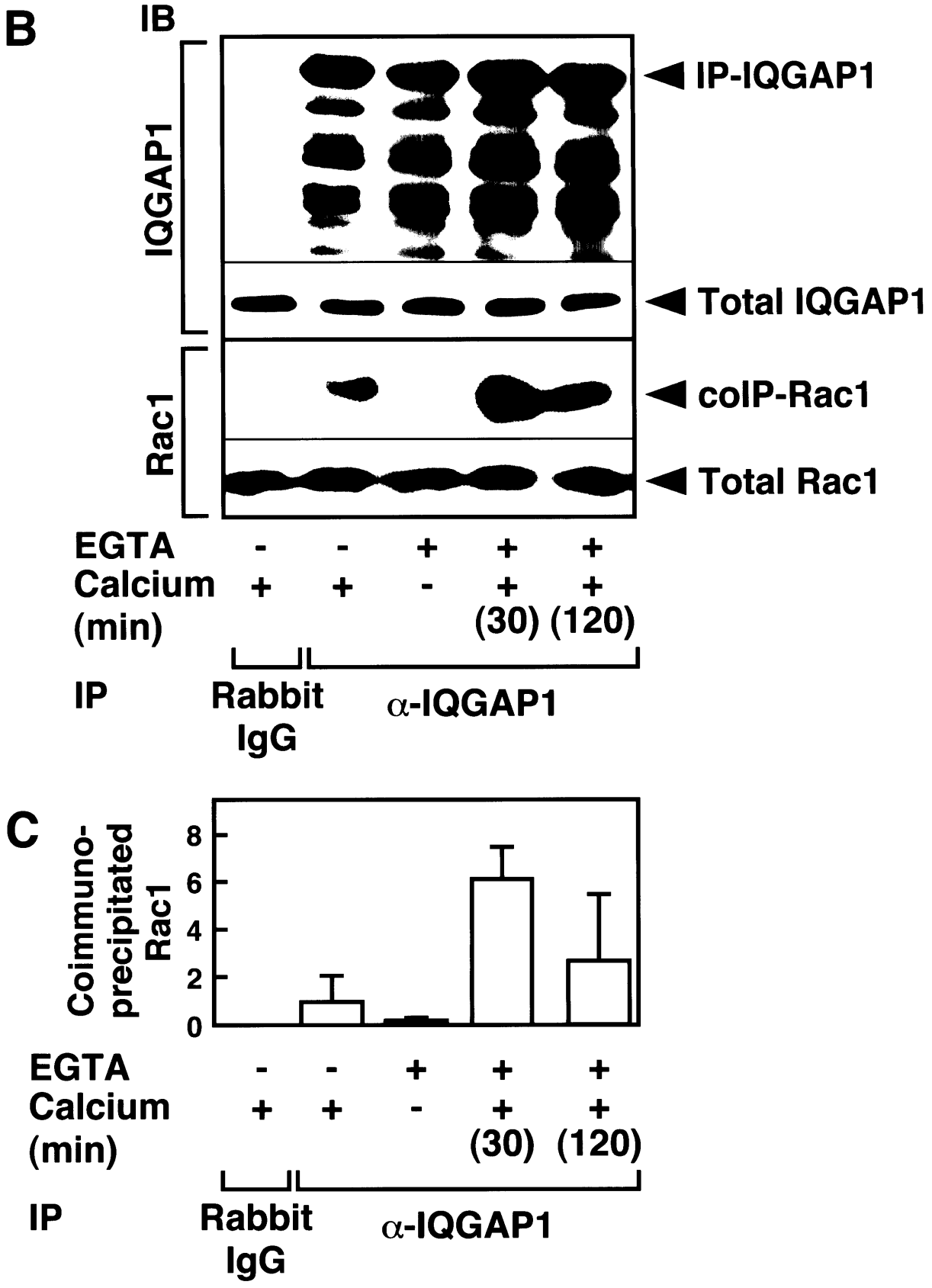
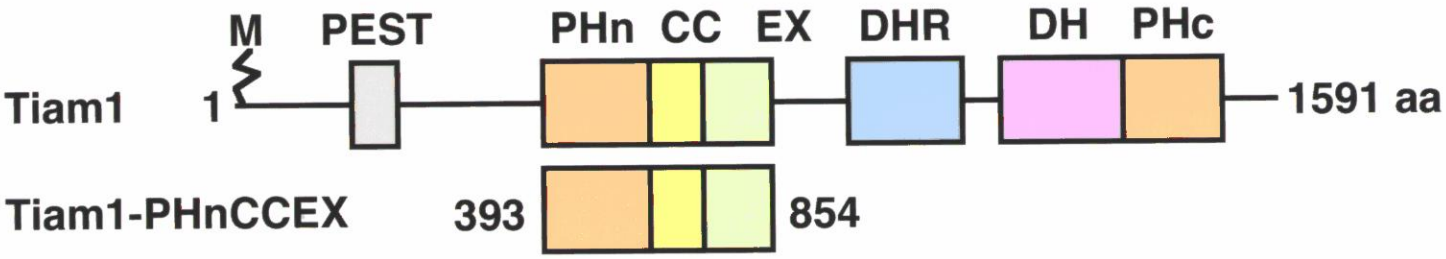
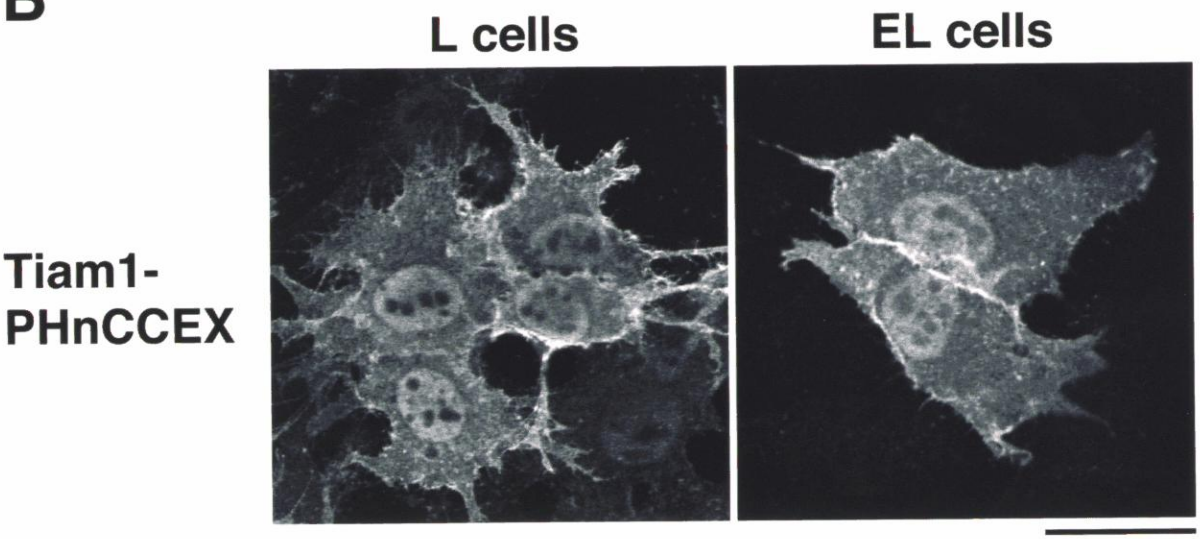

B
IB
IQGAP1
Rac1
IP-IQGAP1
Total IQGAP1
coIP-Rac1
Total Rac1
EGTA - - + + +
Calcium + + - + +
(min) (30) (120)
IP Rabbit IgG α-IQGAP1
C
Coimmuno-precipitated Rac1
0
2
4
6
8
EGTA - - + + +
Calcium + + - + +
(min) (30) (120)
IP Rabbit IgG α-IQGAP1

Fig. 9

A


M
PEST
PHn
CC
EX
DHR
DH
PHc
Tiam1
1
1591 aa
Tiam1-PHnCCEX
393
854

B


L cells
EL cells
Tiam1-
PHnCCEX

C

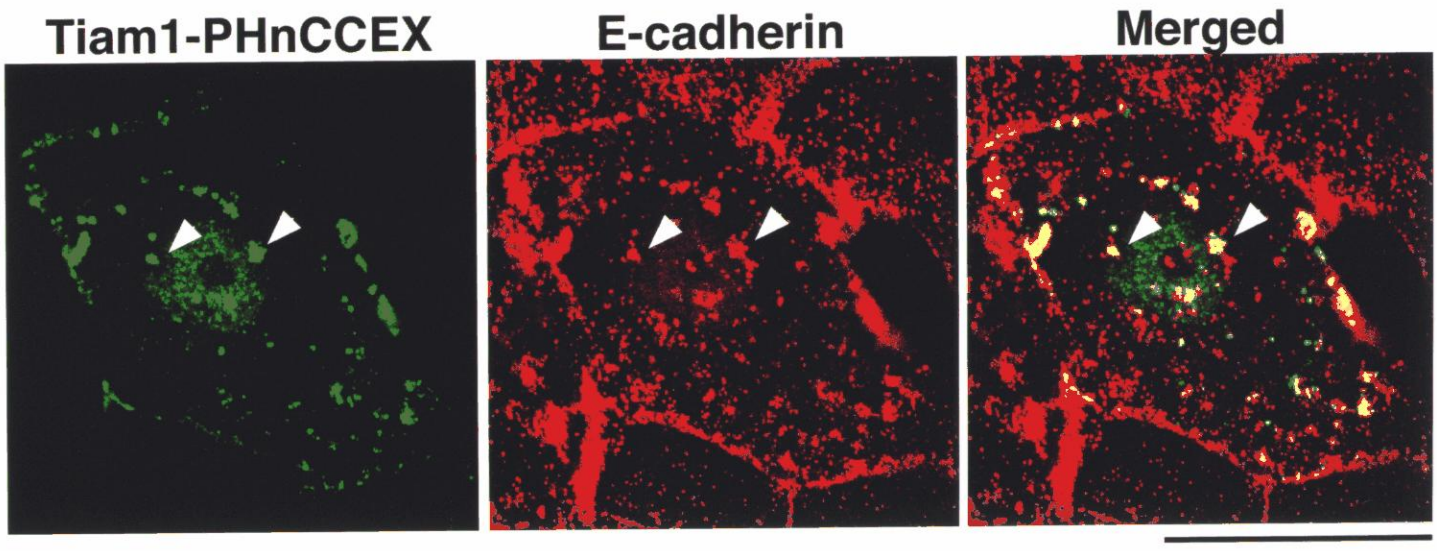
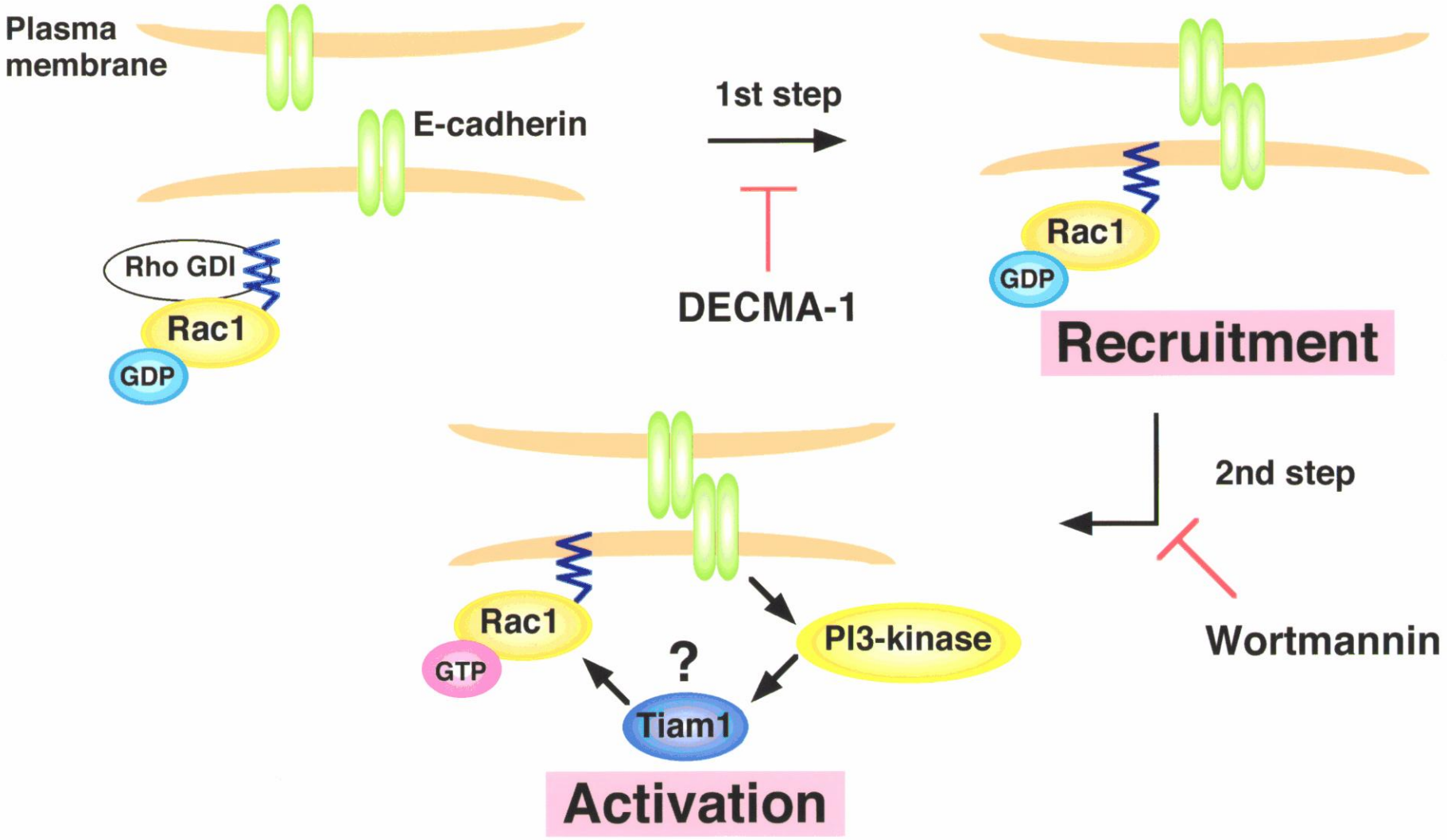

Tiam1-PHnCCEX
E-cadherin
Merged

Fig. 10


Plasma membrane
E-cadherin
Rho GDI
Rac1
GDP
1st step
DECMA-1
Rac1
GDP
Recruitment
2nd step
Wortmannin
Rac1
GTP
?
PI3-kinase
Tiam1
Activation