Endocytosis of E-cadherin regulated by Rac and Cdc42 small G proteins through IQGAP1 and actin filaments

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E-cadherin is a key cell–cell adhesion molecule at adherens junctions (AJs) and undergoes endocytosis when AJs are disrupted by the action of extracellular signals. To elucidate the mechanism of this endocytosis, we developed here a new cell-free assay system for this reaction using the AJ-enriched fraction from rat liver. We found here that non-trans-interacting, but not trans-interacting, E-cadherin underwent endocytosis in a clathrin-dependent manner. The endocytosis of trans-interacting E-cadherin was inhibited by Rac and Cdc42 small G proteins, which were activated by trans-interacting E-cadherin or trans-interacting nectins, which are known to induce the formation of AJs in cooperation with E-cadherin. This inhibition was mediated by reorganization of the actin cytoskeleton by Rac and Cdc42 through IQGAP1, an actin filament-binding protein and a downstream target of Rac and Cdc42. These results indicate the important role of the Rac/Cdc42-IQGAP1 system in the dynamic organization and maintenance of the E-cadherin–based AJs.

Introduction

Adherens junctions (AJs) are the principal mediators of cell–cell adhesion in epithelial cells and highly dynamic structures that turn over rapidly. This is exemplified during epithelial tissue morphogenesis and tumor cell invasion. E-cadherin is the major component of AJs responsible for homophilic cell–cell adhesion (Takeichi, 1988; Yap et al., 1997; Adams and Nelson, 1998; Gumbiner, 2000). E-cadherin first forms cis-dimers on the cell surface of the same cells, followed by formation of trans-dimers on the cell surface of two neighboring cells, causing cell–cell adhesion. As migrating epithelial cells do not always exhibit changes in the protein expression of E-cadherin (Miller and McClay, 1997), it is probable that the cellular processes that regulate the assembly/disassembly of AJs contribute to the acquisition of migratory potential. The endocytosis of E-cadherin represents one such cellular process that could regulate cellular migration (Kamei et al., 1999; Le et al., 1999; Fujita et al., 2002; Palacios et al., 2002). Various modes of the endocytosis for cellular control of cell surface events are known: EGF receptor and NGF receptor are internalized when their ligands bind, such as EGF for the EGF receptor and NGF for the NGF receptor (ligand-dependent endocytosis; Schmid, 1997; Brodsky et al., 2001). In contrast, the cell–matrix adhesion molecule integrin is constitutively internalized in the absence of binding partners, such as collagen for integrin (ligand-independent endocytosis; Lawson and Maxfield, 1995). In the case of E-cadherin, cell–cell contact-dependent regulation of endocytosis has potential implications for understanding the dynamics of the expression of E-cadherin at the cell surface. For example, cell–cell contact-dependent inhibition of endocytosis may contribute to the stabilization of E-cadherin at the cell surface that has been commonly documented to occur as cells grow to confluence (Le et al., 1999). Although the importance of the endocytosis of E-cadherin has been well documented, little is known about the molecular mechanisms of how cell–cell contact regulates the endocytosis of E-cadherin. It is attractive to speculate that the formation of trans-dimers (the trans interaction) of E-cadherin influences the cytoplasmic machinery responsible for the endocytosis of E-cadherin.

To elucidate this molecular mechanism, we have developed a new, general assay to measure the endocytosis of E-cadherin...
from the AJ fraction prepared from rat liver. It can be used to
directly characterize the function of components involved in
the endocytosis of E-cadherin. Here, we use a combination of
biochemical and cell biological approaches to show that the
endocytosis of E-cadherin is regulated by the activity state
of E-cadherin through the activation of the Rac/Cdc42-
IQGAP1 system induced by E-cadherin trans interaction.

Results
A cell-free assay system for the endocytosis
of E-cadherin
We first attempted to develop a cell-free assay system for the
endocytosis of E-cadherin based on in vitro clathrin-coated
vesicle (CCV) formation assay (Schmid and Smythe, 1991;
Miwako et al., 2003). For this purpose, we used the AJ-
enriched fraction from rat liver. The major components of the
Characteristics of the endocytosed vesicles of E-cadherin

Next, we examined if other cell–cell adhesion molecules (CAMs) and their associated peripheral membrane proteins at AJs were recovered in the HSP. We compared the relative amount of each protein between the HSP and MSP (15 µg of protein in each fraction) by immunoblotting. The distribution of E-cadherin in the MSP and the HSP was compared with those of nectin-2, claudin-1, and occludin. Nectin-2 is an Ig-like CAM at AJs (Shimizu and Takai, 2003; Takai and Nakanishi, 2003; Takai et al., 2003). Claudin-1 and occludin are CAMs at TJs (Tsukita et al., 2001). Unlike E-cadherin, all of these CAMs were confined to the MSP and undetectable in the HSP even after the 30-min incubation in the presence of the reaction buffer (Figs. 1 B and 2 A). In addition, the distribution of proteins directly or indirectly associating with E-cadherin was examined in the MSP and the HSP. α- and β-Catenins were recovered in the HSP like E-cadherin. In contrast, p120catenin and IQGAP1 were confined to the MSP and undetectable in the HSP. β-Catenin and p120catenin are E-cadherin–binding proteins (Tsukita et al., 1992; Anastasiadis and Reynolds, 2000), whereas α-catenin is a β-catenin– and actin filament (F-actin)–binding protein (Tsukita et al., 1992). IQGAP1 is an F-actin cross-linking protein known to be a downstream target of Rac and Cdc42 small G proteins (Kuroda et al., 1996; Bashour et al., 1997; Fukata et al., 1997). Moreover, the relative amount of E-cadherin was higher in the HSP than that of the MSP or the original AJs. These results indicate that the recovery of E-cadherin in the HSP is not just due to nonspecific fragmentation of the AJ plasma membranes and suggest that E-cadherin and α- and β-catenins are selectively sorted into the endocytosed vesicles.

We confirmed by immunoelectron microscopy whether E-cadherin is indeed on the endocytosed vesicles budded from the AJ plasma membranes and recovered in the HSP. First, the HSP was observed by electron microscopy. We found that numerous vesicles showed heterogeneous sizes (50–400 nm in diameter) in this fraction, although most were 50–100 nm, as expected for endocytosed vesicles (Fig. 2 Ba). On the assumption that E-cadherin recovered in the HSP formed a complex with β-catenin, E-cadherin was immunoprecipitated from the MSS by the anti–β-catenin mAb-coated magnetic beads. The beads were fixed and examined by immunoelectron microscopy using the anti–E-cadherin mAb and the gold-conjugated secondary antibody. The beads were coated with vesicles on which the immunogolds for E-cadherin were accumulated (Fig. 2 Bb). All the proteins recovered in the HSP, including E-cadherin and α- and β-catenins, were also recovered in the immunopurified vesicles (Fig. 2 Bc). These results indicate that E-cadherin is indeed endocytosed from the AJ plasma membranes and recovered on the endocytosed vesicles.

We examined whether or not E-cadherin undergoes endocytosis in a clathrin-dependent manner. To disturb the clathrin-dependent endocytosis of E-cadherin from the AJ plasma membranes, we used the ENTH domain of epsin. Epsin is a regulatory component of the CCV formation (Brodsky et al., 2001), and its ENTH domain has been shown to be a potent inhibitor of the clathrin-dependent endocytosis (Nakashima et al., 1999). Recombinant GST-ENTH domain of epsin indeed reduced the amount of E-cadherin in the HSP in a dose-dependent manner (Fig. 3 A). Consistently, clathrin-HC, epsin, and β-adaptin were re-
covered in the HSP and the immunopurified vesicles (Fig. 2, A and Bc). β-Adaptin is a component of the CCV (Brody et al., 2001). The vesicles immunopurified by the anti-β-catenin mAb-coated magnetic beads showed diameters of ~80 nm (Fig. 2 Bb), which is consistent with those of previously reported CCVs (Schmid and Smythe, 1991; Miwako et al., 2003). To further validate the results obtained in our assay system, we examined whether or not the ENTH domain of epsin affects the endocytosis of E-cadherin in MDCK cells. We confirmed that this epsin mutant inhibited both the HGF- and low Ca\(^{2+}\)-induced endocytosis of E-cadherin in MDCK cells (Fig. 3 B, a and b). The inconsistency between our results and the former results using MCF7 cells (Paterson et al., 2003) may be due to the variation of mechanisms of endocytosis in different cell types.

Next, we examined if the endocytosis of E-cadherin is caveolin-dependent in our assay system. Caveolin-dependent endocytosis has been shown to be selectively blocked by depletion of cholesterol from the plasma membranes (Ivanov et al., 2003). To deplete cholesterol, we used two reagents, cholesterol oxidase and cyclic heptasaccharide MβCD. We did not find any significant effect of these reagents on the endocytosis of E-cadherin (Fig. 3 C). Our results are consistent with the recent result that the low Ca\(^{2+}\)-induced endocytosis of E-cadherin is caveolin independent in MDCK cells (Ivanov et al., 2003). Moreover, we examined the distribution of caveolin-1 in our assay system. Caveolin-1 was confined to the MSP, but not to the HSP (Fig. 2, A and Bc). Thus, the endocytosis of E-cadherin is likely to be caveolin independent in our assay system. Together, it is likely that E-cadherin undergoes endocytosis via CCVs in this newly developed cell-free assay system.

**Regulation of the endocytosis of E-cadherin through Rac and Cdc42 activated by trans-interacting E-cadherin**

By use of the cell-free assay system, we first attempted to determine which form of E-cadherin, trans-interacting or non-trans-interacting, is endocytosed. For this purpose, we first examined whether or not the rest of E-cadherin is trans interacting after the budding reaction. To examine if the rest of the E-cadherin is trans-interacting after the first cycle of the budding reaction, we added the E-cadherin–blocking antibody in the second cycle of the budding reaction. We found that the E-cadherin–blocking antibody enhanced the budding of E-cadherin in the second cycle reaction (Fig. 4 A). To confirm more directly that non-trans-interacting E-cadherin is preferentially endocytosed in our assay system, we examined if Cef, which is the extracellular fragment of E-cadherin fused to the Fc portion of human IgG, affects the endocytosis of E-cadherin in the presence of 2 mM or 2 μM Ca\(^{2+}\). Cef has been reported to trans interact with cellular E-cadherin in the presence of 2 mM Ca\(^{2+}\), but not in the presence of 2 μM Ca\(^{2+}\) (Kovacs et al., 2002a). To neglect a potential effect of Ca\(^{2+}\), we first determined the Ca\(^{2+}\) effect on the endocytosis of E-cadherin in our assay system because low Ca\(^{2+}\) enhances the dissociation of the trans interaction of E-cadherin. After the preincubation at various concentrations of Ca\(^{2+}\), the membranes were collected and assayed for
The endocytosis of E-cadherin. Preincubation at 2 μM Ca^{2+} for 10 min did not affect the endocytosis of E-cadherin significantly in our assay system (Fig. 4 Ba). The inability of low Ca^{2+} to induce the endocytosis of E-cadherin under these conditions was just due to the short time incubation. We preincubated the AJ plasma membranes with Cef at 2 mM or 2 μM Ca^{2+} for 10 min. After the preincubation, the membranes were collected to remove excess Cef and assayed for the endocytosis of E-cadherin. Cef inhibited the endocytosis of E-cadherin in a dose-dependent manner at 2 mM Ca^{2+}, but not at 2 μM Ca^{2+} (Fig. 4 B b and c). These results indicate that only non-trans-interacting E-cadherin, but not trans-interacting one, undergoes endocytosis in our assay system.

Then, we studied how the endocytosis of trans-interacting E-cadherin is inhibited. It has been shown that trans-interacting E-cadherin induces activation of Rac, but not Cdc42 or Rho small G protein (Van Aelst and Symons, 2002; Shimizu and Takai, 2003; Yap and Kovacs, 2003). Moreover, several studies have revealed that Rac might work positively or negatively in the endocytosis of E-cadherin, depending on both junctional maturity and cellular context (Takaishi et al., 1997; Akhtar and Hotchin, 2001; Palacios et al., 2002). However, the biological significance of Rac activated by trans-interacting E-cadherin is not thoroughly understood. We first examined, by use of Cef, if the trans interaction of E-cadherin induces activation of Rac, Cdc42, and Rho in our assay system. Cef activated Rac and Cdc42, but not Rho, time dependently (Fig. 5 A). Therefore, we examined if Rac and Cdc42 regulate the endocytosis of E-cadherin. For this purpose, we made advantage of Rho GDI, which forms a complex preferentially with the GDP-bound form to the GTP-bound form of the Rho family members, including Rho, Rac, and Cdc42, and extracts them from the membranes (Sasaki and Takai, 1998). Reombinant GST-tagged Rho GDI was added to the AJ plasma membranes in the presence of GDP and a high Mg^{2+}, followed by incubation at 30°C for 20 min. High Mg^{2+} enhances the conversion of the GTP-bound form of the Rho family members to the GDP-bound form. The AJ plasma membranes were chilled on ice and recovered by centrifugation. Rac and Cdc42 were progressively extracted from the AJ plasma membranes by increasing amounts of GST-Rho GDI (Fig. 5 B and not depicted). However, the amount of Cdc42 in the AJ plasma membranes was 10 times lower than that of Rac by quantitative immunoblotting (unpublished data). Thus, Rac is likely to be the major substrate for Rho GDI in our assay system. After the 20 min preincubation of the AJ plasma membranes in the presence of Rho GDI, the amount of E-cadherin recovered in the HSP was measured. Increasing concentrations of Rho GDI increased the amount of E-cadherin in the HSP in proportion to the decreased amount of Rac in the AJ plasma membranes (Fig. 5 B). This Rho GDI-enhanced endocytosis of E-cadherin was inhibited by pretreatment of the AJ plasma membranes with Cef, and conversely the inhibitory effect of Cef was rescued by pretreatment of the AJ plasma membranes with Rho GDI (Fig. 5 C, a and b). These results indicate that the substrate small G proteins for Rho GDI inhibit the endocytosis of E-cadherin. We examined which substrate small G proteins show this effect. The GTPγS-bound form of recombinant Rac, Cdc42, or Rho was added to the AJ plasma membranes pretreated with Rho GDI, and the extent of the endocytosis of E-cadherin was measured. The GTPγS-bound form of Rac and Cdc42, but not that of Rho, inhibited the Rho GDI–enhanced endocytosis of E-cadherin (Fig. 5 Da). We confirmed that added GTPγS-bound form of Rac indeed bound to the AJ plasma membranes pretreated with Rho GDI (Fig. 5 Db). These results indicate that trans-interact-
ing E-cadherin activates Rac and Cdc42, which then inhibits the endocytosis of E-cadherin in our assay system.

Involvement of IQGAP1 in the inhibitory effect of Rac and Cdc42 on the endocytosis of E-cadherin through the actin cytoskeleton

We attempted to search the Rac and Cdc42 target protein(s), which inhibits the endocytosis of E-cadherin. We fractionated the rat brain cytosol by successive column chromatographies. One peak fraction showed an inhibitory effect on the Rho GDI-enhanced endocytosis of E-cadherin. IQGAP1, a downstream target of Rac and Cdc42 (Kuroda et al., 1996; Bashour et al., 1997; Fukata et al., 1997), was enriched in this fraction as estimated by immunoblotting (unpublished data). To confirm this result, we examined the ability of recombinant His-IQGAP1 on the endocytosis of E-cadherin. Recombinant His-IQGAP1 potently inhibited the endocytosis of E-cadherin in a dose-dependent manner (Fig. 6, Aa and B). This inhibitory effect of IQGAP1 was reduced when the AJ plasma membranes pretreated with Rho GDI were used (Fig. 6, Ab and B). In contrast, the inhibitory effect of IQGAP1 was enhanced by the addition of the GTPγS-bound form of Rac and Cdc42 to the AJ plasma membranes (Fig. 6, Ac, Ad, and B). These results indicate that the inhibitory effect of Rac and Cdc42 on the endocytosis of E-cadherin is mediated through at least IQGAP1.

IQGAP1 has been shown to cross-link F-actin into actin bundles (Bashour et al., 1997; Fukata et al., 1997). We generated ΔCHD-IQGAP1 as an IQGAP1 mutant, which had a defect in F-actin binding, but not in Rac binding (Fukata et al., 1997). We found that ΔCHD-IQGAP1 did not show any inhibitory effect on the endocytosis of E-cadherin in the presence or absence of the GTPγS-bound form of Rac (Fig. 6, e and f). Moreover, we confirmed that there is no difference between the Rac GTP-stabilizing activity of IQGAP1 and that of ΔCHD-IQGAP1 (Fukata et al., 1997; unpublished data). These results suggest that IQGAP1 is a target protein for Rac and Cdc42 and plays a role in the endocytosis of E-cadherin presumably through its F-actin cross-linking activity.

We next examined, by the use of an F-actin-disrupting reagent, latrunculin A (Lat-A), if the inhibitory effect of IQGAP1 on the endocytosis of E-cadherin is mediated through the actin cytoskeleton. Lat-A markedly enhanced the endocytosis of E-cadherin from the AJ plasma membranes (Fig. 7 Aa). In contrast, nocodazole, a microtubule-disrupting reagent, did not show any effect on the endocytosis of E-cadherin. The inhibitory effect of Rac and IQGAP1 on the endocytosis of E-cadherin was rescued by the Lat-A treatment (Fig. 7 A, b and c). Moreover, we measured F-actin levels. We found that Rac/IQGAP1 increased the amount of F-actin. In contrast, Rac/ΔCHD-IQGAP1 did not show any significant change in the F-actin level compared with that of the control (Fig. 7 B). These results indicate that IQGAP1 inhibits the endocytosis of E-cadherin through its Rac/Cdc42-dependent F-actin cross-linking activity.

To neglect involvement of other actin regulator proteins, we examined the effect of the Arp2/3 complex on the endocytosis of E-cadherin in our assay system. The Arp2/3 complex has recently been reported to be recruited to the E-cadherin–based adhesion sites (Kovacs et al., 2002b). Moreover, the Arp2/3 complex–mediated actin polymerization has been reported to drive the vesicles away from the plasma membrane (Kaksonen et al., 2003). In our assay system, the Arp2/3 complex did not show any significant effect on the
endocytosis of E-cadherin, even in the presence of the activators, such as the Cdc42-GTPyS-N-WASP complex and WAVE2 (Fig. 7 Ca and not depicted). Moreover, depletion of the Arp2/3 complex from the rat brain cytosol did not show any significant effect on the endocytosis of E-cadherin (Fig. 7 Cb). Thus, the Arp2/3 complex is unlikely to be involved in the endocytosis of E-cadherin in our assay system.

Inhibition of the endocytosis of E-cadherin by trans-interacting E-cadherin or trans-interacting nectin-1 in intact cells

We finally examined if the endocytosis of trans-interacting E-cadherin is indeed inhibited in intact cells. For this purpose, we first used EL cells, L fibroblasts stably expressing E-cadherin, because E-cadherin is constitutively endocytosed and recycled when cells do not contact other cells and E-cadherin does not trans interact (Paterson et al., 2003). We assayed at 18°C to stop this recycling and to measure only the endocytosis of E-cadherin in EL cells. For mimicking the trans interaction of E-cadherin, we incubated EL cells with Cef. We quantified the effect of Cef on the endocytosis of E-cadherin by chasing surface-biotinylated E-cadherin (Le et al., 1999). Cef inhibited the constitutive endocytosis of E-cadherin (Fig. 8 Aa). Immunofluorescence analysis revealed that E-cadherin still remained on the cell surface in the presence of Cef and that IQGAP1 accumulated at the cell periphery where E-cadherin localized in the presence of Cef (Fig. 8 Ab). These results indicate that trans-interacting E-cadherin does not undergo endocytosis in intact cells. It has been shown that trans-interacting E-cadherin induces activation of Rac (Van Aelst and Symons, 2002; Shimizu and Takai, 2003; Yap and Kovacs, 2003). We examined if Rac or Cdc42, which is activated by trans-interacting E-cadherin, is required to induce IQGAP1 translocation to the plasma membrane in EL cells. We transfected cells with N17Rac1, a dominant-negative mutant of Rac, or N-WASP CRIB-CAAX, an inhibitor of activated Cdc42. Both N17Rac1 and N-WASP CRIB-CAAX inhibited the recruitment of IQGAP1 to the plasma membrane in EL cells (Fig. 8 Ab).
Figure 6. Involvement of IQGAP1 in the inhibitory effect of Rac and Cdc42 on the endocytosis of E-cadherin.
(A) Inhibition of the endocytosis of E-cadherin by IQGAP1. (Aa) The AJ plasma membranes were incubated with the indicated concentrations of recombinant His-IQGAP1 and assayed for the endocytosis of E-cadherin. (Ab) The AJ plasma membranes were first incubated with GST-Rho GDI and collected. The collected membranes were incubated with recombinant His-IQGAP1 at the indicated concentrations and assayed for the endocytosis of E-cadherin. The numbers 1 and 2 indicate the sequence of the incubation. (Ac) The AJ plasma membranes were incubated with the GTPyS-bound form of recombinant rac and recombinant His-IQGAP1 at the indicated concentrations and assayed for the endocytosis of E-cadherin. (Ad) The AJ plasma membranes were incubated with the GTPyS-bound form of recombinant Cdc42 and recombinant His-IQGAP1 at the indicated concentrations and assayed for the endocytosis of E-cadherin. (Ae) The AJ plasma membranes were incubated with the indicated concentrations of recombinant His-ΔCHD-IQGAP1 and assayed for the endocytosis of E-cadherin. (Af) The AJ plasma membranes were incubated with the GTPyS-bound form of recombinant Rac and recombinant His-ΔCHD-IQGAP1 at the indicated concentrations and assayed for the endocytosis of E-cadherin. (A, a–f) The quantification of immunoblot is shown as the mean ± SD of duplicate assays in the bottom panel. (B) Endocytosis efficiency (%) is expressed relative to the controls in each experiment in A (a–d). The results shown in all panels are representative of three independent experiments.

Together, it is likely that both Rac and Cdc42 activated by trans-interacting E-cadherin inhibit its endocytosis through IQGAP1 also in intact cells.

We have previously shown that nectins are involved in the formation of AJ in cooperation with E-cadherin (Takai et al., 2003). In the process of the formation of AJ, nectin-based cell–cell adhesion is first formed, followed by recruitment of E-cadherin there, finally forming AJ; and the nectin-based cell–cell adhesion induces activation of Cdc42 and Rac, which facilitates the formation of AJ. Nectins comprise a family consisting of four members: nectin-1, -2, -3, and -4. Like E-cadherin, each nectin forms cis-dimers, followed by formation of trans-dimers, causing cell–cell adhesion. Furthermore, nectin-3 forms hetero-trans-dimers with nectin-1 or -2, and of the many combinations of the trans-dimers, the hetero-trans-dimers between nectin-1 and -3 show the strongest cell–cell adhesion activity. Therefore, we examined whether or not the nectin-induced activation of Cdc42 and Rac is also involved in the inhibition of the endocytosis of E-cadherin. For this purpose, we used EL cells stably expressing nectin-1 (nectin-1-EL cells) and the extra-cellular fragment of nectin-3 fused to the Fc portion of human IgG (Nef-3; Honda et al., 2003). Nef-3, like Cef, inhibited the constitutive endocytosis of E-cadherin in nectin-1-EL cells (Fig. 8 Bb). We examined whether Rac or Cdc42, which is activated by trans-interacting nectin-1, is required to induce IQGAP1 translocation to the plasma membrane in nectin-1-EL cells. We transfected cells with N17Rac1 or N-WASP CRIB-CAAX. Both N17Rac1 and N-WASP CRIB-CAAX inhibited the recruitment of IQGAP1 to the plasma membrane in nectin-1-EL cells (Fig. 8 Bb). Therefore, it is likely that both Rac and Cdc42 activated by trans-interacting nectin-1 inhibits the endocytosis of E-cadherin.

Discussion
In this study, we have described a new biochemical assay that efficiently reconstitutes the endocytosis of E-cadherin using the AJ plasma membranes. We have found here that non-trans-interacting E-cadherin is constitutively endocytosed like integrin (ligand-independent endocytosis), that the formation of endocytosed vesicles of E-cadherin is clathrin dependent, and that E-cadherin, but not other CAMs at AJ and TJ including nectins, claudins, and occludin, is selectively sorted into the endocytosed vesicles. Recent cell-level studies using chemical inhibitors have shown that E-cadherin might be internalized by clathrin- or caveolin-dependent endocytosis (Le et al., 1999; Akhtar and Hotchin, 2001; Palacios et al., 2002; Thomsen et al., 2002; Paterson et al., 2003; Ivanov et al., 2003). However, the results ob-
Inhibiting the endocytosis of E-cadherin results in the stabilization of E-cadherin on the cell surface. When migrating MDCK cells make nascent cell-cell contacts, E-cadherin is recruited specifically to the cell-cell contact sites (Adams et al., 1998). Furthermore, the inhibition of the endocytosis of E-cadherin by the Rac/Cdc42-IQGAP1 system may contribute to the site-specific accumulation of E-cadherin. p120^CTN has recently been shown to be involved in the stabilization of cadherins on the cell surface, suggesting that p120^CTN interacts with an unidentified endocytic machinery to inhibit the endocytosis of cadherins (Davis et al., 2003; Xiao et al., 2003). The p120^CTN-binding site of E-cadherin is shown to be involved in the activation of Rac (Goodwin et al., 2003). Together, p120^CTN and/or other unknown proteins may be upstream regulators of the Rac/Cdc42-IQGAP1 system that inhibits the endocytosis of E-cadherin.

We have previously proposed that nectins, but not E-cadherin, play an important role in recruiting IQGAP1 to very primordial nectin-based cell-cell adhesion sites through the actin cytoskeleton (Katata et al., 2003), in contrast to previous observation that E-cadherin is required for the accumulation of IQGAP1 at AJs solely by binding to β-catenin (Fukata and Kaibuchi, 2001). Nectins are associated with the actin cytoskeleton through afadin, an F-actin- and nectin-binding protein, as E-cadherin is associated with the actin cytoskeleton through α- and β-catenins (Shimizu et al., 2003). We have shown here that Rac and Cdc42 activated by trans-interacting nectin-1 also has a potency to inhibit the endocytosis of E-cadherin. Therefore, together,

![Diagram of endocytosis of E-cadherin](image_url)

Figure 7. Involvement of the actin cytoskeleton in the inhibitory effect of IQGAP1 on the endocytosis of E-cadherin. (A) Prevention of the inhibitory effect of IQGAP1 on the endocytosis of E-cadherin by Lat-A. (Aa) The AI plasma membranes were incubated with Lat-A or nocodazole and assayed for the endocytosis of E-cadherin. (Ab) The AI plasma membranes were first incubated with Lat-A and collected. The collected membranes were incubated with the GTPγS-bound form of recombinant Rac and assayed for the endocytosis of E-cadherin. The numbers 1 and 2 indicate the sequence of the incubation. (Ac) The AI plasma membranes were first incubated with Lat-A and collected. The collected membranes were incubated with recombinant His-IQGAP1 and assayed for the endocytosis of E-cadherin. (A, a–c) The quantification of immunoblot is shown as the mean ± SD of duplicate assays in the bottom panel. The numbers 1 and 2 indicate the sequence of the incubation. (B) F-Actin levels in the AJ plasma membranes. Increase in total F-actin in the AJ plasma membranes treated with the indicated factors was expressed as fold increase to the amount of F-actin in the non-treated control AJ plasma membranes. The mean ± SD of duplicate assays is shown. (C) Effect of the Arp2/3 complex on the endocytosis of E-cadherin. (Ca) The AI plasma membranes were incubated with the indicated concentrations of the Arp2/3 complex and assayed for the endocytosis of E-cadherin. (Cb) The AI plasma membranes were assayed for the endocytosis of E-cadherin in the presence of either control cytosol or Arp2/3-depleted cytosol. The results shown in all panels are representative of at least three independent experiments.
IQGAP1 would be first recruited through the actin cytoskeleton to the cell–cell adhesion sites where Rac and Cdc42 are activated by trans-interacting nectins. IQGAP1 bound to Rac and Cdc42 then induces reorganization of the actin cytoskeleton. In contrast, E-cadherin is recruited to the nectin-based cell–cell adhesion sites and induces the activation of Rac and Cdc42, which then facilitates the recruitment of IQGAP1 and the subsequent reorganization of the actin cytoskeleton. These sequential reorganizations of the actin cytoskeleton are likely to inhibit the endocytosis of E-cadherin on one hand and strengthen the cell–cell adhesion activity of E-cadherin on the other hand.

It has been shown in cultured MDCK cells that HGF induces cell–cell disruption and cell scattering, which is accompanied with endocytosis of E-cadherin. However, it remains unknown that the endocytosis of E-cadherin alone is sufficient for the disruption of the cell–cell adhesion because previous studies have shown that a small pool of E-cadherin...
(20% of total surface E-cadherin) is endocytosed during the HGF actions in MDCK cells (Kamei et al., 1999; Fujita et al., 2002). The endocytosis of a specific portion of E-cadherin may be necessary and sufficient for the HGF action. Another possibility is that an unidentified factor(s), which is essential for the maintenance of the E-cadherin–based cell–cell adhesion, is coendocytosed with E-cadherin. The exact roles and the mechanisms of the endocytosis of E-cadherin in cell migration are important issues to be addressed in the future for our understanding of the signal switching from disruption of cell–cell adhesion to cell scattering.

Materials and methods

Cell-free assay for the endocytosis of E-cadherin

The A1-enriched fraction was prepared from rat livers as described previously (Tsukita and Tsukita, 1989), washed with 0.5 M Tris/HCl, pH 7.5, resuspended in buffer A (20 mM Hepes, pH 7.4, and 125 mM KOAc), and stored at −80°C until use. Thawed A1 plasma membranes (20 μg of protein) were incubated at 30°C in a reaction buffer (36 mM Hepes, pH 7.4, 0.25 M sorbitol, 70 mM KOAc, 5 mM EGTA, 1.8 mM CaCl2, 2.5 mM Mg(OAc)2), an ATP-regenerating system (1 mM ATP, 5 mM creatine phosphate, and 0.2 IU creatine phosphokinase), 100 μM GTP, and 2.5 mg/ml rat brain cytosol). The method for the preparation of the rat brain cytosol is described in the following section. The reaction was stopped by chilling the tube on ice and the membranes were collected by centrifugation at 20,000 g for 10 min. The membranes were resuspended by trituration (20 times pipetting) in 50 μl of 20 mM Hepes, pH 7.2, and 0.25 M sorbitol, and then supplemented with KOAc and Mg(OAc)2 to final concentrations of 150 and 2.5 mM, respectively (final volume of 60.6 μl). Immediately after addition of KOAc and Mg(OAc)2, differential centrifugation was performed at a medium speed (16,000 g) for 2 min. The top 42 μl supernatant fraction (designated MS) was harvested and centrifuged at a high speed (100,000 g) for 20 min. Membrane pellets from the MS and HSP spins were solubilized in an SDS sample buffer at RT for 30 min with vigorously shaking, and proteins were separated by 10% SDS-PAGE. Proteins were transferred to nitrocellulose membrane sheets and immunoblotted with the anti-E-cadherin mAb, followed by the HRP-conjugated secondary antibody (Amersham Biosciences). Blots were developed using the ECL kit and quantitated using a molecular imaging system (Personal Densitometer SI; Amersham Biosciences).

Preparation of the rat brain cytosol

Rat brains were thoroughly homogenized in a homogenizing buffer (20 mM Hepes, pH 7.4, 85 mM sucrose, 100 mM KOAc, and 1 mM Mg(OAc)2). The homogenate was centrifuged at 15,000 g for 10 min and the supernatant was centrifuged at 100,000 g for 1 h. The supernatant was subsequently dialyzed for 4 h in buffer A and frozen at −80°C until use. A2p3/3 complex was depleted from rat brain cytosol using GST-N-WASP affinity column.

Assay for activation of Rho, Rac, and Cdc42

Activation of small G proteins was assayed as described previously (Yamamoto et al., 1990). The A1 plasma membranes (25 μg) and the GDP-bound form of recombinant RhoA, Rac1, or Cdc42 (5 pmol) were treated with Cef or IgG in the presence of [35S]GTPyS. After the incubation for the indicated periods of time, the radioactivity of [35S]GTPyS bound to RhoA, Rac1, or Cdc42 was measured.

Assay for quantification of F-actin

F-Actin was quantified as described previously (Vouret-Craviari et al. 1999). After incubation with various reagents, the A1 plasma membranes were collected and incubated with F-actin buffer (20 mM KPO4, 10 mM Pipes, 5 mM EGTA, 2 mM MgCl2, 0.1% Triton X-100, 3.7% formaldehyde, and 2 μM FITC-phalloidin) at RT for 1 h. The FITC-phalloidin bound membranes were collected by centrifugation 16,000 g for 2 min. The membranes were washed twice (0.1% saponin, 2 mM KPO4, 10 mM Pipes, 5 mM EGTA, and 2 mM MgCl2) and then resuspended in 1.5 ml of ice-cold methanol to extract phalloidin bound to F-actin. After rotating at RT for 1 h, the methanol suspension was collected and the amount of FITC was determined in each sample by using a fluorimeter (fluorescence emission at 520 nm and excitation at 492 nm).

Immunosololation of the endocytosed vesicles of E-cadherin

Immunosololation of vesicles was performed as described previously (Saucan and Palade, 1994). In brief, dynabeads M-500 (Dynal) magnetic beads were covalently coupled with the goat anti-mouse IgG (Fc specific; Sigma-Aldrich) secondary antibody at a density of ~10 μg of antibody per 107 beads according to manufacturer’s instructions. The beads were coated with the anti-β-catenin mAb or the control IgG at a twofold molar excess over the secondary antibody and then washed four times with PBS plus 1 mg/ml BSA. The anti-β-catenin mAb-coated beads (1 mg beads) were added to the MSS (0.15–0.35 ml) and incubated in the presence of buffer B (20 mM Hepes, pH 7.2, 0.25 M sorbitol, 0.15 M KOAc, 5% FBS, and 2 mM EDTA; final volume of 0.4 ml), with continuous rotation at 4°C for 2 h. The immunosololated beads were washed five times with buffer B using a magnet and transferred to fresh tubes. The beads were finally resuspended in 50 μl of the SDS sample buffer for SDS-PAGE, immunoblotting, or EM as described in the following section.

Immunoelectron microscopy

EM was performed to visualize the immunosololated vesicles on the magnetic beads. The samples were fixed in 4% PFA and 0.1% glutaraldehyde in PBS for 45 min, and then in 1% osmium tetroxide in the same buffer for 1 h. The samples were stained in black in 2% uranyl acetate, dehydrated in ethanol and propylene oxide, and embedded in Epon. Thin sections were cut, stained, and examined in a TEM. For immunogold labeling, the vesicles on the beads were first reacted with the anti-pan-cadherin rabbit pAb for 1 h, followed by the goat anti-rabbit IgG conjugated to 10-nm gold (Jackson Immunoresearch Laboratories) for 1 h. The beads were washed, fixed, and examined in a TEM.

Biotinylation assay for the endocytosis of E-cadherin

The assay was performed as described previously with minor modifications (Le et al., 1999). In brief, EL cells were incubated with 0.5 mg/ml sulfo-NHS-s-biotin on ice, and the cells were then incubated at 18°C to allow the endocytosis of E-cadherin for the indicated periods of time in the presence of IgG or Cef. The cells were then incubated in several washes with glutathione buffer (60 mM glutathione, 83 mM NaCl, 83 mM NaOH, and 10% FCS) on ice to remove bound biotinyl groups from remaining cell surface biotinylated proteins. The cells were then lysed in a RIPA buffer. The cell extracts were incubated with streptavidin beads (Sigma-Aldrich) to collect bound biotinylated proteins. Protein bands were then analyzed by SDS-PAGE and immunoblotting with the anti–E-cadherin mAb. For cell staining, the cells were transfected with pEGFP, pEGFP-N-WASP CRIB-CAAX, or pEGFP-N17Rac1 using LipofectAMINE 2000 Reagent. After a 24-h culture, the cells were incubated at 18°C to allow the endocytosis of E-cadherin for 20 min in the presence of IgG or Cef. The cells were then fixed, followed by immunostaining with the anti–E-cadherin mAb (ECCD-2) and the anti-IQGAP1 pAb. Images were captured using a confocal laser scanning microscope (Bio-Rad Laboratories) using a 60x oil immersion objective lens (model Radiance 2000; Bio-Rad Laboratories). Collected data were exported as 8-bit TIFF files and processed using Adobe Photoshop software.

Online supplemental material

Fig. S1 is available in the online supplemental material. Fig. S1 shows the stage specific assay for the endocytosis of E-cadherin and EM pictures of all fractions. Materials and methods for antibodies, reagents, and immunofluorescence microscopic assay for the endocytosis of E-cadherin are available in the online supplemental material. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200401078/DC1.

This work was supported by grants-in-aid for Scientific Research and for Cancer Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (2003). T. Sakisaka is a recipient of a Human Frontier Science Program Career Development Award (2003).

Submitted: 4 June 2004
Accepted: 7 June 2004

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