Involvement of the Ras-Ras-activated Rab5 Guanine Nucleotide Exchange Factor RIN2-Rab5 Pathway in the Hepatocyte Growth Factor-induced Endocytosis of E-cadherin

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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 an extracellular signal, such as hepatocyte growth factor (HGF)/scatter factor. Rab5 small G protein has been implicated in the HGF-induced endocytosis of E-cadherin, but the molecular mechanism for the regulation of Rab5 activity remains unknown. We first studied this mechanism by using the cell-free assay system for the endocytosis of E-cadherin of the AJ-enriched fraction from rat livers. HGF induced activation of Ras small G protein, which then bound to RIN2, a Rab5 GDP/GTP exchange factor with the Vps9-like guanine nucleotide exchange factor and Ras association domains, and activated it. Activated RIN2 then activated Rab5, eventually inducing the endocytosis of E-cadherin. We then studied whether RIN2 was involved in the HGF-induced endocytosis of E-cadherin in intact Madin-Darby canine kidney cells. RIN2 localized at the cell-cell adhesion sites, and its guanine nucleotide exchange factor activity was required for the HGF-induced endocytosis of E-cadherin in Madin-Darby canine kidney cells. These results indicate that RIN2 connects Ras to Rab5 in the HGF-induced endocytosis of E-cadherin.

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 (1–4). 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 the formation of AJs. The complete disassembly of AJs results in a loss of the polarized, poorly motile epithelial phenotype and the acquisition of a migratory or mesenchyme-like phenotype. As migrating epithelial cells do not always exhibit changes in the protein expression of E-cadherin (5), 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 (6–10).

In cultured epithelial cells, some growth factors, such as hepatocyte growth factor (HGF)/scatter factor (SF), epidermal growth factor (EGF), and fibroblast growth factor (FGF), induce disruption of cell–cell adhesion, followed by cell migration (11–14). Actions of HGF/SF, EGF, and FGF are mediated through their receptors (15, 16). HGF/SF receptor, c-Met, EGF receptor (EGFR), and FGF receptor (FGFR) are receptor tyrosine kinases (RTKs) that are linked to the activation of many signaling molecules such as Ras small G protein and phosphoinositide 3-kinase (17, 18). Activation of both Ras and phosphoinositide 3-kinase has been shown to be essential for the HGF-induced cell scattering (17, 18). c-Met or EGFR has been shown to be colocalized with E-cadherin at the lateral membrane of MDCK cells (7, 19, 20). Because signaling from RTKs can induce the internalization of E-cadherin, coendocytosis of activated RTKs and cadherins could be a novel mechanism for regulating cadherin adhesive dynamics (7, 21). The incorporation of RTKs and cadherins into functional complexes (e.g. vascular endothelial growth factor receptor-2 with VE-cadherin; EGFR, insulin-like growth factor 1 receptor FGFR, or c-Met with E-cadherin) is an emerging paradigm (22). The functional consequences of such complexes have the potential to regulate both signaling and adhesion (22).

Signaling pathways of RTKs can modulate the endocytic machinery, as exemplified by the recently uncovered functional connections between Rab5 small G protein and signaling molecules (23–25). Rab5 is a key regulator of transport from the plasma membrane to the early endosomes. Continuous cycles of GDP/GTP exchange and hydrolysis regulate the kinetics of constitutive endocytosis (26), but this nucleotide cycle can also be modulated by extracellular stimuli. Stimulation by EGF enhances the rate of endocytic membrane flow (23) by increasing the fraction of active Rab5 through stimulation of Rab5 guanine nucleotide exchange factor (GEF) RIN1 (25) and down-regulation of the GTPase-activating protein RN-tre (24). Besides regulating receptor internalization (24), RN-tre is also integrated into the EGF signaling pathway via its interaction with the EGFR substrate Eps8 and the adaptor protein Grb2, which links EGFR to mSos, a GEF for Ras (24, 27). In addition, we have shown previously that activation of Rab5 is involved in the HGF-induced disruption of cell-cell adhesion and subsequent cell migration.
through coendocytosis of E-cadherin and c-Met in MDCK cells (7, 12). However, the molecular mechanism of how the activation of Ras is induced by HGF has not fully been understood.

To elucidate this molecular mechanism, we have used a cell-free assay system of the AJ-enriched fraction from rat livers, in which the non-trans-interacting E-cadherin endocytosis is induced. We have shown here that RIN2, a member of the RIN Rab5 GEF family, transduces a signal from Ras to Rab5 in the HGF-induced endocytosis of E-cadherin.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Vectors**—The pCMV-FLAG-RIN2 was provided by Dr. T. Katada (Tokyo University, Tokyo, Japan) (28).

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**FIGURE 1.** Involvement of the Rab family members in the HGF-induced endocytosis of E-cadherin. A, flow diagram of the steps of the cell-free assay system and schematic morphologies of the AJ membrane fraction. Ppt, pellet; Sup, supernatant. B, enhancement of the endocytosis of E-cadherin by HGF stimulation. The AJ membrane was incubated with HGF at the indicated concentrations in the presence of ATP and GTP to induce HGF-induced signaling events and further incubated with rat brain cytosol to induce budding of vesicles. The vesicles carrying E-cadherin were budded from the collected AJ membrane. The AJ membrane was resuspended by trituration (vigorous pipetting) to mechanically pinch off the budded vesicles from the AJ membrane. The sample was then centrifuged at 16,000 \( \times \) g for 2 min to recover the medium speed supernatant (MSS), which might contain vesicles completely pinched off from the AJ membrane, from the medium speed pellet (MSP). To collect these vesicles, the medium speed supernatant was centrifuged at 100,000 \( \times \) g for 20 min, and the high speed pellet (HSP) was obtained. The probable AJ membrane structures in each fraction and procedures are schematically shown in A. We then examined by immunoblotting whether E-cadherin was indeed recovered in the HSP. C, inhibition of the endocytosis of E-cadherin by retrieval of the Rab family members by Rab GDI. The AJ membrane fraction was preincubated with GST-Rab GDI at the indicated concentrations. The membrane was collected by centrifugation and assayed for the endocytosis of E-cadherin as described in A, D, no effect of HGF on the endocytosis of E-cadherin from the AJ membrane fraction pretreated with Rab GDI. After the preincubation with GST-Rab GDI, the membrane was collected, incubated in the presence of HGF at the indicated concentrations, and assayed for the endocytosis of E-cadherin as described in A, 1 and 2 indicate the sequence of the incubation. The amount of E-cadherin in the endocytosed vesicles (70% of total SDS solubilized membrane) was quantitated by immunoblotting with the anti-E-cadherin mAb. The quantification of immunoblot is shown as the mean (\( \pm \) S.D.) of duplicate assays in the lower panel. Asterisks indicate statistical significance (Student’s t test; *, \( p < 0.05 \)). The results shown in all panels are representative of at least three independent experiments.
FIGURE 2. Involvement of Rab5 in the HGF-induced endocytosis of E-cadherin. A, flow diagram of the steps of the cell-free assay system and schematic morphologies of the AJ membrane fraction. Ppt, pellet; Sup, supernatant. B, rescue from the inhibitory effect of Rab GDI on the endocytosis of E-cadherin by Rab5. The AJ membrane fraction was first incubated with GST-Rab GDI and collected. The collected membrane was restored by adding the GTPγS-bound form of Rab5 at the indicated concentrations and assayed for the endocytosis of E-cadherin as described in A. 1 and 2 indicate the sequence of the incubation. C, no rescue from the inhibitory effect of Rab GDI on the endocytosis of E-cadherin by Rab3. The AJ membrane fraction was first incubated with GST-Rab GDI and collected. The collected membrane was restored by adding the GTPγS-bound form of Rab3 at the indicated concentrations and assayed for the endocytosis of E-cadherin as described in A. 1 and 2 indicate the sequence of the incubation. The amount of E-cadherin in the endocytosed vesicles (70% of total SDS solubilized membrane) was quantitated by immunoblotting with the anti-E-cadherin mAb. The quantification of immunoblot is shown as the mean (±S.E.) of duplicate assays in the lower panel. Asterisks indicate statistical significance (Student’s t test; *, p < 0.05). The results shown in all panels are representative of at least three independent experiments.
GEF activity-defective mutant, pCMV-FLAG-RIN2-D696A/P700A, in which aspartic acid at position 696 was replaced with alanine and proline at position 700 was replaced to alanine, was generated using the QuikChange site-directed mutagenesis kit (Stratagene). The pCMV-FLAG-Rab5 and pEF-BOS-Myc-Rab5-S34N were constructed (12).

**Antibodies and Reagents**

The GST fusion fragment of RIN2 (amino acids 334–631) was produced in *Escherichia coli*, purified, and used as an antigen to raise a polyclonal antibody (pAb) in rabbits. The rabbit anti-RIN2 pAb was affinity-purified by using MBP-RIN2 (amino acids 334–631) immobilized on Amino-link agarose beads (Pierce). A mouse anti-E-cadherin (cytoplasmic portion) monoclonal antibody (mAb) (clone 36), a mouse anti-EEA1 mAb (BD Transduction Laboratories), a mouse anti-FLAG mAb (Sigma), a mouse anti-Ha-Ras mAb (clone Y13-259), a mouse anti-c-Myc mAb (Santa Cruz Biotechnology), and a mouse IgG (Jackson ImmunoResearch) were purchased from commercial sources. A rat anti-E-cadherin (extracellular portion) mAb (ECCD-2) was provided by Dr. M. Takeichi (Center for Developmental Biology, RIKEN, Kobe, Japan). Human recombinant HGF/SF was provided by Dr. T. Nakamura (Osaka University, Suita, Japan). Other reagents were purchased from commercial sources.

**Cell-free Assay for the Endocytosis of E-cadherin**

The assay was performed as described (10). Briefly, the AJ membrane fraction was prepared from rat livers as described (29), 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. The thawed AJ membrane fraction (20 μg of protein) was incubated at 30 °C in a reaction buffer (36 mM Hepes/KOH (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 phosphate kinase), 100 μM GTP, and 2.5 mg/ml rat brain cytosol). The reaction was stopped by chilling the tube on ice, and the membrane was collected by centrifugation at 20,000 g for 10 min. The membrane was resuspended by trituration (20 times pipetting) in 50 mM Hepes/KOH (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 (a final volume of 60.6 μl). Immediately after the 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 was harvested and centrifugated at a high speed (100,000 × g) for 20 min. Membrane pellets from the high speed spins were solubilized in an SDS sample buffer at room temperature for 30 min with vigorous shaking, and proteins were separated by 10% SDS–PAGE. The proteins were transferred to nitrocellulose membrane sheets and immunoblotted with the anti-E-cadherin mAb (clone 36), followed by the horseradish peroxidase-conjugated secondary Ab (Amersham Biosciences). The blots were developed using the ECL kit and quantitated using a densitometer Fluorchem™ (Alpha Innotech Corp.).

**FIGURE 3. Induction of activation of Rab5 and Ras by HGF.**

Aa, schematic representation for the assay of the HGF-induced activation of Rab5 using AJ membrane fraction. Ab, the AJ membrane fraction was incubated with [35S]GTP-γ-S and the GDP-bound form of recombinant Rab5 in the presence or absence of HGF for the indicated periods of time. The radioactivity of [35S]GTP-γ-S bound to Rab5 was scintillation-counted and plotted. Bb, schematic representation for the assay of the HGF-induced activation of Ras using AJ membrane fraction. Bb, the AJ membrane fraction was incubated with [35S]GTP-γ-S and the GDP-bound form of recombinant Ha-Ras in the presence or absence of HGF for the indicated periods of time. The radioactivity of [35S]GTP-γ-S bound to Ha-Ras was scintillation-counted and plotted. The mean (± S.D.) of duplicate assays is shown.
Assay for HGF-induced Activation of Ras and Rab5—Lipid-modified Ha-Ras and Rab5 proteins were purified from baculovirus-infected Sf9 cells as described (30). The AJ membrane fraction (25 μg of protein) and the lipid-modified GDP-bound form of recombinant Ha-Ras or Rab5 (5 pmol) were incubated with or without HGF in Buffer B (50 mM Tris/Cl (pH 8.0), 12 mM MgCl2, 2 mM EDTA, 0.4 mM dithiothreitol, 0.06% CHAPS, 100 μM ATP, and 12 μM [35S]GTPγS (6 × 10^3 cpm/pmol)). After the incubation for the indicated periods of time, the radioactivity of [35S]GTPγS bound to Ha-Ras or Rab5 and the AJ membrane was measured. To monitor the amount of [35S]GTPγS bound to Ha-Ras or Rab5, we subtracted the radioactivity of [35S]GTPγS bound to the AJ membrane from the radioactivity of [35S]GTPγS bound to Ha-Ras or Rab5 and the AJ membrane. The method is precisely described in Supplemental Fig. 1.

Assay for GEF Activity for Rab5 of RIN2—GEF assay was performed as described (31). FLAG-RIN2 and FLAG-RIN2-D696A/P700A were purified from transfected HEK293 cells as described (28). The lipid-modified GDP-bound form of Rab5 (3 pmol) was incubated at 30 °C for the indicated periods of time in a reaction mixture (50 μl) containing FLAG-RIN2 (0.5 pmol) with or without the GTPγS-bound form of Ha-Ras (0.5 pmol), 50 mM Tris/HCl (pH 8.0), 12 mM MgCl2, 2 mM EDTA, 0.4 mM dithiothreitol, 0.06% CHAPS, and 12 μM [35S]GTPγS (6 × 10^3 cpm/pmol). The mixture was applied to a nitrocellulose filter, and the radioactivity retained on the filter was measured.

Immunofluorescence Microscopic Assay for the Endocytosis of E-cadherin—MDCK cells (5 × 10^5 cells/35-mm dish) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37 °C for 24 h and then transfected with pCMV-FLAG-RIN2, FLAG-RIN2-D696A/P700A, or both pCMV-FLAG-RIN2 and pEF-BOS-Myc-Rab5-S34N using Lipofectamine 2000 Reagent (Invitrogen). After a 24-h culture, the cells were incubated with 10 ng/ml of HGF for 3 h. The cells were fixed, followed by immunostaining with the anti-E-cadherin mAb (ECCD-2), the anti-FLAG mAb, and the anti-c-Myc mAb. Images were captured using a Carl Zeiss confocal laser scanning microscope with a 63× oil immersion objective lens (model LSM 510-V3.2; Carl Zeiss). X-Z optical sectionings were performed, and the fluorescence intensity of E-cadherin inside the cells and at the cell-cell adhesion sites was measured using the fluorescence intensity profile function of LSM 510 software. Quantification of the endocytosed E-cadherin-positive vesicular structures is presented as the ratio of fluorescence intensity (E-cadherin inside the cell to E-cadherin at the cell-cell adhesion).

Knockdown of RIN2 by the RNA Interference Method—The mammalian expression vector, pBS-H1, was used for expression of small interfering RNA in MDCK cells. The following inserts were used: RIN2 gene-specific insert was a 21-nucleotide sequence corresponding to nucleotides 1177–1197 (5′-AAGAAAGTCATGCTGCTGCTG-3′) of dog RIN2 cDNA, which was separated by a 10-nucleotide noncomple-
mentary spacer (5'-TTGATATCCG-3') from the reverse complement of the same 21-nucleotide sequence.

Other Methods—Protein concentrations were determined with bovine serum albumin as a reference protein (32). SDS-PAGE was done as described by Laemmli (33).

RESULTS

Involvement of Rab Family Members in the HGF-induced Endocytosis of E-cadherin—We have previously developed a cell-free assay system using the AJ-enriched fraction from rat livers, in which the non-trans-interacting E-cadherin endocytosis is induced (10). To gain insight into the mechanism of the HGF-induced endocytosis of E-cadherin, we examined whether HGF affects the endocytosis of non-trans-interacting E-cadherin in this cell-free assay system as schematically shown in Fig. 1A and precisely described under “Experimental Procedures.” HGF enhanced the endocytosis of E-cadherin in a dose-dependent manner (Fig. 1B). We then examined whether Rab family members are involved in the HGF-induced endocytosis of E-cadherin. For this purpose, we took advantage of Rab GDI, which forms a complex preferentially with the GDP-bound form to the GTP-bound form of all the Rab family members and extracts them from the membrane (34). After the preincubation of the AJ membrane fraction with Rab GDI for 30 min, increasing concentrations of Rab GDI decreased the amount of endocytosed E-cadherin (Fig. 1C). This pretreatment of the AJ membrane fraction

FIGURE 5. Involvement of Ras in the HGF-induced endocytosis of E-cadherin. A, flow diagram of the steps of the cell-free assay system and schematic morphologies of the AJ membrane fraction. Ppt, pellet; Sup, supernatant. B and C, regulation of the HGF-induced endocytosis of E-cadherin by Ras. B, the AJ membrane fraction was incubated with HGF in the presence of recombinant GST-RBD or control GST at the indicated concentrations and assayed for the endocytosis of E-cadherin as described in A. C, the AJ membrane fraction was incubated with HGF in the presence of the blocking Ab against Ras at the indicated concentrations and assayed for the endocytosis of E-cadherin as described in A. The amount of E-cadherin in the endocytosed vesicles (70% of total SDS solubilized membrane) was quantitated by immunoblotting with the anti-E-cadherin mAb. The quantification of immunoblot is shown as the mean (± S.D.) of duplicate assays in the lower panel. Asterisks indicate statistical significance (Student’s t test; *, p < 0.05). The results shown in all panels are representative of at least three independent experiments.
FIGURE 6. Identification of RIN2 as a connector between Ras and Rab5 in the HGF-induced endocytosis of E-cadherin. A, binding of Ras to RIN2. In the upper panel, a molecular structure of RIN2 is shown. Domains include SH2, Src homology 2 domain; PR, proline-rich domain; RH, RIN homology domain; Vps9, Vps9p-like domain; and RA, Ras-association domain. In lower panels, the extract of HEK293 cells expressing FLAG-RIN2 was incubated with the GDP-bound or GTPyS-bound form of GST-Ha-Ras or control GST immobilized on glutathione-Sepharose beads. The bound proteins were analyzed by immunoblotting with the anti-FLAG mAb. The quantification of immunoblot is shown as the mean (± S.D.) of duplicate assays in the lower panel. The results shown are representative of three independent experiments. B, enhancement of GEF activity of RIN2 by Ras. The GDP-bound form of Ras, RIN2, Rab5, and Endocytosis of E-cadherin

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with Rab GDI inhibited the HGF-induced endocytosis of E-cadherin (Fig. 1D). These results indicate that the substrate small G proteins for Rab GDI is involved in the HGF-induced endocytosis of E-cadherin.

**Involvement of Rab5 in the HGF-induced Endocytosis of E-cadherin—** We examined which substrate Rab proteins show this effect. The GTPγS-bound form of recombinant Rab5 or Rab3 was added to the AJ membrane fraction pretreated with Rab GDI, and the extent of the endocytosis of E-cadherin was measured (Fig. 2A). The GTPγS-bound form of Rab5, but not that of Rab3, rescued the inhibitory effect of Rab GDI on the endocytosis of E-cadherin in a dose-dependent manner (Fig. 2, B and C). These results indicate that at least Rab5 is involved in the HGF-induced endocytosis of E-cadherin.

**Activation of Rab5 by HGF in a Ras-dependent Manner—** We examined whether HGF induces activation of Rab5 in our assay system. We incubated the AJ membrane fraction with the GDP-bound form of Rab5 and [35S]GTPγS in the presence or absence of HGF. HGF enhanced exchange of Rab5 from the GDP-bound to the GTP-bound form in a time-dependent manner (Fig. 3A). Several studies have revealed that HGF induces activation of Ras, which is required for cell-cell disruption and subsequent cell scattering (17, 18). We then examined whether HGF induces activation of Ras in our assay system. We incubated the AJ membrane fraction with the GDP-bound form of Ha-Ras and [35S]GTPγS in the presence or absence of HGF. HGF enhanced exchange of Ha-Ras from the GDP-bound to the GTP-bound form in a time-dependent manner (Fig. 3B). To understand the relationship between Ras and Rab5, we examined the effect of inactivation of Ras on the HGF-induced activation of Rab5. GST-Ras binding domain (RBD) of Raf, a specific inhibitor of the GTP-bound form of Ras (35), inhibited the HGF-induced activation of Rab5 (Fig. 4A). The blocking Ab against Ras similarly inhibited the HGF-induced activation of Rab5 (Fig. 4B). In addition, we examined the effects of GST-RBD on the HGF-induced endocytosis of E-cadherin as shown in Fig. 5A. GST-RBD inhibited the HGF-induced endocytosis of E-cadherin in a dose-dependent manner (Fig. 5B). The blocking Ab against Ras similarly inhibited the HGF-induced endocytosis of E-cadherin in a dose-dependent manner (Fig. 5C). These results indicate that HGF induces the activation of Rab5 in a Ras-dependent manner.

**Enhancement of the Endocytosis of E-cadherin by Rab5 GEF, RIN2, in a Ras-dependent Manner—** To identify a molecule that connects Ras to Rab5, we conducted a sequence search against the protein data base to find the protein containing Ras association (RA) and Vps9p-like (Rab5 GEF) domains. We found that Ras interaction/interference (RIN) family members contain the RA and Vps9p-like domains in addition to the Src homology 2 domain and the proline-rich domains. We focused on a ubiquitously expressed member of RIN family, RIN2 (28). We confirmed the binding of Ras to Rab5 by a pull-down assay using recombinant proteins. FLAG-tagged full-length RIN2 (FLAG-RIN2) bound the GTPγS-bound form of GST-Ha-Ras preferentially to the GDP-bound form (Fig. 6A). The GTPγS-bound form of Ha-Ras enhanced the RIN2 GEF activity of RIN2 (Fig. 6B). RIN2 alone did not affect the endocytosis of E-cadherin (Fig. 6, C and D). The GTPγS-bound form of Ha-Ras activated RIN2 and enhanced the endocytosis of E-cadherin in a dose-dependent manner, whereas the GDPβS-bound form of Ha-Ras was less effective (Fig. 6, C and E). These results indicate that RIN2 is a direct downstream target of Ras and is involved in the HGF-induced endocytosis of E-cadherin in a Ras-dependent manner, and these results suggest that the RA domain is the negatively regulatory domain for the activity of RIN2.

**Localization of RIN2 at the Cell-Cell Adhesion Sites—** We made an anti-RIN2 pAb and examined subcellular distribution in the rat liver by Western blotting. RIN2 was concentrated in the AJ fraction similar to E-cadherin (Fig. 7A). We then examined the localization of RIN2 in MDCK cells by use of this pAb. The immunofluorescence signal for RIN2 localized at the cell-cell adhesion sites and punctate vesicular structures, which scattered in the cytoplasm in MDCK cells (Fig. 7B). The signal for RIN2 did not overlap with the signal for the early endosomal marker EEA1 at the punctate

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*FIGURE 7. Localization of RIN2 at cell-cell adhesion sites. A, subcellular distribution of RIN2 in rat liver. Subcellular fractionation of rat liver was performed, and each fraction (30 µg of protein each) was subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western blotting with the anti-RIN2 pAb or the E-cadherin mAb. Ho, the homogenate fraction; S, the soluble fraction; P, the pellet fraction; BC, the fraction rich in bile canaliculi; AJ, the fraction rich in AJ; RBC, rat brain cytosol. B, colocalization of RIN2 with E-cadherin at the cell-cell adhesion sites. MDCK cells were doubly stained with the anti-RIN2 pAb (red) and the anti-E-cadherin rat mAb (green). C, colocalization of RIN2 with Rab5, but not EEA1. MDCK cells were triply stained with the anti-RIN2 pAb (red), the anti-EEA1 mouse mAb (green), and the anti-E-cadherin rat mAb (blue). Insets are enlarged images of boxed area. The results shown are representative of three independent experiments. Bars, 10 µm.*
vesicular structures, suggesting that RIN2-positive vesicles are different forms of early endosomes (Fig. 7C). Because we could not detect the signal for Rab5, which is the endocytic vesicular and early endosomal marker (36), with commercially available Abs at our hands, we expressed FLAG-Rab5 in MDCK cells. The signal for RIN2 overlapped with the signal for FLAG-Rab5. These results suggest that RIN2 is involved in a transport pathway from the plasma membrane to early endosomes, not in the homotypic fusion process of early endosomes.

Enhancement of the Endocytosis of E-cadherin by RIN2 in Its Rab5 GEF Activity-dependent Manner in MDCK Cells—To further validate the results obtained in our cell-free assay system, we finally examined whether RIN2 affects the endocytosis of E-cadherin in intact MDCK cells. Activation of c-Met, the cell surface receptor for HGF, results in promoting the endocytosis of E-cadherin (7–9). We examined the effect of RIN2 on the HGF-induced endocytosis of E-cadherin in MDCK cells. Quantitative analysis showed that overexpression of RIN2 enhanced the HGF-induced endocytosis of E-cadherin consistently with our results in the cell-free assay system (Fig. 8, A and E). This enhancement of the endocytosis of E-cadherin by RIN2 was blocked by coexpression of a dominant negative mutant of Rab5. To confirm the GEF activity of RIN2 through the Vps9p-like domain, we generated a GEF activity-defective mutant, RIN2-D696A/P700A double point mutant, as shown in Fig. 8B (37). RIN2-D696A/P700A double point mutant abolished the GEF activity of RIN2 toward Rab5 (Fig. 8C). Overexpression of RIN2—
FIGURE 9. Involvement of RIN2 in the HGF-induced endocytosis of E-cadherin. A, effect of knockdown of RIN2 by the RNAi method. MDCK cells were transfected with control vector or RIN2 small interfering RNA (siRNA). The whole cell lysates (50% transfection efficiency) were subjected to SDS-PAGE, followed by immunoblotting with the anti-RIN2 pAb and the anti-actin mAb. B, quantification of relative immunoblot band intensity of RIN2. C, MDCK cells were cotransfected with green fluorescent protein (GFP) as a morphological marker along with control vector or RIN2 small interfering RNA and cultured in Dulbecco’s modified Eagle’s medium for 48 h. After the culture, the cells were incubated with 10 ng/ml HGF for 3 h. The transfected cells were identified by the expression of green fluorescent protein (blue). Their E-cadherin-positive internal vesicular structures were examined by immunostaining of E-cadherin (green), and the extent of expression of RIN2 was examined by immunostaining of RIN2 (red). Optical X-Y and X-Z sections in transfected cells are presented. Bars, 10 μm. D, quantification of relative fluorescence intensities of E-cadherin at the cell surface and inside cells is presented. Asterisks indicate statistical significance (Student’s t test; *, p < 0.05). The results shown are representative of three independent experiments.

FIGURE 10. A mode of action of HGF in the endocytosis of E-cadherin. HGF induces activation of c-Met receptor tyrosine kinase and induces activation of Ras possibly through Sos. The activated Ras binds to RIN2, which then activates its Rab5 GEF activity, eventually inducing the coendocytosis of E-cadherin together with c-Met receptor. This coendocytosis of E-cadherin and c-Met receptor may shift the equilibrium of trans-interacting state of E-cadherin to non-trans-interacting state and thereby disrupt cell-cell junction gradually.
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D696A/P700A double point mutant reduced the HGF-induced endocytosis of E-cadherin (Fig. 8, D and E). Thus, the GEF activity of RIN2 is required for the HGF-induced endocytosis of E-cadherin.

In the last set of experiments, to further confirm that RIN2 is involved in the HGF-induced endocytosis of E-cadherin, we performed a loss-of-function analysis by use of the RNAi method. We knocked down endogenous RIN2 by the RNAi method (Fig. 9, A and B). Quantitative analysis showed that RIN2-knockdown cells reduced the HGF-induced endocytosis of E-cadherin (Fig. 9, C and D). These data are another line of evidence supporting our argument that RIN2 is involved in the HGF-induced endocytosis of E-cadherin.

DISCUSSION

We have shown previously that Rab5 is involved in the HGF-induced endocytosis of E-cadherin in MDCK cells (7). Here we have first confirmed earlier observations by use of our cell-free assay system for the endocytosis of E-cadherin from the AJ-enriched fraction from rat livers; HGF induces the activation of Rab5; deprivation of all the Rab GDI-sensitive Rab family members reduces the HGF-induced endocytosis of E-cadherin; and this inhibitory effect of Rab GDI is restored by the GTPγS-bound form of Rab5. It has become more evident that Rab5 is involved in the HGF-induced endocytosis of E-cadherin not only in MDCK cells but also in rat livers.

It has been shown that Ras is activated by HGF through the c-Met-Grb2-Sos pathway (17, 18). We have confirmed here by our cell-free assay system that Ras is indeed activated by HGF and that this HGF-induced activation of Ras is required for the HGF-induced activation of Rab5. It may be emphasized here that although many extracellular signals have been shown to induce activation of small G proteins, including Ras and Rab5 in intact cells (25, 38), this is the first description that the extracellular signal molecule HGF induces activation of Ras and Rab5 in a cell-free assay system. Our system includes all the components for the activation of these small G proteins and would be a useful tool for studies on the mechanism of the activation of these small G proteins by the membrane receptor.

We have then shown that Ras induces activation of Rab5 through RIN2, which is a direct downstream target of Ras and a direct upstream regulator of Rab5. RIN2 has the RA domain, and the binding of the GTPγS-bound form of Ras to this domain enhances the GEF activity toward Rab5. It is therefore likely that the RA domain negatively regulates the Rab5 GEF activity. In steady state, RIN2 is likely to form a closed conformation by an intramolecular interaction between the RA domain and the Vps9p-like (Rab5 GEF) domain, and thereby negatively regulates the Rab5 GEF activity. In the active state, the binding of Ras to the RA domain may reduce the intramolecular interaction and stabilize an open conformation of RIN2.

We have shown previously that non-trans-interacting E-cadherin, but not trans-interacting one, undergoes endocytosis and that Rac small G protein activated by the action of trans-interacting E-cadherin inhibits the endocytosis of E-cadherin through the IQGAP-dependent reorganization of the actin cytoskeleton (10). In addition, trans-interacting nectin activates Rac small G protein and inhibits the non-trans-interacting E-cadherin endocytosis through afadin and p120ctn (39, 40). Taken together with the result that activation of Rac5 is required for the HGF-induced endocytosis of E-cadherin, it is likely that inactivation of Rac and Rap is also involved in the HGF-induced endocytosis of E-cadherin in addition to the activation of Rab5. There must be a signal cross-talk among Rab5, Rac, and Rap during the HGF-induced disruption of cell-cell adhesion. Molecular mechanisms for this signal cross-talk are important issues to be addressed in the future for our understanding of cell adhesion.

In cultured MDCK cells, we have shown here that RIN2 enhances the HGF-induced disruption of the cell-cell adhesion in its Rab5 GEF activity-dependent manner, which is accompanied with the endocytosis of E-cadherin. However, it is still not known whether 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 action of HGF in MDCK cells (7, 8). The endocytosis of a specific portion of E-cadherin may be necessary and sufficient for the action of HGF. Another possibility is that an unidentified factor(s), which is essential for the maintenance of 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. In conclusion, we have shown here the signaling pathway from c-Met to Rab5, which is involved in the HGF-induced endocytosis of E-cadherin as schematically shown in Fig. 10.

REFERENCES


**Ras, RIN2, Rab5, and Endocytosis of E-cadherin**

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