Phosphatidylinositol 3-Kinase to Regulate Adhesive Contacts*

E-cadherin Homophilic Ligation Directly Signals through Rac and Phosphatidylinositol 3-Kinase to Regulate Adhesive Contacts*

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Classical cadherins mediate cell recognition and cohesion in many tissues of the body. It is increasingly apparent that dynamic cadherin contacts play key roles during morphogenesis and that a range of cell signals are activated as cells form contacts with one another. It has been difficult, however, to determine whether these signals represent direct downstream consequences of cadherin ligation or are juxtacrine signals that are activated when cadherin adhesion brings cell surfaces together but are not direct downstream targets of cadherin signaling. In this study, we used a functional cadherin ligand (hE/Fc) to directly test whether E-cadherin ligation regulates phosphatidylinositol 3-kinase (PI 3-kinase) and Rac signaling. We report that homophilic cadherin ligation recruits Rac to nascent adhesive contacts and specifically stimulates Rac signaling. Adhesion to hE/Fc also recruits PI 3-kinase to the cadherin complex, leading to the production of phosphatidylinositol 3,4,5-trisphosphate in nascent cadherin contacts. Rac activation involved an early phase, which was PI 3-kinase-independent, and a later amplification phase, which was inhibited by wortmannin. PI 3-kinase and Rac activity were necessary for productive adhesive contacts to form following initial homophilic ligation. We conclude that E-cadherin is a cellular receptor that is activated upon homophilic ligation to signal through PI 3-kinase and Rac. We propose that a key function of these cadherin-activated signals is to control adhesive contacts, probably via regulation of the actin cytoskeleton, which ultimately serves to mediate adhesive cell-cell recognition.

Classical cadherin molecules are critical morphogenetic determinants in metazoan organisms (1, 2). E-cadherin, the prototypical epithelial cadherin, participates in tissue patterning during development and preserves epithelial organization in postembryonic life. In addition to mediating cell-cell cohesion in mature epithelia, E-cadherin has a clear role in cell-to-cell recognition. E-cadherin-expressing cells sort out from tissue culture cells that express different cadherins, while changes in cadherin expression determine tissue segregation in the embryo (1). E-cadherin, like other classical cadherins, therefore plays a central role in determining how cells discriminate like from unlike.

At the cellular level, there is increasing evidence that E-cadherin ligation initiates a cascade of molecular and cellular events that ultimately determine cellular recognition. Conversion of initial homophilic ligation to productive, stable cell-cell adhesion appears to constitute a key step in this recognition process. Studies in migrating cells have shown that E-cadherin-expressing cells make nascent contacts with one another through punctate cadherin contacts (3–6). Productive stabilization of adhesion is distinguished by the extension of these contact zones, leading cells to persist in coherent aggregates with altered migratory activity. Cells that fail to stabilize these initial contacts separate from one another and continue to migrate. Furthermore, a number of cellular mechanisms with the potential to mediate adhesive stabilization have been identified as downstream consequences of cadherin ligation. These include lateral clustering of cadherin molecules (7, 8), recruitment of cadherin-associated junction proteins (9), and reorganization of the actin cytoskeleton (3, 4).

Taken together, these observations raised the attractive possibility that E-cadherin binding may activate signaling pathways to coordinate the cellular cascade that leads from homophilic recognition to productive adhesion. In its simplest form, it is attractive to envisage a scenario where E-cadherin acts as a direct upstream receptor for intracellular signals that ultimately act on the effector mechanisms responsible for adhesive stabilization. Indeed, signaling by PI3-kinase (10) as well as Rho family GTPases (11–13) has been observed to be activated as epithelial cells reestablish contacts after chelation of extracellular calcium. The use of cadherin-blocking antibodies demonstrated that such signals depended on functional E-cadherin.

These studies could not, however, distinguish between signals that were activated as direct downstream consequences of cadherin ligation and juxtacrine signals that required cadherin adhesion to bring cell surfaces together but were not themselves direct downstream consequences of cadherin ligation (2, 14). This distinction has fundamental mechanistic implications for any model of cadherin signaling. For example, gap junction communication requires E-cadherin to bring cell surfaces into contact (15), but connexins do not interact directly with cadherin-expressing cells.
herins. Ultimately, any mechanistic characterization of cadherin signaling must determine whether responses to cell-cell contact represent direct cadherin-activated signals or cadherin-dependent juxtacrine signals.

Nor has it yet been determined whether cadherin-dependent signaling impacts on cell adhesion. Although PI 3-kinase and Rho GTPases have potent effects on the actin cytoskeleton, these signals exert diverse effects on cell behavior, including effects on cell growth and apoptosis, that are independent of adhesion.

In the current report, we therefore aimed to test directly whether E-cadherin functions as an upstream receptor for Rac GTPase signaling and to define its functional significance for cell adhesion. To accomplish this, we developed an assay that allowed us to specifically engage E-cadherin homophilic ligation and determine its effects on cell signaling, adhesion, and cell shape.

MATERIALS AND METHODS

Cell Culture—hE-CHO cells were made by stably transfecting a plasmid expressing full-length human E-cadherin (a kind gift of Dr. M. Takeichi, Memorial Sloan-Kettering Cancer Center, New York) and selecting for transfectants with 0.5 mg/ml G418. Parental CHO and hE-CHO cells were maintained in Ham’s F-12 supplemented with 10% fetal bovine serum, nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. To inhibit PI 3-kinase activity, cells were preincubated for 45 min with wortmannin (60 μM) or LY294002 (100 μM); inhibitors were maintained for the duration of the experiments.

Prioritization of Reconstituent Cadherin Ectodomain-Fc Chimeric Protein (hE/Fc)—hE/Fc was purified by protein A affinity chromatography from conditioned media of CHO cells stably expressing a secreted protein consisting of the complete extracellular region of human E-cadherin fused to the Fc region of IgG (a kind gift of Drs. Carien Niessen and Barry Gumbiner, Memorial Sloan-Kettering Cancer Center, New York). After storage, protein samples were buffer-changed into calcium-containing storage buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM CaCl₂, pH 7.4).

Cadherin Cell Adhesion Assays—Coverslips and glass capillaries were coated with hE/Fc (50 μg/ml) and blocked with 10 mg/ml BSA in Hanks’ balanced salt solution plus 5 mM CaCl₂ (HBSS/Ca). For assays, parental CHO and hE-CHO cells were isolated with 0.01% (w/v) crystal-line trypsin (Sigma) in HBSS/Ca.

To assess adhesive activity, we utilized a previously described laminar flow assay that measures adhesion as resistance to detachment by wall shear stress under laminar flow conditions (7, 16). In brief, freshly isolated cells were infused into glass capillaries coated with hE/Fc and allowed to attach to the inner surface of the capillary for 15 min under stasis. Cells were then subjected to progressively increasing buffer flow rates delivered by a syringe pump (Harvard Instruments). Flow rates were increased at 1-min intervals, and the number of cells remaining attached after each interval was counted by phase-contrast microscopy and expressed as a percentage of the initial number of cells attached. To assess contact formation, freshly isolated cells were seeded onto hE/Fc-coated coverslips at a density prohibiting cell-cell contact. Spreading was quantitated by phase-contrast microscopy, expressed as a percentage of the total number of cells counted, and normalized to the positive controls (hE-CHO cells adherent to hE/Fc). Cells were clasped as spread if they were phase gray and possessed dominant lamellipodia.

Plasmids—Wild-type and mutant Rac1 constructs were generously provided by Dr. A. Hall (MRC Laboratory of Cell Biology), Dr. N. Hotchin (University of Birmingham), and Dr. K. Weber (Institut für Prophylaxie, Munich). The mutant transgenes (17, 18) were RacV12 (constitutively active) and RacN17 (dominantly negative). GFP-p21 was a kind gift of Dr. William Gullick (Imperial Cancer Research Fund, London), GFP-TK a gift from Dr. John Hancock (University of Queensland), and GFP-p85 was provided by Dr. A. Hall (MRC Laboratory of Cell Biology), Dr. N. Hotchin (University of Birmingham), and Dr. K. Weber (Institut für Prophylaxie, Munich). All plasmids were expressed by transient transfection using Levelfectamine (Invitrogen) and following the manufacturer’s instructions. Cells were assayed 24 h after transfection.

Antibodies—Primary antibodies were as follows: 1) mouse monoclonal antibody against the cytoplasmic tail of human E-cadherin (Transduction Laboratories); 2) mouse monoclonal antibody HECD-1 against the extracellular domain of human E-cadherin (a kind gift from Drs. M. Takeichi and M. Wheelock); 3) rabbit polyclonal antibody directed against β-catenin (generously provided by Dr. B. Gumbiner); 4) mouse monoclonal antibody 9E10 directed against the Myc epitope tag; 5) rabbit polyclonal antibody against the p85 subunit of PI 3-kinase (Upstate Biomedical Corp.); 6) rabbit polyclonal antibody directed against GFP (a kind gift of Dr. J. D. T. Unwin); and 7) rabbit polyclonal antibody directed against Akt and phospho-Akt (New England Biochemicals). F-actin was identified using Alexa 488-phalloidin (Molecular Probes, Inc., Eugene, OR).

Immunofluorescence Microscopy and Biochemistry—Cells were fixed in 4% paraformaldehyde for 1 h and permeabilized for 5 min in phosphate-buffered saline containing 0.02% (v/v) Triton X-100. Samples were blocked with 5% (w/v) nonfat dried milk (in phosphate-buffered saline) and then incubated sequentially with primary and secondary antibodies, with extensive washes between antibody incubations. Coverslips were mounted in 50% glycerol, 1% p-propyl-gallate in PBS and viewed using a Bio-Rad MRC600 confocal microscope, Bio-Rad Radiance 2000 confocal microscope, or Olympus AX70 microscope and Hamamatsu Orca camera using Metamorph imaging software. Images were assembled for presentation in Adobe Photoshop. For co-immunoprecipitations, cells were lysed in 1% Nonidet P-40 (plus 10 mM Hepes, 150 mM NaCl, 1 mM EDTA, pH 7.4), immunoprecipitated with HECD-1 bound to Protein G beads, and blotted for E-cadherin, β-catenin, or p85. Akt activation was measured by a modification of established methods (19). Briefly, detergent-soluble membrane-enriched cell lysates were collected after low speed centrifugation and then centrifuged at 100,000 × g for 30 min and separated into cytosolic (S100) and membrane-enriched (P100) fractions. Akt was immunoprecipitated from the P100 fraction; complexes were separated by SDS-PAGE and immunoblotted for either Akt or phospho-Akt.

RESULTS

Recombinant E-cadherin Ectodomain Supports Cadherin-specific Adhesion and Contact Formation—To identify cellular events that arose as specific responses to cadherin adhesive ligation, we utilized a recombinant protein (hE/Fc) consisting of the complete ectodomain of human E-cadherin fused to the Fc region of IgG. Substrata adsorbed with hE/Fc supported cadherin-specific adhesion and contact formation (Figs. 1, 4, and 5). CHO cells stably expressing E-cadherin (hE-CHO cells) adhered robustly to glass capillaries coated with hE/Fc, as assessed in laminar flow adhesion assays, which measure the resistance of cells to detachment forces (Fig. 4A). Adhesion of hE-CHO cells was inhibited by cadherin-blocking antibodies as well as by depletion of extracellular calcium,2 while parental CHO cells that express no detectable endogenous E-cadherin3 readily detached from hE/Fc (Fig. 4A). Adhesion to hE/Fc-coated coverslips was accompanied by rapid and vigorous cell spreading, distinguished by the appearance of prominent cadherin-based lamellipodia (Figs. 1, 2, 4, 5).


3 S. Yap, unpublished results.
and 5). Rounded cells had attached after 15 min (Fig. 1C). Lamellipodial protrusions were apparent at 30 min (Fig. 1E) and became more prominent in the succeeding 60 min (Fig. 1G). Staining with Alexa 488-phalloidin revealed prominent bands of F-actin at the leading edges of cadherin-based lamellipodia (Fig. 4C) as well as long actin bundles that spanned the cells.

Adhesion and spreading on hE/Fc were not affected by integrin-blocking peptides (not shown) (7). Nor was adhesion to hE/Fc associated with assembly of focal adhesions. hE-CHO

Fig. 1. Localization of E-cadherin and Rac as cells adhere to cadherin-coated substrata. A, planar substrate assay for cadherin-specific adhesion. Glass surfaces adsorbed with hE/Fc were used as cadherin-specific adhesive substrata. B, phase-contrast image of hE-CHO cells after 90-min adhesion to hE/Fc. C–H, comparison of β-catenin and Myc-Rac1 localization during cell adhesion to hE/Fc-coated substrata. hE-CHO cells transiently expressing Myc-Rac1 were allowed to adhere to hE/Fc-coated substrata for various times before being immunostained for either β-catenin (to mark the cellular cadherin adhesive complex; C, E, and G) or the Myc-epitope tag (D, F, and H). The adhesive interface between the ventral surfaces of cells and hE/Fc-coated substrata was imaged by confocal microscopy. As cells adhere to hE/Fc, both Myc-Rac1 and cellular β-catenin preferentially localize to the protrusive leading edges of the cells (arrows). Bars, 10 μm.
cells adherent to hE/Fc showed diffuse staining for \( \beta_1 \) integrin (Fig. 2B) and cytoplasmic distribution of focal adhesion kinase (FAK; Fig. 2D). In contrast, adhesion of hE-CHO cells to fibronectin induced the clear recruitment of both \( \beta_1 \) integrin (Fig. 2A) and focal adhesion kinase (Fig. 2C) into focal adhesions at the cell surface. Taken together, these data indicated that the adhesive and cell shape changes observed in this assay were specific responses to cadherin ligation. In particular, the appearance of cadherin-based lamellipodia correlated well with adhesion, providing a convenient assay for productive cadherin adhesion.

**Rac1 Accumulates with the Cadherin-Catenin Complex at the Leading Edges of Cadherin-based Lamellipodia**—As a first step to characterizing the relationship between E-cadherin homophilic ligation and Rac in this assay system, we compared the subcellular localization of wild-type Rac1 and the cadherin-catenin complex as cells adhered to hE/Fc-coated substrata. We used \( \beta_3 \)-catenin to identify the cadherin-catenin complex, since in hE-CHO cells, \( \beta_3 \)-catenin is principally found associated with E-cadherin at the plasma membrane (not shown). hE-CHO cells were transiently transfected with wild-type Myc-Rac1, and confocal microscopy was used to image the distributions of the transgene and of endogenous \( \beta_3 \)-catenin at the adhesive interface (i.e. the ventral surfaces of cells adhering to hE/Fc-coated substrata). At 15 min, when cells had first attached, \( \beta_3 \)-catenin was identified at the margins of contact between the cells and hE/Fc-coated substrata, as well as more diffusely in the body of the cells (Fig. 1C). By 30 min, \( \beta_3 \)-catenin was seen accumulating at the margins of lamellipodial protrusions as well as in clusters under the cell body and in lamellipodial proximal to the leading edges (Fig. 1E). Clustering became more prominent by 90 min (Figs. 1G and 4B), when some residual linear \( \beta_3 \)-catenin staining persisted at the leading edges and in ruffles of cadherin-based lamellipodia (Fig. 1G).

In freshly attached cells, Myc-Rac1 (Fig. 1D) appeared to co-accumulate with \( \beta_3 \)-catenin (Fig. 1C) at the margins of adhesive contacts. As cells spread, Myc-Rac1 staining remained most prominent at the leading edges of cadherin-based lamellipodia (Fig. 1F). Strikingly, little Myc-Rac1 was seen at the adhesive interface proximal to the leading edges, despite the presence of cadherin clusters in this region (Fig. 1E). A cytoplasmic pool of Myc-Rac1 was evident by epi-illumination microscopy (Fig. 7B). By 90 min, when cells had fully spread, co-accumulation of Myc-Rac1 and \( \beta_3 \)-catenin persisted in ruffles, but less was evident at leading edges (Fig. 1, G and H). Similar localization at the leading edges of cadherin-based lamellipodia was observed in cells expressing a GFP-Rac1 construct (not shown), indicating that the localization was not due to the epitope tag. Since the leading edges of cadherin-based lamellipodia represent the sites where the margins of adhesive contact were being extended, this suggested that during adhesion Rac principally accumulated at sites where new cadherin contacts were being formed.

**Binding to hE/Fc Activates Rac Signaling Necessary for Cells to Extend Cadherin-based Adhesive Contacts**—To test the hypothesis that E-cadherin ligation can directly activate Rac signaling, we used pull-down assays (11, 20) to identify changes in GTP-Rac that occurred in response to hE/Fc. A GST fusion protein containing the CRIB domain of PAK was used to isolate active, GTP-bound Rac in lysates from cells as they adhered to hE/Fc (Fig. 3A). We observed a consistent rise in GTP-Rac after hE-CHO cells attached to hE/Fc. Characteristically, this was first evident ∼10–15 min after adhesion to hE/Fc, tending to peak at 30 min. Parallel blots showed no differences in the total Rac content of the cell lysates. GTP-Rac levels did not change when cells were plated onto substrata coated with either poly-L-lysine (PLL) or BSA (Fig. 3A). These findings therefore demonstrate that E-cadherin homophilic ligation alone is sufficient to activate Rac signaling.

To assess the functional significance of such cadherin-activated Rac signaling, we then tested the effect of Rac1 mutants on cadherin-based spreading and lamellipodial formation (Fig. 3B). Transient expression of a dominantly inhibitory N17Rac1 mutant very potently inhibited cell spreading on hE/Fc-coated substrata (Fig. 3B). Cells remained rounded after attachment and displayed no cadherin-based lamellipodia. In contrast, expression of a constitutively active (V12) mutant induced cells to form more extensive, circumferential cadherin-based lamellipodia (Fig. 3B) with clear F-actin bands at the leading edges. Therefore, in this assay system, which measures the ability of cells to form specific cadherin contacts, Rac activity was necessary for extension of the adhesive contacts after initial cadherin ligation.

**PI 3-Kinase Signaling Is Necessary for Cadherin-based Adhesion**—PI 3-kinase is a well documented upstream regulator of Rac (21–24) and has recently been identified to associate with E-cadherin (10). To begin to assess the potential role of PI 3-kinase in cadherin signaling, we first tested whether PI 3-kinase activity was necessary for cadherin-specific adhesion. We incubated cells with either wortmannin (60 nM) or LY294002 (100 μM; not shown), which are reasonably specific inhibitors of type I PI 3-kinases when used at these concentrations (24). Wortmannin significantly inhibited E-cadherin adhesion (Fig. 4A) and largely inhibited cadherin-based cell spreading (Fig. 4, B–D). Adhesive strength was substantially reduced although not completely abolished (Fig. 4A). The vast majority of wortmannin-treated cells remained rounded (Fig. 4D), with no detectable lamellipodia (Fig. 4, B and C, insets). LY294002 had identical effects on adhesion and cadherin-based spreading (not shown). Taken together, these data indicated that PI 3-kinase activity, like Rac, was essential both for adhesion and for cells to extend their zones of adhesive contact after binding to hE/Fc.

**E-cadherin Ligation Recruitment PI 3-Kinase to the Cell Surface**—Pece et al. (10) recently reported that the p85 subunit of type IA PI 3-kinase co-immunoprecipitated with E-cadherin as Madin-Darby canine kidney cell contacts were being reestablished after depletion of extracellular Ca\(^{2+}\). We observed a similar phenomenon in hE-CHO cell monolayers (not shown) and hypothesized that this might reflect direct recruitment of...
PI 3-kinase to E-cadherin in response to homophilic ligation. To test this, we sought evidence for a biochemical interaction between E-cadherin and p85 as hE-CHO cells adhered to hE/Fc (Fig. 5A). Only trace levels of p85 were detectable in immunoprecipitates of E-cadherin from freshly isolated hE-CHO cells prior to plating on hE/Fc. However, p85 was readily detected in E-cadherin immunoprecipitates from cells lysed after adhesion to hE/Fc. Since these experiments did not entail changes in extracellular calcium, nor other adhesive interactions, this indicated that PI 3-kinase was recruited to the cadherin-catenin complex as a specific response to homophilic E-cadherin ligation.

We then sought to test if membrane recruitment of p85 by E-cadherin was associated with changes in PI 3-kinase signaling at the membrane. At various times after attachment to hE/Fc, hE-CHO cells were lysed, and the postnuclear supernatants were separated into plasma membrane-enriched (P100) and cytosolic (S100) fractions by differential centrifugation. As an index of PI 3-kinase activity at the membrane, Akt (PKB; Refs. 25 and 26) was immunoprecipitated from the P100 fractions, and the immune complexes were probed for the active, phosphorylated form of Akt (pAkt). As seen in Fig. 5B, adhesion to hE/Fc was associated with a rise in both total Akt recovered from the P100 fraction and in the levels of pAkt identified in the protein complexes. Immunoblotting the P100 fractions alone confirmed membrane recruitment of Akt, but the pAkt signal was weaker in the whole fractions than when Akt was concentrated by immunoprecipitation (not shown). These findings are consistent with the well accepted notion that Akt is both recruited to and rapidly phosphorylated at the plasma membrane in response to PI 3-kinase activation (25–28). Phospho-Akt was not readily detected in the cytosolic (S100) fraction (not shown). Akt and pAkt were detected in P100 fractions from cells adherent to PLL, but nonspecific adhesion was not accompanied by any change in Akt or pAkt levels (Fig. 5B). This indicated that recruitment of PI 3-kinase upon E-cadherin ligation activated PI 3-kinase signaling at the plasma membrane.

While these biochemical studies clearly indicated that PI 3-kinase interacted with E-cadherin, comparison of p85 levels in the co-immunoprecipitates with those in whole cell lysates (Fig. 5A) suggested that only a small proportion of the total cellular pool of PI 3-kinase associated with the cadherin during homophilic adhesion. To characterize where PI 3-kinase was being recruited to the plasma membrane, we transiently expressed GFP-p85 (29) in hE-CHO cells and compared the subcellular localization of the transgene with that of endogenous E-cadherin as cells adhered to hE/Fc. After 30–45 min, when cells had attached and begun to spread, GFP-p85 (Fig. 5C) was found to localize with cellular E-cadherin (Fig. 5D) at the very leading edges of cadherin-based lamellipodia. A large cytoplasmic pool of GFP-p85 was also observed, as has been previously reported with this construct (29), making it difficult to reliably compare localization of the transgene with that of E-cadherin in the body of the cells. However, it was striking to note that cadherin-based lamellipodia were relatively depleted of p85 in the region immediately proximal to the leading edges (Fig. 5C), despite the presence of clusters of cellular E-cadherin (Fig. 5D). As a control, we also plated hE-CHO cells expressing GFP-p85 on PLL-coated substrata. Although cells spread on this nonspecific adhesive ligand (Fig. 5F), GFP-p85 remained largely in the cytoplasmic pool and showed no accumulation at leading edges (Fig. 5E).

**PI3K Signals Are Generated at Newly Forming Cadherin Contacts**—We then sought to test if the PI 3-kinase observed at the leading edges of cadherin-based lamellipodia was indeed
generating a signal at these sites. PIP₃ is the major product of PI 3-kinase signaling at the plasma membrane and binds specifically to the PH domain of Grp-1 (24, 30, 31). We therefore expressed the Grp-1 PH domain as a GFP fusion protein (GFP-Grp1-PH) to specifically identify sites of PIP₃ production during cadherin-based adhesion (Fig. 6) and used confocal microscopy to image the cadherin adhesive interface (the ventral surfaces of cells) in our assay.

After 30–45 min of adhesion to hE/Fc, GFP-Grp1-PH (Fig. 6A) accumulated with cellular E-cadherin (Fig. 6B) at the leading edges of early cadherin-based lamellipodia. Characteristically, we observed a clear band of GFP-Grp1-PH staining at the leading edges, delimited by a zone of weak staining in the cell protrusion proximal to the leading edge, as well as diffuse cytoplasmic staining in the cell body. Interestingly, E-cadherin was also detected at the adhesive interface in this proximal region of lamellipodia where no GFP-Grp1-PH was seen (Fig. 6A). In contrast, GFP-dynamin 1-PH, which provides a specificity control since it binds only very weakly to PIP₃, remained diffusely distributed (Fig. 6, C and D). Although some staining was seen throughout cadherin-based lamellipodia (Fig. 6C), there was not the pattern of preferential leading edge localization seen with GFP-Grp1-PH (Fig. 6A). Furthermore, when cells were allowed to spread on PLL, GFP-Grp1-PH remained largely in the cytoplasm (Fig. 6E), and only limited staining was observed that was diffusely distributed throughout the cell peripheries. As an additional control, to exclude the possibility that leading edge localization was an edge artifact, we expressed a construct consisting of the membrane anchor domain of K-Ras fused to GFP (GFP-tK), a marker that binds to lipids on the cytoplasmic surface of the plasma membrane (32). As shown in Fig. 6F, GFP-tK distributed diffusely throughout the lamellipodia of hE-CHO cells spreading on hE/Fc, with no evidence of leading edge accumulation. Therefore, the leading edge accumulation of GFP-Grp1-PH seen under identical conditions (Fig. 6A) was unlikely to be a simple edge artifact.

These data therefore indicated that a PIP₃ signal was being generated in a spatially confined region of the cadherin contact zones, namely in the nascent E-cadherin contacts at the leading edges of cadherin-based lamellipodia.

PI 3-Kinase Is Upstream of Rac in Cadherin-activated Signaling—The observation that inhibitors of PI 3-kinase signal-
ing as well as dominant negative Rac1 mutants both blocked cadherin-based spreading suggested the possibility that these molecules might functionally interact in cadherin-activated cell signaling. To explore this, we first compared the subcellular localization of Rac and PI 3-kinase during cadherin-based adhesion. Using doubly transfected hE-CHO cells, we observed that GFP-p85 (Fig. 7A) co-localized with wild-type Myc-Rac1 (Fig. 7B) in the nascent contacts at leading edges of cadherin-based lamellipodia. Therefore, both PI 3-kinase and Rac were spatially organized in a manner that might permit a functional interaction between the two molecules.

If PI 3-kinase acted as an intermediary between cadherin ligation and Rac signaling during adhesion, we then reasoned that expression of constitutively active Rac should rescue cadherin-based lamellipodia formation in cells treated with PI 3-kinase inhibitors. Indeed, transient expression of V12Rac1 induced the robust formation of circumferential cadherin-based lamellipodia (Fig. 7F) despite the presence of wortmannin (60 nm) sufficient to totally inhibit spreading of control hE-CHO cells (Fig. 7D and G). Stimulation of Rac signaling activity could therefore functionally overcome the adhesive block induced by wortmannin, consistent with these molecules being in the same signaling pathway.

Therefore, to directly test the hypothesis that PI 3-kinase and Rac interact in a cadherin-activated signaling pathway, we used pull-down assays (11, 20) to determine the effect of PI 3-kinase activity on Rac signaling during cadherin contact and adhesion (Fig. 3A). Treatment with wortmannin at concentrations that effectively inhibited cadherin-based spreading and adhesion did not inhibit the early rise in GTP-Rac activity seen at 15 min. However, after 30 min, when GTP-Rac levels in control cells (Fig. 3A) were peaking, GTP-Rac levels in wort-
mannin-treated cells showed a significant fall from the levels at 15 min (Fig. 3A), indicating that wortmannin foreshortened the duration of cadherin-activated Rac signaling. This indicated that PI 3-kinase activity was necessary for full stimulation of Rac by E-cadherin homophilic ligation.

**DISCUSSION**

Cadherin cell adhesion molecules are fundamental mediators of cell-cell recognition in metazoan organisms. Homophilic ligation between cadherin ectodomains presented on opposing cell surfaces is postulated to initiate a cascade of cellular responses that ultimately determine whether or not cells form cohesive associations with one another. While contact between cells undoubtedly activates intracellular signaling, the precise role of cadherins in this process is far from comprehensively understood. In this study, we sought to identify intracellular signals that were activated as direct downstream targets of E-cadherin homophilic ligation to control cadherin-based cellular adhesion. Using a recombinant E-cadherin adhesive ligand, we found that cadherin homophilic ligation to hE/Fc was sufficient to activate the Rac GTPase signaling pathway. Adhesion to PLL did not activate Rac, indicating that signaling was a specific response to cadherin homophilic ligation and not a nonspecific consequence of cell spreading. Recent reports that Rac signaling is activated as cultured cells form E-cadherin-dependent contacts with one another (12) could not exclude the possibility that Rac was activated by cadherin-dependent juxtapacin signals. Our current work now clearly demonstrates that E-cadherin is itself an upstream receptor for Rac, which...
signals upon homophilic ligation. Since *Xenopus* C-cadherin has also recently been shown to directly activate Rac (11), it will now be important to assess whether Rac is a downstream signal activated by all classical cadherins.

Our data further identify PI 3-kinase as an intermediary component of the E-cadherin-activated Rac signaling pathway. 1) Inhibitors of PI 3-kinase blocked E-cadherin-based adhesion and lamellipodial extension as effectively as dominant-negative Rac1 mutants. This suggested that PI 3-kinase and Rac signaling were functionally connected elements of the cellular response that allows homophilic ligation of cadherin ectodomains to control cell shape and stabilize adhesive contacts. 2) Expression of a CA Rac1 mutant rescued the inhibition of cadherin-based lamellipodia induced by wortmannin. Therefore, activation of Rac signaling was capable of substituting for loss of PI 3-kinase signaling during cadherin-based adhesion. 3) E-cadherin-activated Rac signaling was significantly shortened in cells treated with wortmannin (60 nM) before being allowed to adhere to hE/Fl-coated substrata. The ability of cells to form cadherin-based lamellipodia was assessed by immunofluorescence microscopy after co-staining for the epoxide tag and F-actin and quantified as the percentage of cells displaying clear lamellipodial extension.

PI 3-kinase has been identified as an upstream regulator of Rac in models of growth factor signaling (21–24). Our data, taken with a recent study of cell-cell contact formation in Madin-Darby canine kidney cells (12), indicate that this pathway also plays a role in E-cadherin-activated cell signaling. However, we also identified an early, previously unidentified (12), component of cadherin-activated Rac signaling that was not blocked by wortmannin and which may therefore be independent of PI 3-kinase. Consistent with this, the peak of pAkt accumulation at the plasma membrane occurred ∼30 min after cells adhered to hE/Fe and coincided with the wortmannin-sensitive phase of cadherin-activated Rac signaling. Potentially two distinguishable pathways may then link E-cadherin to Rac, the molecular details of which remain to be characterized. The p85 subunit of PI 3-kinase can interact with β-catenin both in vivo and in vitro (33, 34). It is therefore attractive to postulate that β-catenin may recruit PI 3-kinase to the cadherin-catenin complex upon ligation, leading to the generation of PIP_3, that we observed in early cadherin contacts. PIP_3 has been reported to recruit exchange factors for GTPases (33), such as Tiam-1 (35), a potential pathway for PI 3-kinase to potentiate Rac activity downstream of E-cadherin. Additionally, p120^Caten^ has been reported to activate Rac signaling (36), making this protein a possible candidate to mediate PI 3-kinase-independent activation of Rac by E-cadherin.

Given the pleotropic effects of PI 3-kinase and Rac (24, 37), what function may be served by these E-cadherin-activated
signals? Our data implicate this signaling pathway in the cellular adhesive response to initial cadherin homophilic recognition. A characteristic feature of productive cadherin recognition is the expansion of the zones of contact between cells, from punctate initial contacts to continuous linear regions of contact (3, 4, 38). This is replicated in our hE/Fc assay system, since initial adhesive binding is rapidly followed by cell spreading, a process that entails cadherin-based lamellipodial protrusion of the cell margins, which expand the zones of adhesive contact. Importantly, both PI 3-kinase inhibitors and expression of a DN Rac1 mutant blocked the formation of cadherin-based lamellipodia, preventing extension of the initial zones of adhesive contact. Similarly, PI 3-kinase inhibitors prevent cadherin-based cell-cell contacts from forming, while DN Rac mutants disrupt epithelial integrity (39–41). Taken together, we propose that PI 3-kinase and Rac form a signaling pathway that is activated by homophilic ligation of E-cadherin in nascent adhesive contacts, leading to extension of these zones of contact and ultimately to productive cell-cell recognition.

The extension of nascent cadherin contacts to form stable regions of cell-cell cohesion is likely to involve coordinated changes in cadherin distribution and cytoskeletal organization at the cell surface. Of note, complex changes in actin cytoskeletal activity accompany the formation of cadherin-based cell-cell contacts (4, 38). We recently found that E-cadherin can interact physically with the Arp 2/3 actin nucleation complex and identified the leading edges of cadherin-based lamellipodia as principal sites for actin assembly. Insofar as Rac is well known to activate actin assembly, we propose that E-cadherin-activated Rac signaling serves to promote adhesive stabilization by stimulating actin assembly to convert limited nascent contacts into more expansive zones of adhesion (Fig. 8).

Interestingly, several observations suggested that cadherin-directed signaling was restricted to specific regions of adhesive contact. Thus, we found that PIP3 was generated at the very leading edges of cadherin-based lamellipodia, where GFP-p85 co-localized with the cadherin-catenin complex. Similarly, although we did not image GTP-loading of Rac, we observed that at the cadherin adhesive interfaces Rac-1 co-accumulated with E-cadherin principally at leading edges. Strikingly, in regions of cadherin-based lamellipodia proximal to the leading edges, we observed relatively little PIP3, GFP-p85, or Rac, despite the presence of cadherin clusters at the adhesive interface. Since cadherins cluster upon homophilic engagement (7, 8, 42), these observations suggest strongly that not all ligand-occupied cadherin molecules engage in signaling to PI 3-kinase or Rac. Instead, cadherin signaling in this pathway may be confined to regions of the cell where new contacts are forming and cadherin molecules are in the process of undergoing homophilic ligation. This would be consistent with increasing evidence for strict subcellular compartmentalization of 3-phosphoinositide (43) and Rac signaling (44). Identifying the molecular mechanisms responsible for such spatio-temporal restriction will be an interesting challenge for the future.

In conclusion, we propose that the E-cadherin-activated Rac signaling pathway described in this report acts as a local, membrane-based regulator that controls the adhesive response to initial cadherin homophilic ligation (Fig. 8). We envisage that the spatial restriction of cadherin-activated Rac signaling allows actin assembly to be directed specifically to nascent adhesive sites. In the planar adhesion assay that we utilized, these sites are represented by the leading edges of cadherin-based lamellipodia. In native cell-cell contacts, such cadherin-activated signaling would occur in the punctate or filopodial contacts that cells first make with one another, thereby directing actin assembly to drive the extension of cell surfaces upon one another, expanding the zones of intercellular contact. This model does not exclude potential roles for other cell signals; indeed, it is probable that following initial homophilic ligation further signals may be initiated to reinforce adhesion and remodel the actin cytoskeleton (45, 46). Instead, cadherin-activated Rac signaling is likely to be an initial point in a cascade of signaling events that are spatially and temporally regulated in the process of cadherin-based cell-cell recognition.

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REFERENCES
E-cadherin Homophilic Ligation Directly Signals through Rac and Phosphatidylinositol 3-Kinase to Regulate Adhesive Contacts
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