Immediate-Early Signaling Induced by E-cadherin Engagement and Adhesion*\textsuperscript{**}

Received for publication, June 25, 2007, and in revised form, November 15, 2007 Published, JBC Papers in Press, December 17, 2007, DOI 10.1074/jbc.M705209200

Tomas D. Perez\textsuperscript{+1}, Masako Tamada\textsuperscript{+2}, Michael P. Sheetz\textsuperscript{§}, and W. James Nelson\textsuperscript{+§3}

From the Departments of \textsuperscript{+}Molecular and Cellular Physiology and \textsuperscript{§}Biological Sciences, Stanford University, Stanford, California 94305-5430 and the \textsuperscript{§}Department of Biological Sciences, Columbia University, New York, New York 10027

Epithelial cell-cell interactions require localized adhesive interactions between E-cadherin on opposing membranes and the activation of downstream signaling pathways that affect membrane and actin dynamics. However, it is not known whether E-cadherin engagement and activation of these signaling pathways are locally coordinated or whether signaling is sustained or locally down-regulated like other receptor-mediated pathways. To obtain high spatiotemporal resolution of immediate-early signaling events upon E-cadherin engagement, we used laser tweezers to place beads coated with functional E-cadherin extracellular domain on cells. We show that cellular E-cadherin accumulated rapidly around beads, reaching a sustained plateau level in 1–3 min. Phosphoinositides and Rac1 co-accumulated with E-cadherin, reached peak levels with E-cadherin, but then rapidly dispersed. Both E-cadherin and Rac1 accumulated independently of Rac1 GTP binding/hydrol-

ysis, but these activities were required for Rac1 dispersal. E-cadherin accumulation was dependent on membrane dynamics and actin polymerization, but actin did not stably co-accumulate with E-cadherin; mathematical modeling showed that diffusion-mediated trapping could account for the initial E-cadherin accumulation. We propose that initial E-cadherin accumulation requires active membrane dynamics and involves diffusion-mediated trapping at contact sites; to propagate further contacts, phosphatidylinositol 3-kinase and Rac1 are transiently activated by E-cadherin engagement and initiate a new round of membrane dynamics, but they are subsequently suppressed at that site to allow maintenance of weak E-cadherin mediated adhesion.

Members of the cadherin family of Ca\textsuperscript{2+}-dependent cell adhesion proteins initiate cell adhesion by \textit{trans} pairing of cad-

herins on opposing cell surfaces (1, 2) and then stabilize adhe-

sion by accumulating in the plane of the membrane (3–6) by a process thought to involve the actin cytoskeleton. Live cell analysis of asynchronous cell-cell contacts indicates that initial interactions occur opportunistically between opposing lamellipo-

dia formed as cells crawl. Subsequent expansion of the con-

act occurs through interactions between additional lamellipodia confined to the perimeter of the contact, whereas lamellipodia activity generally decreases within the contact (6–8). However, many questions remain. It is unclear whether E-cadherin engagement results in or is the consequence of downstream activation of membrane and actin dynamics, whether E-cadherin accumulation is an active process driven by actin assembly, or how lamellipodia activity is locally regulated during cell-cell adhesion.

Actin and membrane dynamics mediated by Rho family GTases have been implicated in regulating lamellipodia activity and cadherin adhesion (5, 9–12). Reports show that Rac1 is activated upon E-cadherin adhesion (9, 13, 14) and co-localized with E-cadherin after 30 min (11), and that products of PI\textsuperscript{3-kinase (8, 15–17), PI(3,4)-bisphosphate and PI(3,4,5)-triphasphate (PI phosphates) that may recruit guanine exchange factors and activate Rac1 (10–12), are also localized to new cell-cell contacts. Analysis of intracellular sites of Rac1 activation during \textit{de novo} cell-cell adhesion revealed that active Rac1 is mainly localized to the periphery of the spreading cell-cell contact (18) where lamellipodia and PI 3-kinase activity (measured by localization of PH-Akt-GFP) are highest (8). However, it remains unclear whether activation and accumulation of these signaling proteins are coincident with E-cadherin engagement. It was thought that Rac1 activation induces interactions between cadherins and the cortical actin cytoskeleton, although more recent direct studies of the binding of actin to the cadherin-catenin complex revealed that the interaction is not direct (19, 20). These new results raise the question of whether actin polymerization and dynamics are important for initiating E-cadherin contacts or stabilizing/clustering E-cadherin adhesions or both.

A difficulty in determining the relationships between initial E-cadherin accumulation, activation of Rac1, and the distribution of the actin cytoskeleton in cell-cell adhesion is the analysis of asynchronous adhesions between migrating cells. Under...
these conditions, it is difficult to determine unambiguously whether changes in the organization of E-cadherin, Rac1 and associated signaling proteins, and actin are hierarchical, coordinate, or coincidental and to what extent actin dynamics associated with ongoing cell migration obscures its role(s) in E-cadherin engagement and accumulation. Moreover, stimulation of different cell adhesion proteins and juxtaeocrine signaling molecules can further complicate understanding downstream effects of E-cadherin engagement. One approach to overcoming these problems has been to locally activate E-cadherin with beads coated with the extracellular domain of E-cadherin (16, 21). Previous studies using this approach, however, generally examined protein distributions in fixed samples after beads had attached for 15–90 min to nonadherent cells expressing exogenous E-cadherin (17, 21–24). Because of the long time frame of bead attachment and the use of fixed samples, the results do not provide direct insight into the spatiotemporal regulation of initial events involving E-cadherin engagement and local recruitment of signaling proteins and actin organization. To overcome these problems, we used laser tweezers to place individual beads coated with functional E-cadherin extracellular domain (25) (Ecad-bead) onto lamellipodia of single MDCK epithelial cells, and then protein dynamics were immediately and rapidly imaged for very short times. Our results show for the first time that E-cadherin engagement results in co-ordinate but transient recruitment of Rac1 and PI phosphates. Significantly, we find that actin reorganization around sites of E-cadherin engagement is erratic and not co-ordinate with E-cadherin recruitment. Our results indicate a dynamic model of how cell adhesion is initiated by transient accumulation of Rac1 upon E-cadherin engagement to cause further E-cadherin accumulation on active membranes and subsequently a loss of Rac1 to maintain weak E-cadherin adhesion on quiescent membranes.

**EXPERIMENTAL PROCEDURES**

**Tissue Culture**—MDCK cells were maintained in Dulbecco’s modified Eagle’s medium with 1g/liter sodium bicarbonate and 1g/liter glucose supplemented with 10% fetal bovine serum and penicillin, streptomycin, and kanamycin. Prior to imaging experiments, the MDCK cells were replated and grown for 1 day at low density to minimize cell-cell contacts.

**Plasmid Construction and Transfection**—Ecad-RFP was made by replacing the enhanced GFP coding sequence from pEGFP-E-cadherin (20) with the coding sequence for tandem dimer DsRed (26), a mutated DsRed variant that undergoes intramolecular dimerization. Coding sequences for PH-Akt-GFP and GFP-Rac1WT (8) were subcloned into pIRES-puro3, and actin-GFP was constructed as previously described (20). For transfections, the cells were plated in 35-mm2 or 6-well dishes at 106 cells on coverslips for at least 1 h before imaging. 20 μl of Ecad/Fc suspension were washed twice in 1 ml bead buffer, resuspended in 20 μl of bead buffer, vortexed for 30 s, and sonicated for ~15 s.

**Fluorescence Time Lapse Imaging Experiments**—Low density cultures of MDCK cells were trypsinized, and 0.5–1.0 × 106 cells were plated on 22-mm square collagen-coated coverslips in 35-mm dishes. The cells were allowed to attach and spread on coverslips for at least 1 h before imaging. 20 μl of Ecad/Fc-coated beads were mixed with 400 μl of phenol red-free Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) supplemented with penicillin, streptomycin, and kanamycin and with 10% fetal bovine serum (Atlas Biologicals) (imaging medium). If used, 0.2 μM cytochalasin D (Sigma-Aldrich) or 20 μM LY294002 (LY; Calbiochem) was added to the imaging medium in Me2SO; as a control for the Me2SO vehicle, imaging was also performed in the presence of Me2SO only. A coverslip with cells was fixed to a holder, and imaging medium with beads was added. Imaging was performed on an Olympus IX81 microscope controlled with SimplePCI software (Compix, Inc.). Fluorescence excitation was provided by a Coherent Innova 70C ion laser emitting at 488 nm at ~70 milliwatts and 560 nm at ~18 milliwatts. A Coherent 890 laser outputting 800 nm at ~180 milliwatts was used for the laser tweezers. Trapped beads were placed on cells and held for 15 s. If the bead stuck, time lapse imaging was started at 10-, 20-, or 30-s intervals. The beads were imaged up to 15 min unless E-cadherin accumulated at the beads and then only for a maximum of 30 min.

**Data Processing and Analysis**—Tiff stacks of RFP, GFP, and brightfield were assembled and processed using ImageJ software (National Institutes of Health). The brightfield image was used to position a circular region of interest around the bead at each time point. The mean fluorescence intensity in the region of interest in the corresponding RFP and GFP stacks was measured. The region of interest was expanded radially around the center by 5 pixels, and the region immediately around the bead was measured as background. There was less than 2-fold difference in the bead area and background area. Raw, background-subtracted intensities were analyzed in Prism (Graphpad Software) or first linearly interpolated in Matlab (The MathWorks, Inc.) to 10 s before analysis. Signal intensities of each trace were normalized to 0 and 100% for the minimum and maximum intensity, respectively, and individual intensity traces were aligned to the peak of E-cadherin accumulation. Individual time traces were averaged to compute the standard accumulation profile for each protein.
Protein Dynamics at E-cadherin Contacts

The effects of cytochalasin D on the proportion of beads sticking to cells and accumulating Ecad-RFP were compared using Wald Chi-square statistics, and standard errors for the proportions and the difference of proportions were compared using the profile likelihood method. These calculations were performed using the GENMOD procedure of the SAS statistical package.

To compare the effects of LY or Rac1 mutant expression with controls, Mann-Whitney two-tailed t tests were used in all cases except for PH-Akt-GFP accumulation. Because PH-Akt-GFP localization could be expected to decrease upon LY treatment (as shown by us in Ref. 8), a one-tailed t test was used.

The peak accumulation intensity was determined as a percentage of increase of the peak intensity over the background cell/cytoplasmic intensity (termed cell bg, determined by measuring the fluorescence intensity in the cell outside of the contact site). In the frame showing the highest accumulation intensity for each protein, the area average intensity at the bead (peak intensity) was measured in regions adjacent to the bead. The percentage of increase was calculated by: \( \frac{\text{[peak intensity] (cell bg)}}{\text{[cell bg]}} \times 100 \).

K-means clustering was performed in R (R Project for Statistical Computing). The logs of the accumulation times for Ecad-RFP were clustered into two groups. The variance of the set assuming two means was determined by the following,

\[
\sigma^2 = \frac{\sum_{i=1}^{n} (X_i - \mu)^2 + \sum_{j=1}^{m} (Y_j - \mu)^2}{n + m - 2} \quad \text{(Eq. 1)}
\]

where \( X \) and \( Y \) are the two clusters, \( n \) and \( m \) are the sizes of the clusters, and \( \mu \) is the mean of the respective cluster. The variance assuming one mean was calculated by the following,

\[
\sigma^1 = \frac{\sum_{i=1}^{n} (X_i - \mu_0)^2 + \sum_{j=1}^{m} (Y_j - \mu_0)^2}{n + m - 1} \quad \text{(Eq. 2)}
\]

where \( \mu_0 \) is the mean of both clusters. The likelihood ratio (LR) determined by

\[
LR = \frac{\sigma^2}{\sigma^1} \quad \text{(Eq. 3)}
\]

would reject the model of a single mean according to a chi-square distribution with one degree of freedom.

Calculation of E-cadherin Accumulation by Diffusion—Diffusion-limited accumulation of E-cadherin was calculated in MatLab. Diffusion across an area of uniform E-cadherin concentration (62, 87, or 400 per \( \mu m \)) with a radius of 8 \( \mu m \) to perfectly absorbing sphere with radius 0.5 \( \mu m \) for times up to 280 s was calculated using the built-in routines for solving partial differential equations, in this case, the diffusion equation \( \nabla \cdot \sigma \nabla C = D \times \nabla^2 C. \) Calculation of the bead binding capacity was performed based on the manufacturer’s specified binding capacity for human IgG for a specific lot of beads with a specified average diameter. The molar equivalent of E-cad/Fc protein was doubled to account for the two E-cadherin extracellular domains present in the dimeric E-cad/Fc protein.

RESULTS

Placement of Ecad beads by laser trap on cells allowed us to precisely determine the timing and location of initiation events involving E-cadherin-mediated engagement by rapidly imaging fluorescent-tagged cellular proteins accumulating around the Ecad-bead. In all cases, a single bead was placed and analyzed per cell (\( n = 94 \)), and imaging was performed for 15 min and then continued for up to 15 min more if accumulation of cellular E-cadherin was detected; bead endocytosis did not occur during this short time course.

We used E-cadherin fused to tandem dimer DsRed (26) (Ecad-RFP) (Fig. 1, A and B) to mark the distribution of cellular E-cadherin around the bead. Ecad-RFP accumulated rapidly around Ecad-beads within 40–200 s of attachment of the bead on a lamellipodium; the highest fluorescence was often observed on the side of the bead facing the leading edge of the cell, although accumulation was considered specific only if fluorescence accumulated completely around the bead. Similar results were obtained with \( \alpha \)-catenin-GFP (supplemental Fig. S1), indicating that the accumulation of Ecad-RFP represented the distribution of the E-cadherin-catenin complex. Note also that both of these RFP- and GFP-tagged proteins accumulated and reached a plateau level that was maintained subsequently, indicating little or no photobleaching of either RFP or GFP over the time course of the experiment.

Because the time of onset of E-cadherin accumulation varied, Ecad-RFP fluorescence intensity around individual Ecad-beads was normalized to the peak intensity level (100%) and aligned according to the peak accumulation of Ecad-RFP (set to \( t = 0 \)). Accumulation times were measured, and K-means clustering was used to assign each accumulation time into groups. A likelihood ratio test revealed two statistically significant, independent fast and slow rates of E-cadherin accumulation (Fig. 1C). An average trace showed mean accumulation times of 60 s for the fast rate and 180 s for the slow rate. In the case of slow accumulations, there appeared to be an early, slow process that was followed by quicker accumulation similar to that of the “fast” rate. At present the significance of these different rates of E-cadherin accumulation are unknown, although they may reflect differences in local membrane activity (see below). Upon reaching a maximal level, Ecad-RFP persisted around the Ecad-bead. That these events were specific to E-cadherin engagement was confirmed by examining laser trap placed bovine serum albumin-coated beads, which attached to lamellipodia but did not induce E-cadherin or \( \alpha \)-catenin accumulation (supplemental Fig. S1). Cells expressing Ecad-RFP and GFP alone were also used to confirm that the accumulation of proteins around Ecad beads was specific because GFP alone did not accumulate around beads (supplemental Fig. S1). Occasionally, GFP was diffusely localized around beads in a pattern distinct from the specific, focused accumulation of Ecad-RFP and other GFP-tagged proteins; in these cases, GFP accumulation appeared to be due to an increase in cytoplasmic volume.
PI 3-kinase and Rac1 are thought to be involved in regulating E-cadherin accumulation at cell-cell contacts (9, 11, 12, 15, 16, 27, 28). The regulatory subunit of PI 3-kinase localizes to cell-cell contacts and associates with E-cadherin/β-catenin (29), and the PH domain of Akt, which binds lipid products of PI 3-kinase (30), localizes to newly forming cell-cell contacts (8). Therefore, in the present studies we analyzed the accumulation of PH-Akt-GFP as a marker for active PI 3-kinase (8).

We observed precise co-accumulation of Ecad-RFP with PH-Akt-GFP and GFP-Rac1WT. The time traces of PH-Akt-GFP and GFP-Rac1WT accumulation were separated according to whether Ecad-RFP accumulated at the slow or fast rate. Both PH-Akt-GFP and GFP-Rac1WT accumulated in parallel with rapid Ecad-RFP accumulation and with the quick phase that occurred later in the slow Ecad-RFP accumulation (Fig. 1D). Strikingly, peak levels of PH-Akt-GFP and GFP-Rac1WT accumulation were separated according to whether Ecad-RFP accumulated at the slow or fast rate. Both PH-Akt-GFP and GFP-Rac1WT accumulated in parallel with rapid Ecad-RFP accumulation and with the quick phase that occurred later in the slow Ecad-RFP accumulation (Fig. 1D). Strikingly, peak levels of PH-Akt-GFP and GFP-Rac1WT accumulation were separated according to whether Ecad-RFP accumulated at the slow or fast rate. Both PH-Akt-GFP and GFP-Rac1WT accumulated in parallel with rapid Ecad-RFP accumulation and with the quick phase that occurred later in the slow Ecad-RFP accumulation (Fig. 1D).

Averaged PH-Akt-GFP time traces observed with fast rate Ecad-RFP accumulation showed little difference from those with the slow Ecad-RFP accumulation (Fig. 1D). A small difference, however, could be detected between the averaged GFP-Rac1WT time traces observed with fast and slow E-cadherin accumulations. GFP-Rac1WT accumulation at a slower rate during slow E-cadherin accumulation; however,
Protein Dynamics at E-cadherin Contacts

FIGURE 2. Effect of PI 3-kinase inhibition on protein accumulation at E-cadherin contacts

A and B. fast (A) or slow (B) accumulation kinetics of Ecad-RFP in the presence (+LY) or absence (control) of 20 μM LY294002. Averaged traces with S.E. control: as in Fig. 1C. +LY: fast, n = 4 – 8; slow, n = 3 – 4.

C. GFP-Rac1WT accumulation traces are divided according to corresponding fast or slow Ecad-RFP traces. GFP-Rac1WT accumulation in the presence of LY aligns with the untreated slow GFP-Rac1WT profile. The profile could not be significantly distinguished from fast GFP-Rac1WT accumulation. Untreated, slow: n = 5 – 7; untreated, fast: n = 2 – 5; +LY: n = 4 – 5. D. PI 3-kinase inhibition does not affect the level of Ecad-RFP or GFP-Rac1WT accumulation. The peak accumulation intensities of Ecad-RFP, PH-Akt-GFP, and GFP-Rac1WT were determined in each movie of cells treated with (+LY) or without (−LY) PI 3-kinase inhibitor. Analysis of vehicle-only control (dimethyl sulfoxide, DMSO) was performed on E-cad-RFP and PH-Akt-GFP expressing cells only. Peak accumulation measured from cells expressing GFP alone is shown for comparison. The peak accumulation was normalized to the expression level by dividing by the cytoplasmic background fluorescent intensity adjacent to the bead-contact. The mean with S.E. is shown;

n for Rac − LY = 10, Rac + LY = 7; PH − LY = 10, PH + LY = 9, PH + MeSO = 5; E-cad − LY = 21, E-cad + LY = 10, E-cad + MeSO = 5; GFP = 5. t test between GFP and indicated sets: *, p < 0.05; **, p < 0.01; ***, p < 0.005.

t test between PH − LY and PH + LY, #, p < 0.001, one-tailed t test.

because its accumulation began earlier with respect to the peak E-cadherin accumulation, both averaged GFP-Rac1WT time traces show maximum accumulation coincident with the peak of Ecad-RFP.

Actin and active membrane dynamics are thought to be involved in cadherin-mediated cell-cell adhesion between migrating cells (3, 6, 8, 22, 23, 31, 32), although cadherin/catenin-actin interactions are not direct (19, 20), and Rac1 is a well characterized regulator of actin polymerization and lamellipodia formation in migrating cells (33). Because previous studies of cell-cell adhesion were performed on migrating cells, it has been difficult to distinguish actin dynamics involved in E-cadherin engagement and cell movements. Therefore, we analyzed the distribution of actin-GFP and Ecad-RFP around Ecad-beads. The normalized fluorescence intensity time traces of actin-GFP were aligned with the peak of Ecad-RFP accumulation (Fig. 1F). Although the accumulation of Ecad-RFP correlated with an increased likelihood of actin accumulation around the Ecad-bead, the level of actin-GFP accumulation was highly variable with no evidence of periodicity (supplemental Fig. S2); note that high peaks of actin fluorescence intensity occurred up to 40 s before Ecad-RFP peaked. In addition, actin accumulation around the Ecad-beads was not stable with some spikes lasting less than 20 s. The averaged, normalized fluorescence intensity of actin also show flat profiles around small peaks at t = 0, indicative of uncorrelated increases and decreases of actin-GFP intensity (Fig. 1F). Arp3 has also been reported to accumulate at cell-cell contacts (23), but we found only inconsistent accumulation at early time points (supplemental Fig. S2).

Given the surprisingly disordered nature of actin accumulation at E-cadherin contact sites, we considered whether E-cadherin accumulation was independent of actin filament organization. Therefore, we analyzed Ecad-RFP accumulation in cells treated with 0.2 μM cytochalasin D. Under this condition, filamentous actin-GFP was decreased, the cells began to round-up, and lamellipodia activity was greatly reduced. Although actin filaments were disrupted, we detected no difference in Ecad beads adhering to cytochalasin D-treated cells compared with control cells (58 ± 8% versus 44 ± 8% for cells untreated or treated with cytochalasin D, respectively; p = 0.18). However, Ecad-beads placed on these cells did not accumulate Ecad-RFP during the course of imaging (38 ± 7% for untreated versus none for treated cells). Taken together, these results show that actin levels fluctuate in the vicinity of E-cadherin engagement, but actin does not stably accumulate at contacts, consistent with the lack of direct binding of actin to the cadherin-catenin complex (19, 20). However, actin filament organization may play a role in local membrane dynamics that mobilize E-cadherin around the point of contact (see “Discussion”).

To further examine roles of PI 3-kinase and Rac1 in E-cadherin accumulation, cells stably expressing Ecad-RFP and either PH-Akt-GFP, GFP-Rac1WT, or actin-GFP were treated with the PI 3-kinase inhibitor LY at a concentration previously shown to completely inhibit PI 3-kinase activity at cell-cell contacts as measured by PH-Akt-GFP localization (8). LY inhibited the accumulation of PH-Akt-GFP at bead contact sites (supplemental Fig. S3), showing that accumulation of PH-Akt-GFP at Ecad beads is dependent on PI 3-kinase activity. Ecad-RFP accumulated at the same slow and fast rates and persisted around Ecad-beads in the presence or absence of LY (Fig. 2, A and B). LY also did not affect the organization of actin-GFP around Ecad-beads (supplemental Fig. S3).

Because some Rac1 guanine exchange factors require PI phosphates for localization and activation, it is thought that local activation of PI 3-kinase at E-cadherin contacts localizes...
and activates Rac1. Therefore, accumulation of GFP-Rac1WT was analyzed in the presence of the PI 3-kinase inhibitor. GFP-Rac1WT accumulated around Ecad-beads in the presence of LY. With the exception of one event, GFP-Rac1WT accumulation in the presence of LY corresponded to fast Ecad-RFP accumulation. We averaged the majority of events and compared that with the average fluorescence time traces of GFP-Rac1WT observed with fast and slow Ecad-RFP accumulations from untreated cells (referred to as fast and slow Rac1 accumulation, respectively; Fig. 2C). Because PI 3-kinase activity could mediate localization of Rac1 to contact sites, we assessed the accumulation rate of GFP-Rac1WT in the presence of LY. Ecad-RFP accumulated at the fast rate, but GFP-Rac1WT appeared to accumulate more like the slow rate of Rac1 in untreated cells; however, there was not a statistically significant difference from the fast rate of Rac1 accumulation (Fig. 2C, inset). In addition, we did not detect differences in the subsequent dispersal of GFP-Rac1WT after peaking in the presence and absence of LY (Fig. 2C). We determined the percentage of increase of the peak accumulation intensity of Ecad-RFP and GFP-Rac1WT with respect to the cellular background level (see "Experimental Procedures"). The mean peak accumulations of Ecad-RFP and GFP-Rac1WT in the presence or absence of LY were not significantly different, whereas the mean peak accumulation of PH-Akt-GFP decreased from 29.2 ± 7.8 to 5.8 ± 1.1% in the presence of LY, demonstrating that PI 3-kinase was inhibited (Fig. 2D). PH-Akt-GFP accumulation in the presence of LY was similar to the background fluorescence measured for GFP alone. The addition of Me2SO vehicle alone had no effect on either Ecad-RFP or PH-Akt-GFP accumulation around Ecad beads. These results show that PI 3-kinase activity is not required for either E-cadherin or Rac1 accumulation (see also Refs. 11 and 14) or the fast or slow rates of E-cadherin accumulation. It remains possible, however, that PI 3-kinase activity is required for downstream events such as increasing membrane activity at the edges of the expanding contact (see below) or recruitment of other cell-cell adhesion proteins and vesicle targeting machinery to the cell-cell contact necessary to expand the contact.

We tested directly whether E-cadherin accumulation required active, GTP-bound Rac1. MDCK cells stably expressing Ecad-RFP were transiently transfected with either dominant negative GFP-Rac1N17 or constitutively active GFP-Rac1V12. Ecad-RFP accumulated around Ecad-beads in cells expressing GFP-Rac1N17 (Fig. 3A). GFP-Rac1N17 also accumulated at contact sites with kinetics similar to those of GFP-Rac1WT (Fig. 3B). However in the case of slow accumulation, GFP-Rac1N17 did not disperse after peaking (mean intensity, ~75% after >120 s following completion of accumulation), although GFP-Rac1N17 dispersal was observed after fast accumulation; recall that GFP-Rac1WT dispersed during both slow and fast accumulation. We did not detect differences in the rates of fast or slow Ecad-RFP accumulation in the presence of GFP-Rac1N17 (Fig. 3D and Table 1), and the peak levels of Ecad-RFP in the presence of GFP-Rac1WT or GFP-Rac1N17 (Fig. 3E) were also not significantly different (two-tailed t test, p = 0.14). Thus, GFP-Rac1N17 affected neither the rate nor the absolute level of E-cadherin accumulation. These results appear to contradict previous studies showing that Rac1N17 disrupted cell-cell contacts (8, 9, 34). However, it is important to note that we examined only very early E-cadherin-mediated adhesion events and not adhesion involving other proteins or long term adhesion that might be susceptible to Rac1N17 expression. The rate and maximal level of Ecad-RFP accumulation were not affected by expression of GFP-Rac1V12 except that it accumulated predominantly at the fast rate (Fig. 3D). In addition, GFP-Rac1V12 had an identical accumulation profile as that of GFP-Rac1WT during fast accumulation (Fig. 3C). We conclude that Rac1 recruitment to E-cadherin sites is independent of GTP binding but appears to require GTP binding and/or hydrolysis for dispersal. The rate of E-cadherin recruitment can be altered by increasing the level of active Rac1-GTP but is unaffected by depleting Rac1-GTP.

**DISCUSSION**

By examining individual cadherin clustering events at high temporal and spatial resolution, we have for the first time directly quantified the kinetics of E-cadherin, PI 3-kinase, and Rac1 distributions immediately following E-cadherin engagement. Previous studies have examined cell-cell adhesion between asynchronous contacts formed by migrating cells (6–8) where it is difficult to specify the timing and location of initial E-cadherin engagement and distinguish roles of actin and membrane dynamics with those involved in cell migration. Alternatively, protein distributions have been analyzed around Ecad beads in fixed samples after very long periods of bead attachment (17, 21–23) when immediate and dynamic changes in protein distributions could not be examined.

We showed that E-cadherin engagement caused the local recruitment and concentration of PI 3-kinase activity (as measured by the distribution of PH-Akt-GFP) and Rac1, but not actin. However, E-cadherin accumulation itself was dependent on actin filament organization but independent of PI 3-kinase activity or Rac1 accumulation. How then does E-cadherin accumulate? Because we know from this study the amount and rate of accumulation of cadherin at a contact, we tested with mathematical modeling whether E-cadherin could accumulate by simple diffusion-mediated trapping (35). We used micro- and macroscopic diffusion coefficients (D = 0.2–0.6 to 3.0 × 10−10 cm2/s) (6, 36, 37) and cell surface density measurements (E.S.D. = 2.5–16 × 104 molecules/cell) (38) of E-cadherin determined from previous studies. Based on the manufacturer’s specification of maximum binding of human IgG to beads, we calculated that ~45,000 molecules of E-cad/Fc extracellular domain fusion protein attached to a bead. We assumed, however, that only 20% of the bead surface (~9000 molecules) was in contact with cellular E-cadherin because the latter accumulated around the bead in a width-limited ring rather than a uniform cup shape (Fig. 1, A and B), and once it bound to the bead E-cadherin we considered that it became immobilized with respect to the bead surface.

With a D = 0.2 × 10−10 cm2/s and E.S.D. = 2.5 × 104 molecules/cell, we calculated (see “Experimental Procedures”) that <7500 molecules would accumulate over 280 s; with an E.S.D. = 16 × 104/molecules, ~9000 molecules would accumulate in only 30 s (Table 2). Higher diffusion coefficients (D = 0.6
or $3 \times 10^{-10} \text{ cm}^2/\text{s}$ would result in accumulation of $\sim 9000$ molecules in 240 and 30 s, respectively, at E.S.D. of $2.5 \times 10^4$ molecules/cell, and 11 and 2 s, respectively, at

### Table 1

$p$ values determined by t test comparison of E-cadherin accumulation rates in cells expressing different Rac1 constructs

| Type of Rac1 expressed | Mean ± S.E. | Wt: 127.9 ± 9.744 |
|------------------------|-------------|-------------------|
| N17                    | 127.7 ± 16.10 | $p = 0.9913$ |
| V12                    | 83.53 ± 8.615 | $p = 0.0159$ |
| V12                    | 83.53 ± 8.615 vs. wt-fast: 71.11 ± 3.749 | $p = 0.1413$ |

or $3 \times 10^{-10} \text{ cm}^2/\text{s}$ would result in accumulation of $\sim 9000$ molecules in 240 and 30 s, respectively, at E.S.D. of $2.5 \times 10^4$ molecules/cell, and 11 and 2 s, respectively, at

### Table 2

Modeled E-cadherin accumulation by diffusion-mediated trapping

E-cadherin accumulation to a bead by diffusion was simulated, and the time to accumulate $\sim 9000$ molecules was determined for each diffusion coefficient at each E-cadherin surface density (E.S.D.). All simulations assumed a bead with a 0.5-μm radius on a cell with a 400-μm$^2$ surface area with E-cadherin uniformly distributed over the surface.

| Diffusion coefficient | E.S.D. | Time to accumulate |
|-----------------------|--------|--------------------|
| $\mu$m$^2$/s | $\times 10^9$ molecules/cell | $S$ |
| 0.2                  | 2.5    | 280$^*$            |
|                      | 3.5    | 234               |
| 0.6                  | 2.5    | 141               |
|                      | 3.5    | 78                |
| 1.0                  | 2.5    | 16                |
|                      | 3.5    | 11                |
| 3.0                  | 2.5    | 29                |
|                      | 3.5    | 16                |
| 6.0                  | 2.5    | 2                  |

$^*$ 17194 molecules accumulated in the time indicated.
3-kinase activity would then locally decrease actin and membrane dynamics at sites of new E-cadherin accumulation, allowing consolidation of E-cadherin clustering, thereby strengthening adhesion in the more quiescent center of the laterally expanding contact (Fig. 4).

Recruitment of Rac1 to E-cadherin contacts appears to be linked indirectly to E-cadherin accumulation. Wild type, constitutively active, and dominant negative Rac1 all localized to E-cadherin contacts with similar rates. If E-cadherin accumulation were dependent on actin polymerization and membrane dynamics, increased levels of active Rac1 in the form of GFP-Rac1V12 should locally increase actin and membrane dynamics at nascent contacts, thereby stimulating faster E-cadherin accumulation. This is indeed what we found. However, the lack of a detectable change in the rates of E-cadherin accumulation in cells expressing GFP-Rac1N17 indicates either that basal actin dynamics are sufficient to promote fast E-cadherin accumulation without active Rac1 signaling because some actin polymerization machinery can work outside of Rac1 regulation or that the suppression of Rac1 activation may be incomplete in the lamellipodia on which beads were placed (Rac1N17 may not compete with endogenous Rac1 for all guanine exchange factors).

A previous study reported that Rac1 tended to localize at the dynamic, expanding edges of a forming contact, but not where contacts had already formed and the membranes had become more quiescent (8). Although both PI 3-kinase and Rac1 reached peak levels at the same time as E-cadherin, we found that the levels of PI 3-kinase activity and Rac1 rapidly decreased, whereas E-cadherin levels were maintained. Thus, the recruitment and activation of PI 3-kinase and Rac1 upon E-cadherin engagement is transient. Moreover, retention of E-cadherin at early contact sites does not require sustained high levels of PI 3-kinase and Rac1. Because GFP-Rac1N17 is stable at E-cadherin contacts during slow accumulation, we suggest that under normal conditions endogenous Rac1 must be GTP-bound and then locally inactivated by a mechanism that is currently unknown. Interestingly, direct analysis of subcellular sites of Rac1 activation between contacting MDCK cells using the Raichu FRET-based biosensors (40, 41) revealed that Rac1 activity is high in a restricted zone at the edges of expanding cell-cell contacts but is very low within the established cell-cell contact where E-cadherin has accumulated (18). Thus, the co-accumulation of E-cadherin and Rac1 around Ecad beads but then rapid dispersal of Rac1 upon E-cadherin peak accumulation shown in the present study is consistent with our analysis of contacts between pairs of adhering MDCK cells. Further studies are needed, however, to define how PI 3-kinases and Rac1 are locally activated and then inactivated at early contact sites.

Acknowledgments—We thank Roger Tsien (University of California, San Diego) for supplying the tandem dimer DsRed coding sequence and Soichiro Yamada (University of California, Davis) for transfrecting and subcloning the Ecad-RFP and Ecad-RFP/actin-GFP cells. We thank Olivier Rossier at Columbia University for advice and subcloning the Ecad-RFP and Ecad-RFP/actin-GFP cells. We thank Olivier Rossier at Columbia University for support in aligning and maintaining the lasers for the microscope and laser tweezers and Daniel Rabinowitz at Columbia University for aid with statistical analysis. We thank Lance Kam at Columbia University for advice and assistance with the diffusion modeling.

E.S.D. = 16 × 10⁴ molecules/cell (Table 2). These modeling data show that diffusion-mediated trapping satisfies our experimentally derived average, maximal accumulation times of cellular E-cadherin (60–180 s). Although we cannot rule out the possibility of direct delivery of E-cadherin to Ecad-bead/cell contacts, in vesicles for example, we conclude that diffusion-mediated trapping alone may be sufficient to explain how initial E-cadherin accumulation occurs. In support of this model, another report also showed that membrane diffusion in neurons is sufficient to supply adhesion receptors to coated beads (39).

Because actin was not stably associated with Ecad-beads and proteins accumulated only when beads were on lamellae or lamellipodia,⁵ we propose that E-cadherin accumulation depends on local membrane dynamics caused by actin polymerization. Thus, diffusion-mediated clustering of single E-cadherin molecules might be due to intrinsic plasma membrane activity in the vicinity of contacts between neighboring cells (or an Ecad bead in these experiments). This might be mediated by the release of regional restrictions in protein distributions by local changes in actin organization, active clustering of E-cadherin by membrane dynamics, or a combination of both. Subsequent transient activation of membrane dynamics by Rac1 and PI 3-kinase activity at those sites might temporally increase local membrane dynamics at contacts and hence the probability of additional, new E-cadherin engagements; these new contacts would in turn activate another cycle of Rac1 activation and E-cadherin engagement and hence lateral expansion of the contact (Fig. 4). Immediate down-regulation of both Rac1 and PI 3-kinase activity would then locally decrease actin and membrane dynamics at sites of new E-cadherin accumulation, allowing consolidation of E-cadherin clustering, thereby strengthening adhesion in the more quiescent center of the laterally expanding contact (Fig. 4).

A previous study reported that Rac1 tended to localize at the dynamic, expanding edges of a forming contact, but not where contacts had already formed and the membranes had become more quiescent (8). Although both PI 3-kinase and Rac1 reached peak levels at the same time as E-cadherin, we found that the levels of PI 3-kinase activity and Rac1 rapidly decreased, whereas E-cadherin levels were maintained. Thus, the recruitment and activation of PI 3-kinase and Rac1 upon E-cadherin engagement is transient. Moreover, retention of E-cadherin at early contact sites does not require sustained high levels of PI 3-kinase and Rac1. Because GFP-Rac1N17 is stable at E-cadherin contacts during slow accumulation, we suggest that under normal conditions endogenous Rac1 must be GTP-bound and then locally inactivated by a mechanism that is currently unknown. Interestingly, direct analysis of subcellular sites of Rac1 activation between contacting MDCK cells using the Raichu FRET-based biosensors (40, 41) revealed that Rac1 activity is high in a restricted zone at the edges of expanding cell-cell contacts but is very low within the established cell-cell contact where E-cadherin has accumulated (18). Thus, the co-accumulation of E-cadherin and Rac1 around Ecad beads but then rapid dispersal of Rac1 upon E-cadherin peak accumulation shown in the present study is consistent with our analysis of contacts between pairs of adhering MDCK cells. Further studies are needed, however, to define how PI 3-kinases and Rac1 are locally activated and then inactivated at early contact sites.

Acknowledgments—We thank Roger Tsien (University of California, San Diego) for supplying the tandem dimer DsRed coding sequence and Soichiro Yamada (University of California, Davis) for transfrecting and subcloning the Ecad-RFP and Ecad-RFP/actin-GFP cells. We thank Olivier Rossier at Columbia University for advice and assistance with the diffusion modeling.
Protein Dynamics at E-cadherin Contacts

REFERENCES

1. Gumbiner, B. M. (1996) Cell 84, 345–357
2. Takeichi, M. (1991) Science 251, 1451–1455
3. Adams, C. L., Nelson, W. J., and Smith, S. J. (1996) J. Cell Biol. 135, 1899–1911
4. Yap, A. S., Brieher, W. M., Pruschy, M., and Gumbiner, B. M. (1997) Curr. Biol. 7, 308–315
5. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (2002) J. Cell Biol. 157, 469–479
6. Kovacs, E. M., Goodwin, M., Ali, R. G., Paterson, A. D., and Yap, A. S. (2002) Curr. Biol. 12, 379–382
7. Takeichi, M. (1991) Science 251, 1451–1455
8. Ehrlich, J. S., Hansen, M. D., and Nelson, W. J. (2002) Curr. Biol. 12, 367–381
9. Adams, C. L., Nelson, W. J., and Smith, S. J. (1996) J. Cell Biol. 135, 1899–1911
10. Chavrier, P., and Sheetz, M. P. (2000) Cell 100, 209–219
11. Nakagawa, M., Fukata, M., Yamaga, M., Itoh, N., and Kaibuchi, K. (2001) J. Cell Sci. 114, 1829–1838
12. Malliri, A., van Es, S., Huveneers, S., and Collard, J. G. (2004) J. Biol. Chem. 279, 30092–30098
13.准入者, M., Nakagawa, M., Kawajiri, A., Itoh, N., Shojo, I., Matsuura, Y., Yonehara, S., Fujisawa, H., Kikuchi, A., and Kaibuchi, K. (1999) J. Biol. Chem. 274, 26044–26050
14. Betson, M., Lozano, E., Zhang, J., and Braga, V. M. (2002) J. Biol. Chem. 277, 36962–36969
15. Rimm, D. L., Koslov, E. R., Kebriaei, P., Cianci, C. D., and Morrow, J. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8813–8817
16. Goodwin, M., Kovacs, E. M., Thoreson, M. A., Reynolds, A. B., and Yap, A. S. (2003) J. Cell Biol. 159, 1227–1240
17. Thoreson, M. A., Reynolds, A. B., and Yap, A. S. (2003) J. Cell Biol. 159, 1227–1240
18. Atanassova, M., and Takeda, J. (2002) Curr. Biol. 12, 379–382
19. Tookey, P. A., Babcock, A. J., and Sheetz, M. P. (2000) J. Cell Biol. 150, 903–915
20. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
21. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
22. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
23. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
24. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
25. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
26. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
27. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
28. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
29. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
30. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
31. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
32. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
33. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
34. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
35. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
36. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
37. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
38. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
39. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
40. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
41. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
42. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
43. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
44. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
45. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
46. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
47. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
48. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
49. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351
50. Vasioukhin, V., Bauer, C., Murga, C., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 19347–19351