Mechanism for Transition from Initial to Stable Cell–Cell Adhesion: Kinetic Analysis of E-Cadherin–Mediated Adhesion Using a Quantitative Adhesion Assay

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Abstract. A centrifugal force–based adhesion assay has been used to quantitatively examine the kinetics of formation of cell–cell contacts mediated specifically by expression of E-cadherin under the control of a glucocorticoid-inducible promoter in mouse fibroblasts. Analysis of cells expressing maximal or minimal levels of E-cadherin showed that the strength of E-cadherin–mediated adhesion developed in a single exponential step over a short time (half-maximal adhesion, 13–17 min). At 37°C, adhesion strength increased rapidly in the first 20 min without an apparent lag phase. After 90 min, adhesion strength reached a plateau. Differences in final strengths of adhesion were commensurate with the level of E-cadherin expression. Strengthening of adhesion was temperature dependent. At 19°C, strengthening of adhesion was delayed and subsequently developed with a slower rate compared to adhesion at 37°C. At 4°C, adhesion was completely inhibited. Strengthening of adhesion was absolutely dependent on a functional actin cytoskeleton since adhesion did not develop when cells were treated with cytochalasin D. Together, our current and previous (McNeill et al., 1993. *J. Cell Biol.* 120:1217–1226) studies indicate that the rate of initial strengthening of E-cadherin–mediated adhesion is neither dependent on the amount of E-cadherin expressed nor on long-range protein diffusion in the membrane to the adhesion site. However, initial strengthening of adhesion is dependent on temperature-sensitive cellular activities that may locally couple clusters of E-cadherin to the actin cytoskeleton.

CELL–CELL adhesion is critical for normal development of multicellular organisms, tissue regeneration, immunological responses, and tumor metastasis (Takeichi, 1991). During these cellular processes, cell–cell adhesion is dynamic. For example, cell–cell contacts must be transient during cell movements in morphogenesis and establishment of cell boundaries and after activation of immune response. However, maintenance of cell boundaries and the structural integrity of tissues and organs requires strong, stable cell–cell contacts. Defining mechanisms that initiate and strengthen cell–cell contact is key to understanding these complex cellular processes.

A family of membrane proteins, termed cadherins, are regulators of Ca^{2+}-dependent cell–cell adhesion in vertebrates (for reviews see Takeichi, 1991; Kemler, 1992; Geiger and Ayalon, 1992; Grunwald, 1993; Ranscht, 1994) and invertebrates (Mahoney et al., 1991; Oda et al., 1993). Cell aggregation assays have been used to examine the specificity and adhesive function of cadherins (Nose et al., 1988; Steinberg and Takeichi, 1994). Cell–cell recognition is mediated by the extracellular domain of cadherin which specifies homophilic interactions between the same class of cadherins on opposing cell surfaces. In addition, the interaction between the cytoplasmic domain of cadherins and cytosolic proteins termed catenins is important for cell adhesion (for reviews, see Gumbiner, 1993; Kemler, 1993; Nagafuchi et al., 1993).

Although these studies have defined protein–protein interactions that are important for cell adhesion, mechanisms involved in initiating, strengthening, and then stabilizing cell–cell contacts remain poorly understood. Previously, we showed that cell–cell contacts are initially transient and then become stabilized (McNeill et al., 1993). During initial formation of cell–cell contacts, E-cadherin is readily extracted from cells in buffers containing Triton X-100. However, 10–15 min after stabilization of the contact, E-cadherin becomes resistant to extraction with Triton X-100, although there is little increase in the absolute amount of E-cadherin at the contact. We interpreted these observations as evidence that stabilization of cell–cell adhesion is dependent on linkage of E-cadherin to the cytoskeleton, rather than an increase in the absolute amount of E-cadherin at the contact site.

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We have now sought to directly test this hypothesis. We have adopted a centrifugal force–based adhesion assay (McClay et al., 1981) to quantitatively measure the strengthening of cell–cell adhesion mediated specifically by E-cadherin. To examine cadherin-mediated adhesion in the absence of other junctional complexes (e.g., desmosomes, tight junctions) that are normally present in epithelial cells, we used fibroblasts which do not normally exhibit Ca^{2+}-dependent cell–cell adhesion and a glucocorticoid-inducible promoter to regulate E-cadherin expression. Using these cells, we measured, for the first time, the kinetics of strengthening of E-cadherin–mediated cell adhesion and examined the effect of E-cadherin expression, integrity of the cytoskeleton, and temperature on the rate and final strength of cell adhesion.

Materials and Methods

Cell Culture

Cells were routinely cultured in Dulbecco's modified Eagle's medium (DME) supplemented with 5% fetal bovine serum. Low passage aliquots of L cells expressing E-cadherin (LE cells)[1] were used for only 3 wk to ensure the same quality of cells for the study. To induce E-cadherin expression, cells were cultured overnight in the presence of different concentrations of dexamethasone (DEX); DEX was added in the appropriate dilution from a stock solution of 1 mM in ethanol.

Cell Transfection

Full length mouse E-cadherin cDNA was derived from pSUM (Stappert and Kemler, 1994) by enzymatic cleavage with KpnI/BglII and cloned into the KpnI/BamHI sites of the expression vector pLK-neo (Hirt et al., 1992). Mouse L-M(TK-) cells were transfected using lipofectamine protocol (Gibco BRL, Gaithersburg, MD). G418-resistant clones were isolated after 4 d of selection in 1 μg/ml G418 and 10 d of selection in 300 mg/ml G418 and then analyzed for expression of E-cadherin by immunofluorescence and immunoblotting with DECMA-1.

Immunofluorescence Microscopy

Cells were grown overnight to half confluency on glass coverslips. Cells were extracted in ice-cold CSK buffer (50 mM NaCl, 300 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM $MgCl_2$, 0.5% Triton X-100, 10 μg/ml leupeptin, and 1 mM Pefabloc [Boehringer Mannheim Biochemicals, Indianapolis, IN]) for 15 s before fixation. Aldehyde fixation was quenched for 10 min in a PBS buffer containing 25 mM glycine, 25 mM NH_4Cl, and 25 mM lysine. For double labeling with DECMA-1 and either anti-α- or β-catenin antibodies, cells were fixed in 100% methanol at −20°C for 10 min and then washed three times in PBS. After incubation in blocking buffer (PBS buffer containing 0.2% BSA, 1% normal goat serum), cells were incubated with antibodies diluted in blocking buffer: anti–E-cadherin (rabbit polyclonal or rat monoclonal DECMA-1; a generous gift from Dr. Rolf Kemler, Max Planck Institute for Immunology, Freiburg, Germany); anti–α-catenin, β-catenin, plakoglobin (all rabbit polyclonal and described in Hinck et al., 1994); fodrin (rabbit polyclonal; Nelson and Veshnock, 1987) or vinculin (mouse monoclonal 1:400; Sigma Chemical Co., St. Louis, MO). After washing three times in PBS, cells were incubated in a 1:200 dilution in blocking buffer of FITC- or rhodamine-conjugated secondary goat anti-rabbit, goat anti-rat, or goat anti-mouse antibodies (Boehringer Mannheim Biochemicals), depending on the species used to generate the primary antibody. Cells were mounted in Elvanol containing 1 mg/ml *p*-phenylendiamine and examined with a microscope (Axioplan; Carl Zeiss, Inc., Thornwood, NY) equipped for epifluorescence microscopy.

1. *Abbreviations used in this paper*: BWB, blot washing buffer; CD, cytochalasin D; DEX, dexamethasone; HCM and LCM, high and low calcium medium; LE cells, L cells expressing E-cadherin; LP cells, untransfected L cells.

SDS-PAGE and Immunoblotting

Cells were grown to confluency overnight in 6 cm petri dishes. Cells were washed once with PBS buffer containing 2 mM $CaCl_2$ and then lysed on the petri dish in 450 μl of boiling SDS buffer (2% SDS in 40 mM Tris/HCl, pH 6.8). After further boiling for 10 min, lysates were centrifuged at 100,000 *g* for 30 min at room temperature. The protein concentration of the lysate was determined with the BCA protein assay according to the manufacturer's instruction (Pierce, Rockford, IL). Volumes were adjusted to equal protein concentrations by addition of SDS buffer, and proteins were separated by SDS-7.5% PAGE (Laemmli, 1970). Proteins were transferred electrophoretically to nitrocellulose membranes. Membranes were blocked in blot washing buffer (BWB; 15 mM Tris/HCl [pH 7.5], 120 mM NaCl, 2 mM $CaCl_2$, 0.1% Tween-20) containing 10% dried milk, followed by incubation for 2 h with DECMA-1 supernatant diluted 1:2 in BWB containing 5% dried milk. Blots were washed twice for 15 min each with BWB and then incubated with horseradish peroxidase-conjugated secondary goat anti-rat antibody for 1 h. After washing twice for 15 min each with BWB, immunolabeling was detected by the ECL detection system according to the manufacturer's instructions (Amersham Corp., Arlington Heights, IL).

Centrifugal Force-Based Adhesion Assay

The assay was adopted from the adhesion assay developed and described by McClay et al. (1981) and Lotz et al. (1989) (Fig. 1). Cells were grown overnight in the presence or absence of DEX. To prepare the substratum monolayers, cells were removed from the culture dish in 137 mM NaCL, 5.4 mM KCL, 5.6 mM glucose, 4.2 mM $NaHCO_3$, 0.5 mM EDTA for 20 min at room temperature. Cells were pelleted at low speed and then resuspended in low calcium medium (LCM; DME containing 10 mM Hepes, pH 7.4, and only 5 μM $CaCl_2$, lacking phenol red and $NaHCO_3$) at a density of 2×10^6 cells/ml. 1.6×10^5 cells were added to poly-D-lysine (PDL, Sigma Chemical Co.) coated wells of flexible 96-well plates (model 3912 Microtiter Test III; Falcon Plastics, Cockeysville, MD). After 5 min at room temperature, cells were centrifuged onto the bottom of the well at 50 *g* for 8 min in the swinging bucket microtiter plate holder of the centrifuge (CPR; Beckman Instruments, Fullerton, CA), and then incubated at 37°C for 20 min. The substratum monolayer was washed gently with LCM and kept at 4°C until single radioactive-labeled cells (top cells) were added. Under these conditions, E-cadherin–mediated cell–cell contacts are not formed in the substratum monolayer.

Top cells were metabolically labeled by incubating low density cultures of 2×10^6 cells in DME containing 10 μCi/ml ^{35}S-methionine (Amersham Corp.) for 1 h at 37°C. Cells were then washed with HDF and removed from the culture dish by incubating for 10 min in HDF without trypsin at room temperature. Cells were placed on ice, pelleted, and resuspended at

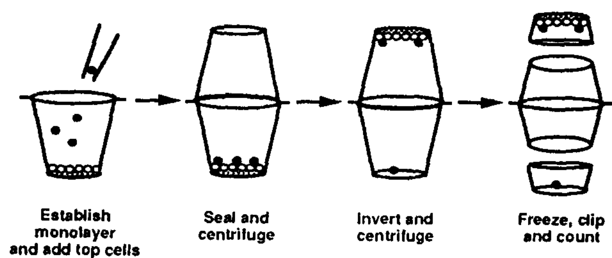

Figure 1. Centrifugal force–based adhesion assay. A confluent monolayer of cells is formed at the bottom of a microtiter well. Radioactively labeled cells (top cells) are added to the well and brought into contact with the monolayer synchronously by centrifugation at 4°C. A second microtiter well filled with medium is mounted on top of the first yielding a sealed chamber. After incubation, the assembled chamber is inverted and centrifuged to remove unbound, labeled cells from the monolayer. After centrifugation, the chamber is quick-frozen in an ethanol-dry ice bath and the bottom of each chamber is clipped. The radioactivity in all three compartments (bottom, top and middle) is determined by scintillation counting, and the percentage of bound cells is calculated.

a density of 10^6 cells/ml in high calcium medium (HCM; LCM containing 1.8 mM $CaCl_2$) at 4°C. Medium on the substratum monolayer was exchanged for HCM and 10^5 of the ^{35}S-methionine–labeled cells were added to each well. The maximum number of cells that could be added to the substratum monolayer while keeping cell–cell contacts within this population to a minimum was determined by phase contrast microscopy. Another 96-well plate, filled with medium, was inverted on the plate containing the cells. The plates were held together by a layer of double-sided carpet tape (Scotch/3M, St. Paul, MN) attached to the top surface of each plate, which allowed liquid continuity between opposite wells without leaking. Plates were centrifuged (see above) at 16 *g* for 8 min at 4°C, which resulted in gentle contact between the top ^{35}S-methionine–labeled cells and the substratum monolayer. According to the requirements of the given experiment (see Results), cells were incubated at different temperatures for different times. In the case of a 37°C or 19°C incubation, plates were assembled after the incubation period. After incubation, plates were inverted and centrifuged at room temperature at the indicated *g*-force (see Results) for 8 min. In initial experiments, a range of *g*-forces (44 *g*–600 *g*) was tested for removing top cells in order to find a *g*-force at which background adhesion between untransfected (LP) cells was low (≤20%) and increases in E-cadherin-mediated adhesion between LE cells became detectable within a linear range. At low *g*-forces (44, 115, and 300 *g*), background adhesion was between ~40% and ~30% (data not shown). Acceptable levels of background adhesion (i.e., ≤20%) were obtained at *g*-forces above 500-600 *g*. The plates were frozen in a slurry of dry ice-ethanol and the bottom 3 mm of both wells containing the substratum monolayer and cells that did not adhere, respectively, were clipped. The well containing the substratum monolayer (and adherent top cells), the opposing well, and the middle of the communicating chambers (containing free cells) were counted separately in a liquid scintillation spectrometer. Percent cells bound was calculated as cpm bound cells/(cpm bound cells + cpm free cells). Results are reported as the mean percent bound of three to four experimental replicates ±SD if not indicated differently. Note that scanning electron microscopy revealed that when cells were centrifuged off the substratum monolayer, the latter remained intact and no cell membrane remnants from the second layer of cells were detected on the substratum monolayer (data not shown). Thus, loss of ^{35}S-methionine–labeled top cells from the substratum monolayer was due to insufficient cell–cell adhesion and not cell rupture.

In experiments in which the effect of cytochalasin D (CD) on cell–cell adhesion was tested, radioactively-labeled top cells were resuspended in HCM containing 4 μM CD (CD/HCM). Untreated cells were resuspended in HCM containing the same amount of DMSO contained in CD/HCM following dilution of the CD stock solution (2 mM CD in DMSO). The assay was then carried out as described above. The immediate effect of CD on disruption of the actin cytoskeleton was confirmed by fixation and phalloidin staining of cells after 3 min of CD incubation (data not shown). In experiments in which the effect of nocodazole on cell–cell adhesion was tested, top cells were incubated at 4°C for 30 min, then for another 30 min at 4°C in the presence of HDF containing either 1 μM or 33 μM nocodazole (HDF/noco). Cells were then incubated at room temperature in HDF/noco for 20 min. The procedure for isolating top cells was the same as the standard assay except that nocodazole was present during the labeling procedure and the centrifugation and incubation steps with the substratum monolayer. Disruption of microtubules under these experimental conditions was confirmed by immunofluorescence microscopy (data not shown).

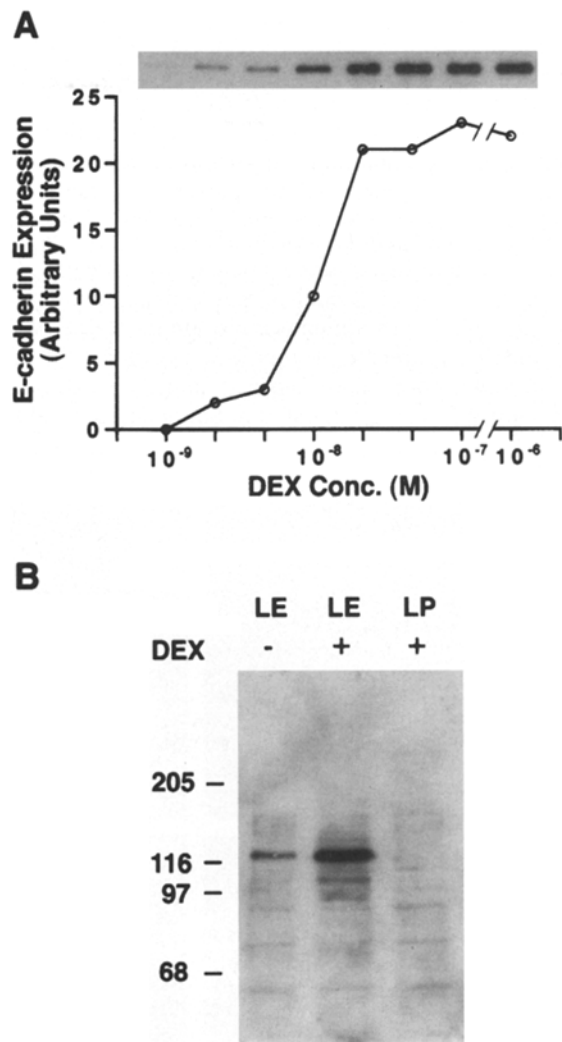

Figure 2. Differential expression of E-cadherin in LE cells under the control of an inducible glucocorticoid promoter. (*A*) LE cells were cultured for 19 h in the presence of different concentrations of DEX as indicated. Cell lysates containing equal amounts of protein were immunoblotted with the E-cadherin–specific monoclonal antibody DECMA-1. Relative amounts of E-cadherin were determined by scanning densitometry and plotted against the DEX concentration used in the corresponding cell culture. (*B*) Lysates of LE and LP cells cultured overnight with or without 10^{-6} M DEX were prepared and equal amounts of protein were immunoblotted with the E-cadherin–specific antibody DECMA-1. Numbers on the left indicate molecular mass in kD.

Results

Inducible Expression of E-cadherin in Fibroblasts (LE Cells)

The murine fibroblast cell line, L-M (TK-), neither expresses cadherins nor exhibits Ca^{2+}-dependent cell–cell adhesion in culture (see below). L-M (TK-) cells were transfected with full length murine E-cadherin cDNA (LE cells) under the control of a glucocorticoid-inducible promoter (pLK-neo; Hirt et al., 1992). Maximal expression of E-cadherin was achieved by incubating transfected cells for ~16 h in the presence of DEX; different levels of protein expression were obtained by varying the concentration of DEX. Immunoblotting of whole cell lysates with E-cadherin antibody followed by scanning densitometry showed that the amount of protein increased over 100-fold in the range of 10^{-9} to 10^{-6} M DEX (Fig. 2 *A*). Control cells (LP), which were transfected with empty vector, do not express E-cadherin in the presence or absence of DEX (Fig. 2 *B*).

E-cadherin also accumulated in LE cells in the absence of exogenous DEX (Fig. 2 *B*), probably due to leakiness of the glucocorticoid-inducible promoter (Hirt et al., 1992). This level of E-cadherin expression was similar to that in cells incubated in 10^{-9} M DEX and was ~100-fold less than that in maximally induced cells (Fig. 2 *A*). In the ex-

periments described below, we limited our adhesion analysis to cells incubated in 10^{-6} M DEX (maximal E-cadherin expression) or without DEX (minimal E-cadherin expression).

Characterization of E-cadherin Distribution in LE Cells

Small colonies of LE cells were incubated in 10^{-6} M DEX for ~16 h. Immunofluorescence microscopy revealed a clustered distribution of E-cadherin at cell–cell contacts (Fig. 3 *A*). Intense focal staining was also on the apical cell surface, which may indicate protein localization in microspikes (data not shown). E-cadherin staining was not observed in control LP cells incubated in the presence or absence of DEX (data not shown). Alpha-catenin and β-catenin co-localized precisely in clusters that contained E-cadherin (Fig. 3, *A* and *A'*, *B* and *B'*). Fodrin also localized to cell–cell contacts in E-cadherin expressing cells and could be found in E-cadherin containing clusters (Fig. 3, *C* and *C'*, *arrowheads*). Vinculin localized to focal adhesions at cell-substrate contacts, but not in E-cadherin clusters (Fig. 3 *D*). Immunofluorescent staining for plakoglobin revealed only background staining (Fig. 3 *E*).

Establishing a Quantitative Analysis of E-cadherin–mediated Adhesion

To directly quantify E-cadherin–mediated adhesion, we adopted a centrifugal force-based adhesion assay (McClay

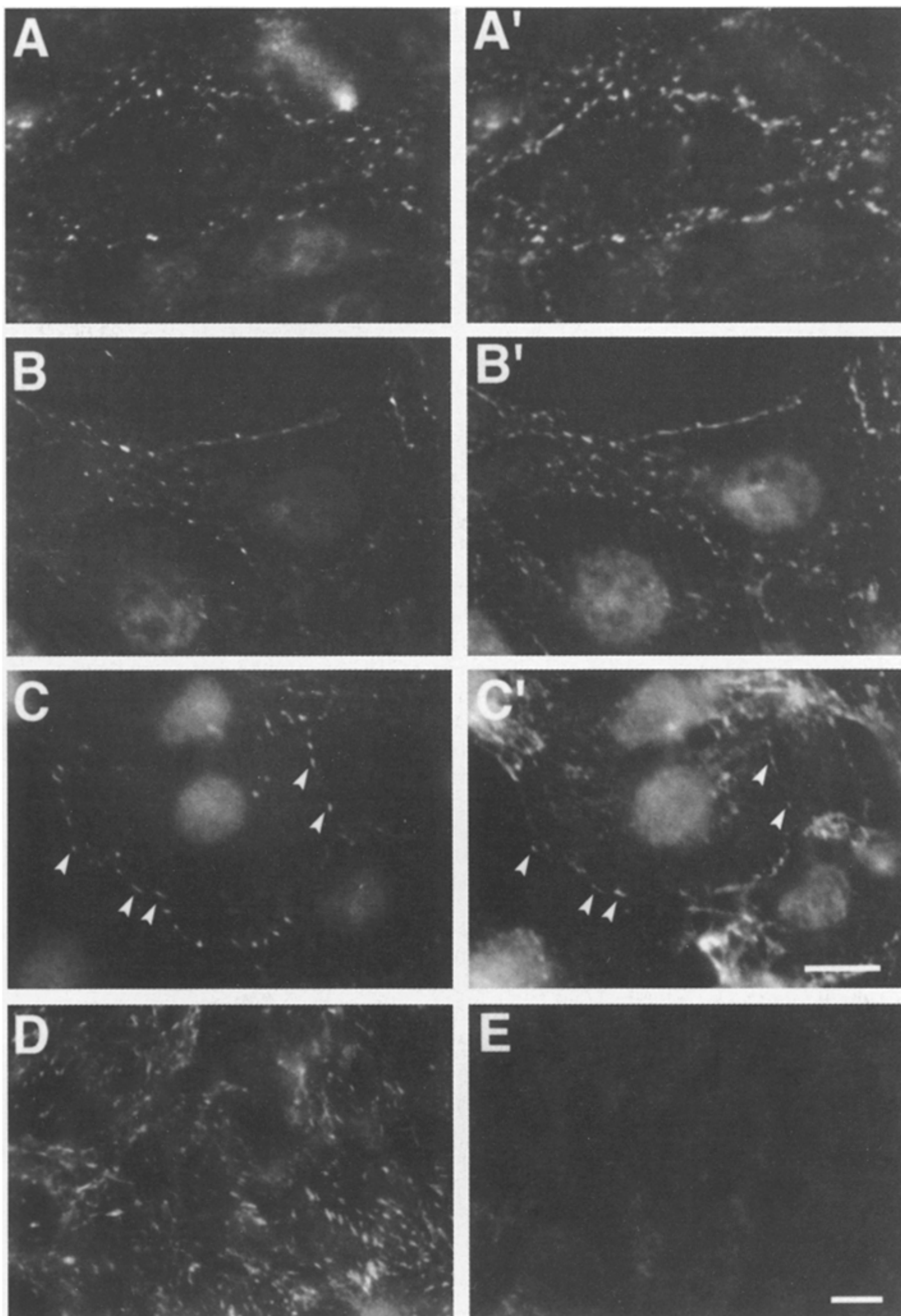

Figure 3. Immunofluorescent staining of E-cadherin and the intracellular proteins α–catenin, β–catenin, fodrin, plakoglobin, and vinculin in DEX-induced LE cells. LE cells cultured overnight with 10^{-6} M DEX were extracted with buffer containing 0.5% Triton X-100 prior to fixation. The photographs show cells double-stained with E-cadherin (*A*) and α–catenin (*A'*), E-cadherin (*B*) and β–catenin (*B'*) and E-cadherin (*C*) and fodrin (*C'*) antibodies. Alpha-catenin and β–catenin clearly co-localize with E-cadherin in clusters at the cell–cell contact. Fodrin staining overlaps with all E-cadherin clusters (*arrowheads*) but not all fodrin co-localizes with E-cadherin. (*D*) Immunostaining of vinculin. (*E*) Immunostaining of plakoglobin. Bar, 10 μm.

et al., 1981; see Fig. 1). Three experiments were performed to determine the specificity of E-cadherin–mediated adhesion in this assay. In all three types of experiments, percentage of bound cells was determined after 10 min of cell–cell contact. First, adhesion was compared between the same type of cells (LE or LP cells) with or without DEX pretreatment. Similar mean values were measured between untreated (20%) and treated (17%) LP cells (Fig. 4 *A*). LE cells that expressed low amounts of E-cadherin (DEX-untreated) exhibited a small but statistically significant (P = 0.008) increase in cell binding compared to adhesion between LP cells (DEX-untreated; 28 and 20%, respectively). Significantly, when LE cells were pretreated with 10^{-6} M DEX to induce high levels of E-cadherin expression (see Fig. 2 *B*) the percentage of bound cells increased dramatically compared to that between LP cells (47 and 17%, respectively; Fig. 4 *A*) and between LE-DEX cells (47 and 28%, respectively; Fig. 4 *A*).

Second, adhesion between LP and LE cells was compared with adhesion between LE and LE cells (Fig. 4 *B*). All cells in this series of experiments were pretreated with DEX. Whereas adhesion between LE cells increased significantly when compared with LP cells (67 and 24%, respectively), adhesion between LE and LP cells tested in each combination (monolayer and single cells of different cell types) did not deviate significantly from the amount of adhesion exhibited by control (LP) cells alone (22, 28, and 24%, respectively; Fig. 4 *B*).

Third, adhesion between LE cells expressing high levels of E-cadherin (+DEX) was tested in the presence of a physiological concentration of Ca^{2+} (1.8 mM) and compared with adhesion at a low concentration of Ca^{2+} (5 μM) which inhibits cadherin-mediated adhesion (Nelson and Veshnock, 1987). As observed in the previous experiments, cell binding between E-cadherin–expressing cells increased significantly in the presence of 1.8 mM Ca^{2+} when compared with LP cells (50 and 20%, respectively; Fig. 4 *C*). However, in the presence of 5 μM Ca^{2+}, the number of bound cells did not increase significantly above the background value measured between LP cells (16 and 14%, respectively). Adhesion between LP cells did not change when the Ca^{2+} concentration was diminished (20 and 14%, Fig. 4 *C*).

E-cadherin–mediated Adhesion Develops in a Single Exponential Step

The kinetics of E-cadherin–mediated adhesion between LE cells (±DEX) was measured over a time course of 150

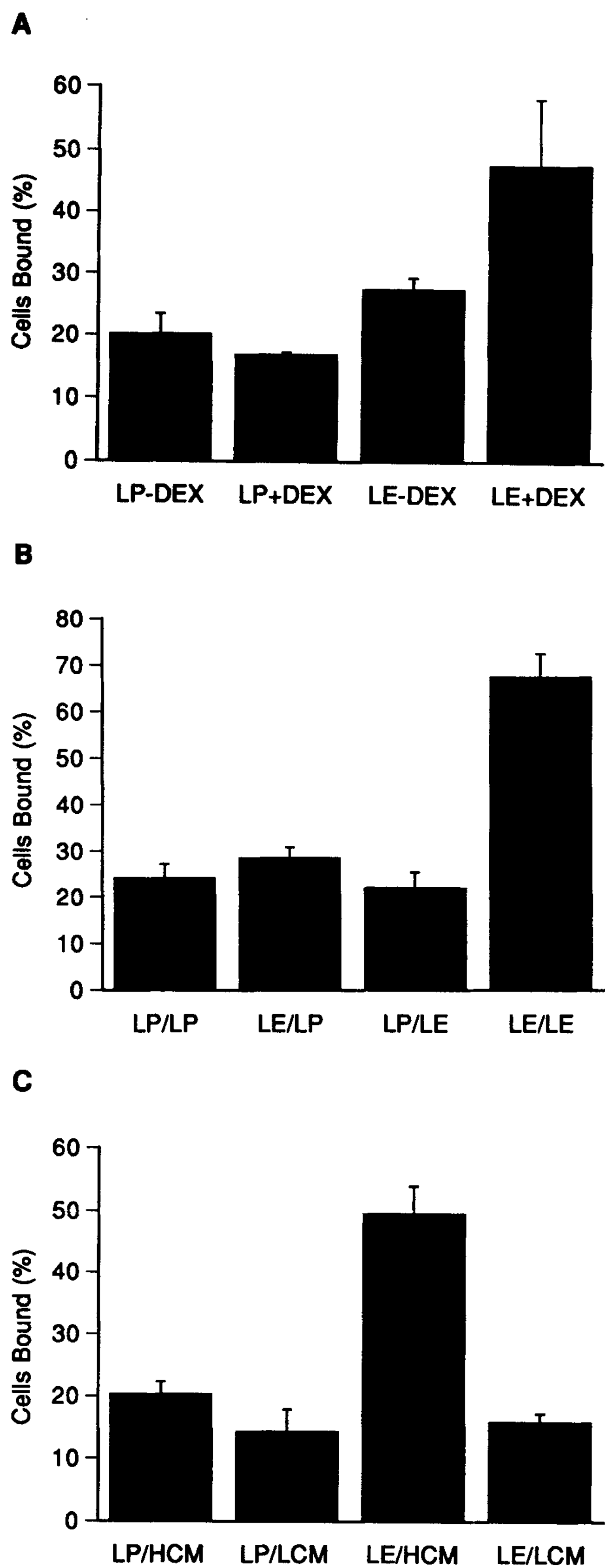

Figure 4. Specific quantification of E-cadherin–mediated adhesion using the centrifugal force-based adhesion assay. Single, radioactively labeled cells (top cells) were incubated on substratum monolayers at 37°C for 10 min. Percentages of cells bound to the monolayer were determined after centrifugation with 600 *g*. Error bars represent SDs. (*A*) LP and LE cells were cultured overnight in the presence or absence of 10^{-6} M DEX as indicated. Adhesion between the same types of cells was measured. (*B*) LP and LE cells were cultured overnight in the presence of 10^{-6} M DEX. Combinations of types of cells used in the adhesion assay (monolayer, first position; top cells, second position) are indicated for each bar. (*C*) LP and LE cells were cultured overnight in the presence of 10^{-6} M DEX. Adhesion between the same type of cells was tested at a physiological concentration of Ca^{2+} (2 mM; HCM) or at a low Ca^{2+} concentration (5 μM; LCM).

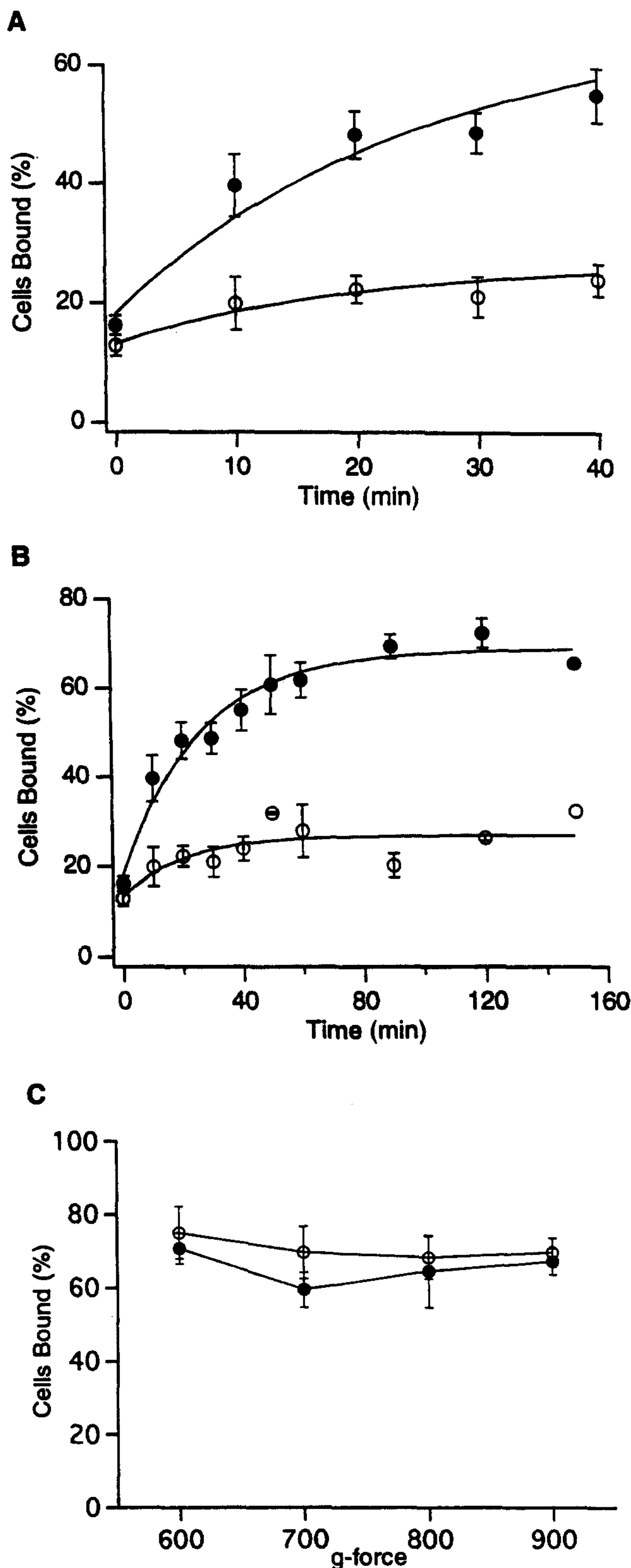

Figure 5. Kinetics of the development of E-cadherin–mediated adhesion. LE cells were treated overnight with 10^{-6} M DEX (–●–) or left untreated (–○–). Top cells were added to monolayers and incubated at 37°C for the indicated times. Adhesion was measured with a centrifugal force of 600 *g*. (*A*) Kinetics of the initial phase of adhesion. (*B*) Same data set as shown in A, but with additional points at later times to show the final level of adhesion. Data points represent the means of the means (total of 39 for −DEX; total of 41 for +DEX) of seven independent experiments and were fit to a single exponential function; error bars in A and B represent the SEM. (*C*) LE cells were treated overnight with 10^{-6} M DEX. Top cells were added to monolayers and adhesion was measured after 90 min (–●–) and 120 min (–○–) of incubation at 37°C at different g-forces. Error bars represent SDs.

min using 600 *g* to remove nonadherent cells (see Methods). At both maximal and minimal levels of E-cadherin expression, adhesion developed very rapidly in a single exponential step (Fig. 5 *A* and *B*); Fig. 5 *A* shows adhesion kinetics during the initial phase of strengthening of adhesion, and Fig. 5 *B* shows the kinetics of adhesion during the complete time course. Half maximal binding of cells was reached within similar times for both maximal and minimal levels of E-cadherin (17 min [+DEX] and 13 min [−DEX]; Fig. 5 *A*). After 90 min of cell–cell contact, the amount of bound cells approached a maximum. Maximum percentage of cells bound after 150 min was significantly lower between LE cells that were not pre-treated with DEX than between LE cells that were pretreated with DEX (32 and 65%, respectively; Fig. 5 *B*), although the kinetics of increase in numbers of bound cells were similar ±DEX (Fig. 5 *A*). To determine whether a maximum number of bound cells was reached after 90 min (plateau phase; Fig. 5 *B*), higher g-forces were applied to cells expressing the maximum amount of E-cadherin (LE+DEX). Increasing the centrifugal force from 600 *g*–900 *g* did not reveal a difference in numbers of bound cells after 90 min and 120 min of cell–cell contact (Fig. 5 *C*).

Temperature Dependent Development of Cadherin-mediated Adhesive Strength

We measured adhesion at 4 and 19°C to examine the dependence of the adhesion machinery on lateral mobility of proteins in the plane of the membrane and on the metabolic activity of the cell. Development of E-cadherin–mediated adhesion was followed over a 40 min incubation period at 37 and 4°C (Fig. 6 *A*). As shown in the previous set of experiments (see Fig. 5 *A* and *B*), the number of bound cells increased rapidly at 37°C. In contrast, the number of bound cells remained at a background level at 4°C for the whole time course (Fig. 6 *A*) and up to 130 min (data not shown). LE (−DEX) cells expressing minimal amounts of E-cadherin (see Fig. 2 *B*) developed comparably less adhesion than LE (+DEX) cells at 37°C (Fig. 6 *A*).

In experiments conducted at 19°C, adhesion between LE (+DEX) cells developed with kinetics that were distinctly different from those at 37°C (Fig. 6 *B*). While the number of bound cells at 37°C increased in a rapid, single exponential step, the numbers remained similar to background levels at 19°C for 50–60 min. After this lag period, adhesion increased but at a rate that was significantly slower than that at 37°C.

Cadherin-mediated Adhesion is Dependent on an Intact Actin Cytoskeleton but Not on Microtubules

The requirement of the cytoskeleton for initial strengthen-

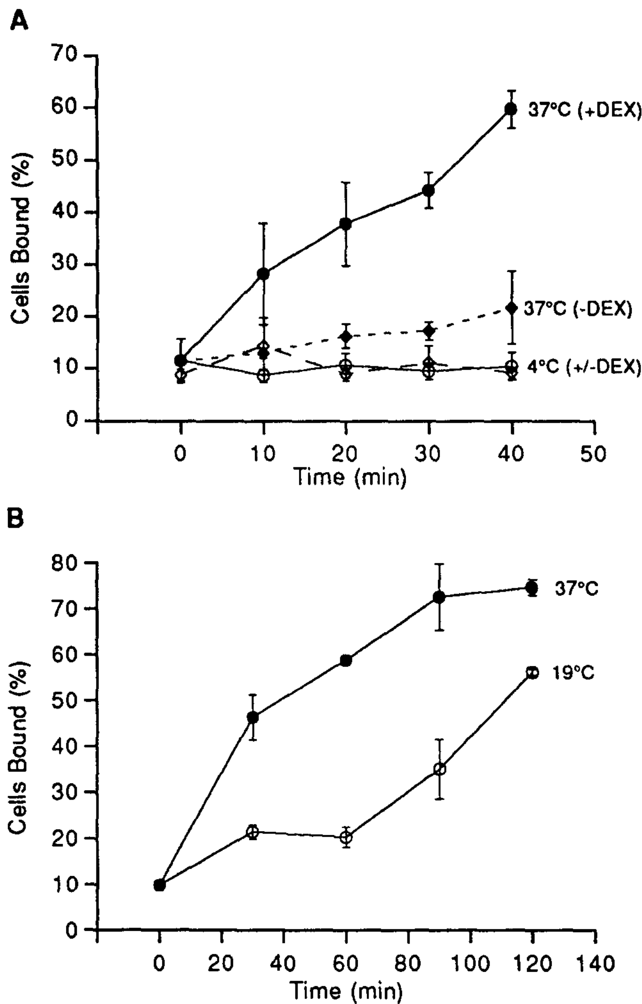

Figure 6. Effect of temperature on E-cadherin–mediated adhesion. (*A*) Development of E-cadherin–mediated adhesion at 4°C and 37°C. LE cells were treated overnight with 10^{-6} M DEX or left untreated as indicated. Top cells were added to equally treated monolayer cells and incubated for the indicated times at 37 or 4°C. The centrifugal force applied was 600 *g*. Error bars represent SDs. (*B*) Development of E-cadherin–mediated adhesion at 19 and 37°C. LE cells were treated overnight with 10^{-6} M DEX. Top cells were added to monolayers and incubated for the indicated times at 37 or 19°C. The centrifugal force applied was 600 *g*. Error bars represent SDs.

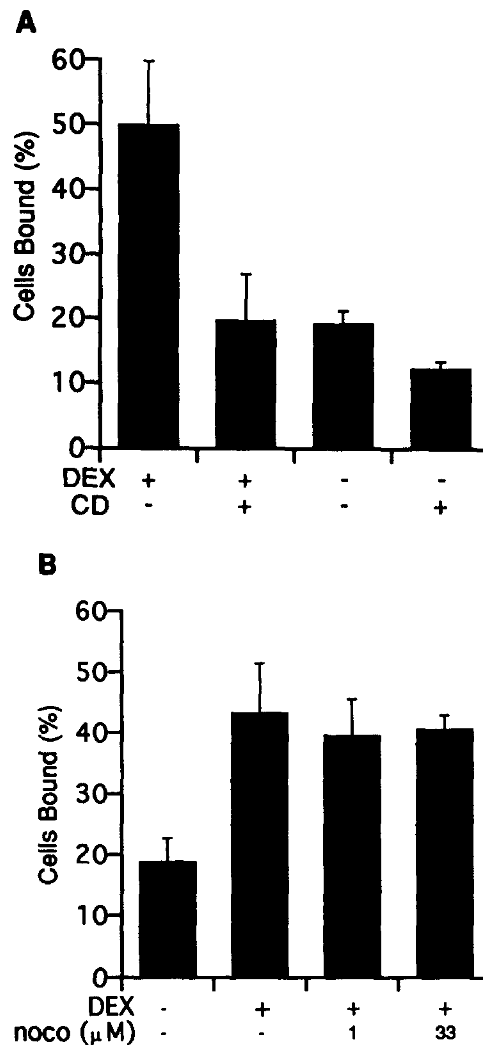

Figure 7. Effects of cytochalasin D and nocodazole on E-cadherin–mediated adhesion. LE cells were cultured overnight in the presence or absence of 10^{-6} M DEX as indicated. Top cells were added to monolayer cells and incubated for 10 min at 37°C before a centrifugal force of 600 *g* was applied. Only top cells, but not monolayer cells, were treated with CD (*A*), or nocodazole (noco) (*B*) as indicated. Error bars represent SDs.

ing of E-cadherin–mediated adhesion was examined by disrupting either actin filaments (CD) or microtubules (nocodazole). The concentrations of inhibitors used in these experiments completely disrupted cytoskeleton organization as determined by immunofluorescence microscopy (data not shown). LE cells pretreated with CD did not develop cadherin-mediated adhesion compared to untreated cells, and adhesion remained at a background level throughout the time course of the experiment (Fig. 7 *A*). The same effect of CD on adhesion was found in LE cells (−DEX), indicating that the relatively weak adhesion mediated by low levels of E-cadherin expressed in these cells is also dependent on the actin cytoskeleton. As shown in Fig. 7 *B*, the amount of adhesion developed by LE cells pretreated with nocodazole was similar to that of cells with an intact microtubule cytoskeleton.

Discussion

In the present study, we adopted a centrifugation force–based cell–cell adhesion assay to examine how initial contacts are made and then strengthened between cells expressing E-cadherin. This centrifugal force–based adhesion assay provides a sensitive, quantitative measurement of very small binding strengths (McClay et al., 1981). Previous studies of cadherin-mediated cell–cell adhesion have used an assay involving aggregation of cells in suspension culture. Nose et al. (1988) and Steinberg and Takeichi (1994) also showed that formation of cell aggregates is rapid. The half-time for aggregation was determined to be 15–30 min, which is similar to the time required for half-

maximal development of adhesion measured with the centrifugal force–based adhesion assay (see Fig. 5).

When cells expressing E-cadherin were brought into contact with each other, kinetics of cell binding developed in a single, exponential step. This kinetic behavior of cell binding was characterized by a rapid increase in the first 20 min after initial cell–cell contact. Within the limitations of our experimental system we determined that after 90 min of cell–cell contact, maximum cell binding was reached. The time to reach half-maximal binding was similar when E-cadherin was either maximally (17 min, LE+DEX) or minimally expressed (13 min, −DEX). Although our study is limited to these two extremes of E-cadherin expression, results indicate that differences in the final level of adhesion strength attained correspond to the relative amount of E-cadherin expressed.

Strengthening of cell–cell adhesion was dependent on an intact actin cytoskeleton. In the presence of CD, cells exhibited little or no strengthening of E-cadherin–mediated adhesion (Fig. 7; see also, Hirano et al., 1987; Friedlander et al., 1989; Jaffe et al., 1990; Matsuzaki et al., 1990). Thus, extracellular interactions between E-cadherin molecules are not sufficient to provide adhesion strength above background levels but mainly serve in the initial recognition event (see below). Candidate proteins for regulating actin filament association with E-cadherin are α-catenin (Rimm et al., 1995) and fodrin (Nelson and Veshnock, 1987), both of which co-localize with E-cadherin in clusters during cell–cell adhesion (see Fig. 3).

What is the role of the actin cytoskeleton in strengthening of E-cadherin–mediated cell adhesion? Analysis of the final strength of adhesion at maximal (10^{-6} M DEX) and minimal (−DEX) levels of E-cadherin expression showed that the kinetics of initial strengthening of cell–cell adhesion were similar. Furthermore, strengthening of cell adhesion over this range of E-cadherin concentrations absolutely required an intact actin cytoskeleton. Thus, initial formation of cell–cell contacts appears to be independent of the amount of E-cadherin at the contact but is dependent on another cellular activity, such as linkage of cadherin to the actin cytoskeleton or cross-linking of preformed cadherin-catenin-actin complexes into higher-ordered structures. In our previous study of early events in the formation of cell–cell contacts in MDCK cells (McNeill et al., 1993), we found that during the initial phase of formation of stable cell–cell contacts, the amount of E-cadherin at the contact did not change, and all of the protein could be solubilized in a buffer containing Triton X-100. However, after ~10 min, there was a rapid, 4–6-fold increase in the amount of E-cadherin at cell–cell contacts that was resistant to extraction with Triton X-100, indicating that E-cadherin had become associated with the cytoskeleton. Significantly, this increase in the detergent insoluble pool of E-cadherin was not matched by a corresponding increase in the total amount of E-cadherin at cell–cell contacts. Together, our previous and current results indicate that the initial stabilization and strengthening of E-cadherin–mediated adhesion is independent of the amount of E-cadherin at the contact site but is dependent on linkage of E-cadherin to the actin cytoskeleton.

How is linkage of E-cadherin to the actin cytoskeleton initiated, and how does this linkage affect the kinetics of strengthening of cell adhesion? The development of cell adhesion mediated by cytoskeletal-linked adhesion molecules is generally characterized by two steps: a recognition event and a subsequent stabilization phase (Carter et al., 1981; McClay et al., 1981; Lotz et al., 1989). The transition between these two steps is poorly understood in any of the known cell adhesion systems. The transition phase may involve a nucleation event that is required before physical linkage to the cytoskeleton occurs. Such a nucleation event may consist of trapping adhesion molecules at the cell–cell or cell-substrate contact (McCloskey and Poo, 1986; Po-Ying et al., 1991). Subsequent linkage of adhesion molecules to the cytoskeleton could occur either through mass action of preassembled protein complexes or require enzymatic activity (e.g., kinases, phosphatases) to catalyze the association of the components.

To gain further insight into the mechanisms of E-cadherin–mediated adhesion, we examined the effect of reduced temperatures on the strengthening of cell adhesion. The effects of reduced temperature on living cells are at least twofold: a reduction in membrane fluidity and a reduction in enzymatic activity. Results revealed that E-cadherin adhesion is strongly temperature dependent. At 4°C, we detected no strengthening of adhesion in cells expressing maximum amounts of E-cadherin. At 19°C, however, measurable adhesion developed after a lag period of 60 min, although the increase in adhesion occurred at a rate slower than that observed at 37°C. One interpretation of the lag period prior to the onset of measurable cell adhesion at 19°C is that a critical amount of cadherin must diffuse laterally in the plane of the membrane to the contact site before adhesion can be strengthened. However, we consider that E-cadherin diffusion over long distances is unlikely. First, strengthening of adhesion in cells expressing minimal levels of E-cadherin (LE-DEX) was not preceded by a lag phase, and the kinetics of adhesion developed in a single exponential step over a 100-fold range in E-cadherin expression level. Second, in MDCK cells, we did not detect an increase in the total amount of cadherin at cell–cell contacts during stabilization of initial cell adhesion (~85 min; McNeill et al., 1993). Note also that single particle tracking studies have shown that the majority of E-cadherin on a free cell surface is in a stationary or restricted diffusion mode (Kusumi et al., 1993). However, we cannot rule out the possibility that short-range clustering of E-cadherin occurs. The clusters of E-cadherin observed by immunofluorescence microscopy (see Fig. 3; see also, Näthke et al., 1994) may be representative of this type of localized diffusion. In addition, it is possible that E-cadherin exists as preformed clusters in the plasma membrane before being linked to an actin cytoskeleton. Clusters of densely packed E-cadherin could facilitate rapid strengthening of cell adhesion by potentiating the association of cadherin complexes on opposite cell membranes (Shapiro et al., 1995) and may promote rapid linkage of cadherin to the actin cytoskeleton upon formation of cell–cell contacts. Finally, the decreased rate of adhesion at 19°C suggests the involvement of a delayed cellular activity in the strengthening of adhesion that may include changes in phosphorylation of cadherin and catenins (Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993; Shibamoto et al., 1994; Stappert and Kemler, 1994),

or in the dynamics of actin assembly with the cadherin/catenin complex.

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